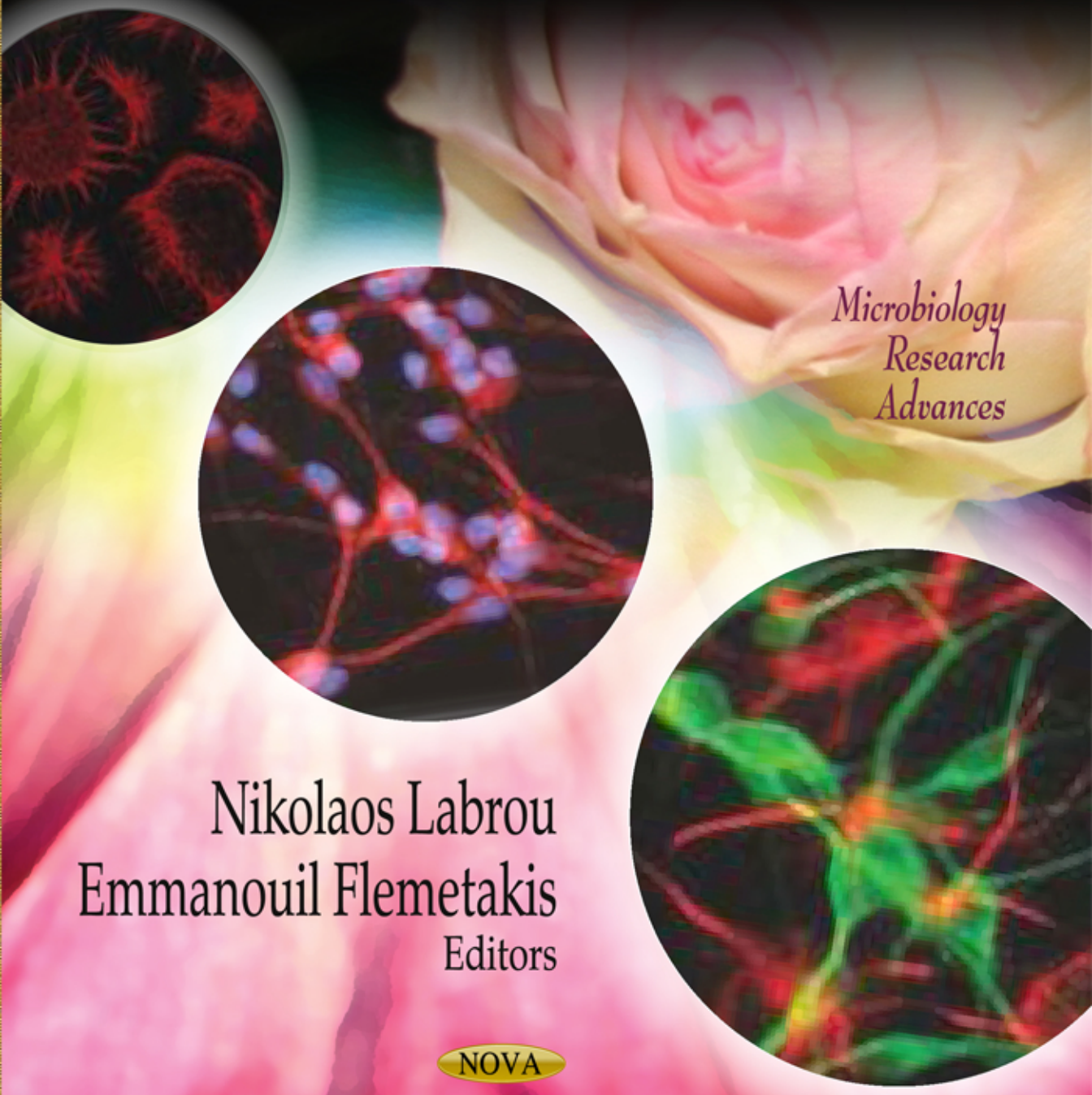
Glutathione

Biochemistry, Mechanisms of Action and Biotechnological Implications



Microbiology Research Advances

Nikolaos Labrou
Emmanouil Flemetakis
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GLUTATHIONE

BIOCHEMISTRY, MECHANISMS OF ACTION AND BIOTECHNOLOGICAL IMPLICATIONS

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MICROBIOLOGY RESEARCH ADVANCES

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BIOCHEMISTRY, MECHANISMS OF ACTION AND BIOTECHNOLOGICAL IMPLICATIONS

NIKOLAOS LABROU
AND
EMMANOUIL FLEMETAKIS
EDITORS

New York

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Additional color graphics may be available in the e-book version of this book.

Library of Congress Cataloging-in-Publication Data

ISBN: 978-1-62417-503-9 (eBook)

Library of Congress Control Number: 2012954195

Published by Nova Science Publishers, Inc. † New York

Contents

Preface		**ix**
Part I:	**Overviews**	**1**
Chapter I	Glutathione: Biosynthesis and Mechanism of Action *Érika Cristina Pavarino, Anelise Russo, Ana Lívia Silva Galbiatti, Wanda Pereira Almeida and Eny Maria Goloni Bertollo*	**3**
Chapter II	Central Functions of Glutathione, and Peptides and Amino Acids Related to Glutathione Synthesis and Metabolism, on Stress Responses *Mitsuhiro Furuse and D. Michael Denbow*	**35**
Chapter III	Glutathione: Disease-related Changes and Biomarkers of Oxidative Stress *Hugo Juárez Olguín and David Calderón Guzmán*	**55**
Part II:	**Glutathione in Mammalian Cell and Clinical Implications**	**77**
Chapter IV	Glutathione Studies and its Applicability in Animal Reproduction: An Alternative Therapy against Infertility? *Maria Madalena Pessoa Guerra, Sildivane Valcácia Silva and Diogo Ribeiro Câmara*	**79**
Chapter V	The Significance of Astrocytic Glutathione System in Neuroprotection: A Potential Role for Curcumin *Jagdeep K. Sandhu, Caroline Sodja, Angele Byrd, Christian Cadieux, Patricia Lanthier, P. Roy Walker and Marianna Sikorska*	**95**
Chapter VI	N-Acetylcysteine Amide (NACA), a Novel GSH Prodrug: Its Metabolism and Implications in Health *Shakila Tobwala and Nuran Ercal*	**111**

Chapter VII Role of Glutathione in Infection and Cardiovascular Diseases **143**
Dennis Gray, Beatrice Saviola, Frederick Guilford, Melissa Khurasany and Vishwanath Venketaraman

Chapter VIII Glutathione and Glutathione S-Transferases: Risk Factors in Multifactorial Diseases **165**
Ana Cristina Ramalhinho and Luiza Breitenfeld

Chapter IX Structure and Catalytic Properties of Human Glutathione Transferase P1-1 **187**
Evangelia Chronopoulou, Marianna Chatzikonstantinou, Panagiotis Madesis, Irini Nianiou-Obeidat and Nikolaos E. Labrou

Chapter X Risk of Cancer Associated with Measures of Placental Glutathione S-Transferase (GST-P 7-7): A Comprehensive Review **199**
Daniel Araki Ribeiro, Juliana Noguti, Augusto César, Camilo Dias Seabra Pereira, Rodrigo Brasil Choueri, Carla Cristina Medalha, Cristina dos Santos Cardoso de Sá, Rogério Aparecido Dedivits, Luis Fernando Barbisan and Regina Celia Spadari-Bratfisch

Chapter XI Roles of Glutathione S-Transferases in Cancer and Aging: A Friend or Foe? **207**
Misa Imai, Megumu Ito, Junwen Qin, Naomi Yamakawa and Yuji Takahashi

Chapter XII Glutathione S-Transferase Gene Polymorphisms in Multiple Myeloma **223**
Judit Várkonyi,Lívia Jánoskúti, Dóra Szakály, Barbara Újvári, Pál Pánczél, Nóra Hosszúfalusi, Peter Farkas, Gábor Mikala, Attila Tordai, Bernadette Schoket and István Karádi

Part III: Glutathione in Plants **231**

Chapter XIII Glutathione/Ascorbate Cycle and Its Interrelation with Lipoic Acid: Could It Explain Why Plants Do Not Get Cancer? **233**
Cristina Sgherri and Usue Pérez-López

Chapter XIV The Use of Highly Specific GSTs towards the Development of Stress Tolerant Transgenic Plants **263**
Panagiotis Madesis, Irini Nianiou-Obeidat, Nikolaos E. Labrou and Athanasios Tsaftaris

Chapter XV Glutathione: An Important Player in Plant-Microbe Interactions **275**
Sotirios E. Tjamos and Emmanouil Flemetakis

Part IV: Glutathione in Microorganisms **293**

Chapter XVI Glutathione Metabolism in Protozoan Parasites: Making the Difference **295**
Diego G. Arias, Vanina E. Márquez, Matías S. Cabeza, Alberto A. Iglesias and Sergio A. Guerrero

Chapter XVII Glutathione and Programmed Cell Death in Kluyveromyces Lactis **327**
Ana García-Leiro, Andreia S. Fernandes, Ana Castro, Arnaldo Videira, M. Esperanza Cerdán and M. Isabel González-Siso

Chapter XVIII Biotechnological Production of Glutathione in Yeast **341**
Zhi-Qiang Xiong, Mei-Jin Guo and Ying-Ping Zhuang

Index **355**

Preface

Glutathione (γ-glutamyl-cysteinyl-glycine; GSH) is the most abundant low-molecular-weight thiol found in both prokaryotic and eukaryotic organisms. GSH has potent electron-donating capacity. Its high redox potential renders GSH both a potent antioxidant and a conventional cofactor for enzymatic reactions that require readily available electron pairs. GSH participates in many cellular functions such as redox control, stress defence, drug detoxification and cell signalling including regulation of DNA and protein synthesis, gene expression, apoptosis and cell proliferation. GSH concentration in the cell is homeostatically controlled, being continually self-adjusting with respect to the balance between GSH synthesis, its recycling from GSSG and its utilization by other enzymes. Alterations in its concentration contribute to oxidative stress, which plays a key role in aging and the pathogenesis of many diseases associated with redox processes modulation.

The field of 'glutathione' research have become increasingly important and it is growing at a constant rate. This is emphasized by the increasing number of publications on this subject. For example, analysis of the articles in the scientific literature published during the past twenty years (1991 and 2011), using the Scopus (www.scopus.com) bibliographic database, showed that the publication rate of 'glutathione'-related articles has increased steadily during the past two decades. In particular, the anual number of published articles has raised from 2,228 in 1991 to about 8,234 in 2011, with an annual rate of about 263 articles/year (R^2 0.989) (Figure 1). The total number of articles that have been published between 1991-2011 have reached the impressive number of 130,417.

It is impossible for a single book volume to cover all of the different areas of glutathione research in which scientists have made significant progress. Thus, we have selected a few key-examples covering a wide range of diverge scientific disciplines and state-off-the-art experimental approaches, in order to provide the reader with a representative sample of current status of the field.

This book consists of sixteen reviews and two research chapters. Each chapter mainly discusses cutting-edge developments and deals with the information gained over the last few years on the physiological, biochemical and molecular roles of the GSH and its significance in different organisms and cells. Therefore, the book is divided in three sections each of which dealing with the biological processes that glutathione is involved in mammalian, microbial and plant cell.

The present book would definitely be an ideal source of scientific information to the advanced students, junior researchers, and scientists involved in health and plant sciences, cellular and molecular biology, biochemistry, biotechnology and other related areas.

We sincerely hope that the readers will enjoy the information provided in this issue, and find its contents interesting and scientifically stimulating. We also hope that we have established a successful compilation of chapters within the exciting area of glutathione research. Finally, we would like to thank the authors for taking the time to contribute articles to this book.

Dr. Nikolaos Labrou and Dr. Emmanouil Flemetakis
Editors

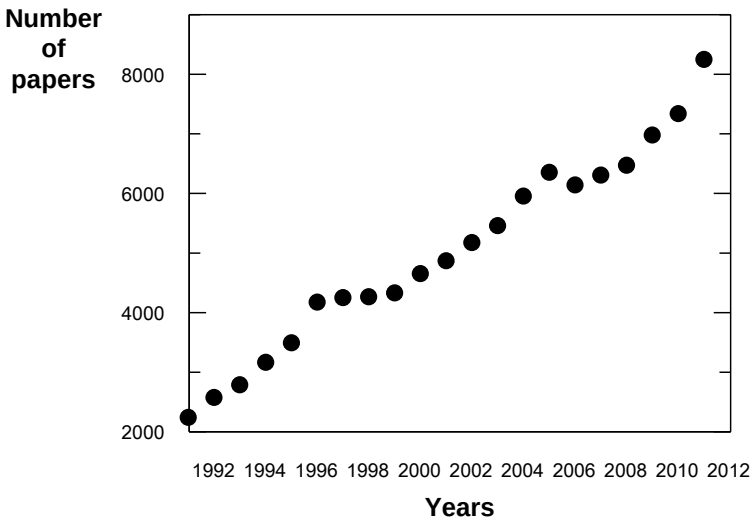

Figure 1. Publication rate of the ‘glutathione’-related articles in biomedical literature between 1991-2011. The analysis was achieved using the Scopus (www.scopus.com) bibliographic database. The figure shows the total number of articles published per year that contain the key word ‘glutathione’ at their titles or abstracts.

Part I: Overviews

In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter I

Glutathione: Biosynthesis and Mechanism of Action

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Abstract

Glutathione (GSH) is an antioxidant composted of three amino acids - cysteine, glycine, and glutamate and can be found in virtually every cell of the human body. Its biosynthesis occurs intracellularly (except in epithelial cells), and the highest concentration of glutathione is in the liver cells, making it critical in the body's detoxification process. The Glutathione has a central role in biotransformation and elimination of xenobiotics and protects cells against oxidative stress. According to catalytic cycle we can identify the activity of three groups of enzymes: glutathione oxidase (GO), glutathione peroxidase (GSH-Px) and glutathione reductase (GR). The first two enzymes, GO and GSH-Px, catalyze the oxidation of GSH to GSSG and the last, GR, is responsible for the regeneration of GSH from GSSG in the presence of NADPH. The glutathione transferases (GSTs), also known historically as glutathione S-transferases comprise a family of multifunctional enzymes that catalyze the nucleophilic attack of reduced glutathione (GR) to compounds that have a carbon, a nitrogen or a sulfur atom electrophilic. The GSTs of mammals can be divided into three large families: cytosolic GST, mitochondrial GST and microsomal GST.

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The mammalian cytosolic GSTs denomination are based on the similarity of the amino acid sequence, in seven classes (designated by Greek letters) Alpha, Mu, Pi, Sigma, Theta, Omega and Zeta. The GST-mitochondrial is composed by the class of Kappa.

The GSTs enzymes act in one of the phases of the xenobiotic-metabolizing and have two main types of enzymes: the oxidative metabolism or Phase I mediated by cytochrome P450 (CYPs) enzymes and Microsomal epoxide hydrolase (mEHs) that convert many compounds to highly reactive metabolites in carcinogenic; and the enzyme conjugate or Phase II reactions that involves GSTs among other enzyme families that act inactivating the products of Phase I. There are the phase III biotransformation that may activate membrane transporters that function to shuttle drugs and other xenobiotics across cellular membranes.

Alterations in genes that encode enzymes involved in xenobiotic metabolism can modify the function of these enzyme resulting in incorrect activation or detoxification of smoke and alcohol metabolites and this can influence several types of cancer developing, including bladder, testis, prostate, lung, breast, esophageal, liver, colorectal and head and neck cancer.

Several human GST are polymorphic, containing variant alleles with altered enzyme function. Their ability to conjugate electrophiles makes these enzymes critical in the detoxification of a wide range of epoxides and certain other agents of environmental concern, including pesticides, therapeutic drugs such as chemotherapeutic agents, or dietary components.

Abbreviations

A:	adenine
ABC:	ATP-binding cassette
ADP:	adenosine diphosphate
AhR:	nuclear receptor
Ala:	alanine
ARDS:	acute respiratory distress syndrome
ATP:	adenosine triphosphate
C:	citosine
CAR:	constitutive androstane receptor
CAT:	catalase
cGST:	cytosolic glutathione S-transferase
COPD:	chronic obstructive pulmonary diseases
CYPs:	cytochrome P450 family enzymes
Cys:	cysteine
DN:	diabetic nephropathy
DNA:	desoxirribonucleic acid
FAD:	flavin-adenine-dinucleotide
FADH:	flavin-adenine-dinucleotide phosphate-oxidase
G:	guanine
Glu:	glutamate
Gly:	glycine
GO:	glutathione oxidase

GPX: glutathione peroxidase
GR: glutathione reductase
GSH: glutathione
GSHR: mixed disulfides of GSH
GSO_2H:glutathione sulfinic acid
GSO_3H:glutathione sulfonic acid
GSSG: glutathione disulfide
$GSSO_3H$: S-sulfoglutathione
GST: glutathione S-transferase
GSTA: glutathione S-transferase alpha
GSTD: glutathione S-transferase delta
GSTK: glutathione S-transferase kappa
GSTM: glutathione S-transferase mu
GSTO: glutathione S-transferase omega
GSTP: glutathione S-transferase pi
GSTS: glutathione S-transferase sigma
GSTs: glutathione transferases
GSTT: glutathione S-transferase theta
GSTZ: glutathione S-transferase zeta
H_2O: water
H_2O_2: hydrogen peroxide
His: histidine
HIV: Human Papiloma Virus
HNSCC:head and neck squamous cell carcinoma
Ile: isoleucine
kDa: kilodalton
MAPEG:membrane-associated proteins in eicosanoid and glutathione metabolism
MDRs: multiple drug resistances
mEHs: microsomal epoxide hydrolase enzymes
MGST: microsomal glutathione S-transferase
mGST: mitochondrial glutathione S-transferase
NADP+:nicotinamide adenine dinucleotide phosphate
NADPH:nicotinamide adenine dinucleotide phosphate-oxidase
NATs: N-acetyltransferases
NSAIDs:non-steroidal anti-inflammatory drugs
OATPs: organic anion-transporting polypeptides
OATs: organic anion transporters
OCTs: organic cation transporters
OH: hydroxyl
PAHs: polycyclic Aromatic Hydrocarbons
PXR: pregnane X receptor
ROS/RNS:reactive oxygen and nitrogen species
ROS: Reactive oxygen species
SLTs: sulfotransferase enzyme
SNPs: single nucleotide polymorphisms
SOD: superoxide dismutase

T: tirosine
TPMTs: thiopurine S-methyltransferases
Tyr: tyrosine
UDP: uridine 5'-diphospho-glucuronosyltransferase
UGTs: UDP-glucuronosyltransferases
Val: valine

1. Introduction

Glutathione (GSH) is a non tripeptide thiol compound found in both prokaryotes and eukaryotes, except for some organisms which use different thiol cofactors [1]. Also known as "super defender" or "master antioxidant anioxidanty", it participates in the detoxification of reactive oxygen species (ROS) and xenobiotics, plays a major role in cell proliferation and death, DNA synthesis and repair, regulation of protein synthesis, prostaglandin synthesis, amino acid transport and enzyme activation. Moreover GSH mantains essential thiol status, regulates immune functions and the regulation of pathways essential for whole body homeostasis [2-4]. The biosynthesis and catabolism of GSH occurs by a regulated series of enzymatic and plasma membrane transport steps that are collectively referred to the γ-glutamyl cycle [5].

There are numerous associations involving cellular GSH dishomeostase and some pathological states and the interface between GSH certain signaling pathways that are key underlying regulators of cellular responses to many stresses. These alterations include upregulation or downregulation of GSH biosynthesis and changes in membrane transporters that play roles in cellular and tissue distribution of GSH. Despite the many roles played it has been difficult to ascribe causal relationships between changes in GSH levels or redox state and development of disease [6].

The importance of GSH to human diseases is perhaps best illustrated by the multitude of disorders that are observed in patients with errors in GSH metabolism. Glutathione deficiency contributes to oxidative stress, and, therefore, may play a key role in aging and the pathogenesis of many diseases [7]. Studies on GSH metabolic disorders have led to the free radical theory of human diseases and to the advancement of nutritional therapies to improve GSH status under various pathological conditions [8,9]. Thus, good knowledge regarding GSH biosynthesis is critical for the development of effective therapeutic strategies to prevent and treat a wide array of human diseases, including cancer [10]. It may in the future be considered as important to health as an alkaline diet, exercise and lifestyle [11,12].

2. Biochemistry, Biosynthesis and Mechanisms of Action

Glutathione (GSH- the tripeptide γ-L-glutamyl-L-cysteinylglycine) is the major antioxidant agent of the body responsible for the inactivation of various metabolites, and it has a central role in the biotransformation and elimination of xenobiotics and the protection of

cells against oxidative stress [3,13,14]. In addition, glutathione is essential for cell proliferation [15-16] and maintaining the thiol redox potential in cells by keeping the sulfhydryl groups of proteins in the reduced form [4, 17]. GSH is the major non-protein thiol in tissues and has been accepted as one of the major determinants of cellular redox homeostasis. The roles of GSH include providing a mobile pool of reduced sulfur, assisting in the detoxification of xenobiotics, protecting against heavy metal toxicity, source of reductant in enzymatic reactions, affecting growth and development, regulating gene expression, and aiding in the resistance to pathogen infection and the tolerance to environmental perturbations that promote oxidative stress [18]. Moreover, studies suggest that GSH plays a role in the regulation of apoptosis [19].

GSH is an antioxidant found in every cell of the human body (Figure 1). Glutathione is the major cellular thiol and is present in mammalian cells at concentrations up to 12 mM. The functions of GSH include the regulation of GSH turnover, maintenance of the thiol redox status of the cell, acid biosynthesis, metal transport and excretion [2, 20].

GSH biosynthesis occurs intracellularly (except in epithelial cells), and the highest concentration of the glutathione is present in liver cells, where GSH aids the body's detoxification process. GSH is synthesized by the consecutive actions of two enzymes, *γ*-glutamylcysteine synthetase (reaction 1) and GSH synthetase (reaction 2), that is Mg+2 and ATP-dependent [21].

$$\text{L-Glu} + \text{L-Cys} + \text{ATP} \xrightarrow{E_1} \text{L-Glu—L-Cys} + \text{ADP} + \text{Pi}$$

$$\text{L-Glu—L-Cys} + \text{Gly} + \text{ATP} \xrightarrow{E_2} \text{GSH} + \text{ADP} + \text{Pi}$$

First, cysteine and glutamate are combined by γ-glutamylcysteine synthetase (reaction 1) to generate L-*γ*-glutamylcysteine-L-cysteine. This enzyme is regulated by GSH synthetase, which prevents excessive GSH production. The L-*γ*-glutamylcysteine-L-cysteine may be transported into the cells or may be reduced to cysteine and *γ*-glutamylcysteine. The *γ*-glutamylcysteine can be used directly by GSH synthetase (reaction 2) to produce GSH. However, if the activity of *γ*-glutamylcysteine synthetase is impaired, *γ*-glutamylcysteine can be converted to 5-oxoproline, a cyclic form of glutamate, by γ-glutamylcyclotransferase [22].

In the second reaction (2), GSH synthetase catalyzes the ATP-dependent formation of GSH from *γ*-glutamylcysteine [21,22]. Due to an increase in glutathione levels, GSH synthesis is self-regulated; otherwise, cysteine availability is usually rate-limiting. Fasting, protein-energy malnutrition, or other dietary amino acid deficiencies limit glutathione synthesis. After synthesis, intracellular GSH is exported from most cells [22,23].

Once outside of the cell, the *γ*-glutamyl-cysteine bond of GSH may be hydrolyzed by *γ*-glutamyl transpeptidase. The *γ*-glutamyl transpeptidase may function in translocation, and *γ*-glutamylcysteine synthetase and GSH- synthetase may catalyze energy-requiring recovery steps in transport. These and other considerations suggest that GSH may be a carrier in amino acid transport by other cells. This process requires two enzymes commonly found on the cell surface, *γ*-glutamyl transpeptidase and cysteinylglycine dipeptidase. The enzyme *γ*-glutamyl transpeptidase transfers a glutamate to other amino acids, releasing cysteinylglycine, which in turn can be broken by a dipeptidase to produce cysteine and glycine. Cysteine and glycine as

well as γ-glutamyl amino acids are then taken into cells by specific amino acid transporters and used for GSH biosynthesis [24].

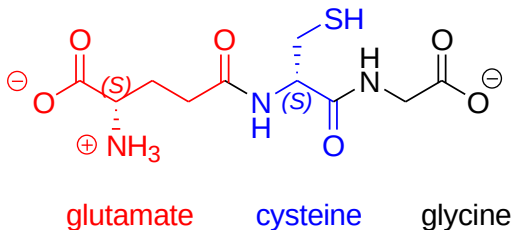

Figure 1. Glutathione structure: a tripeptide formed by glutamate, cysteine and glycine.

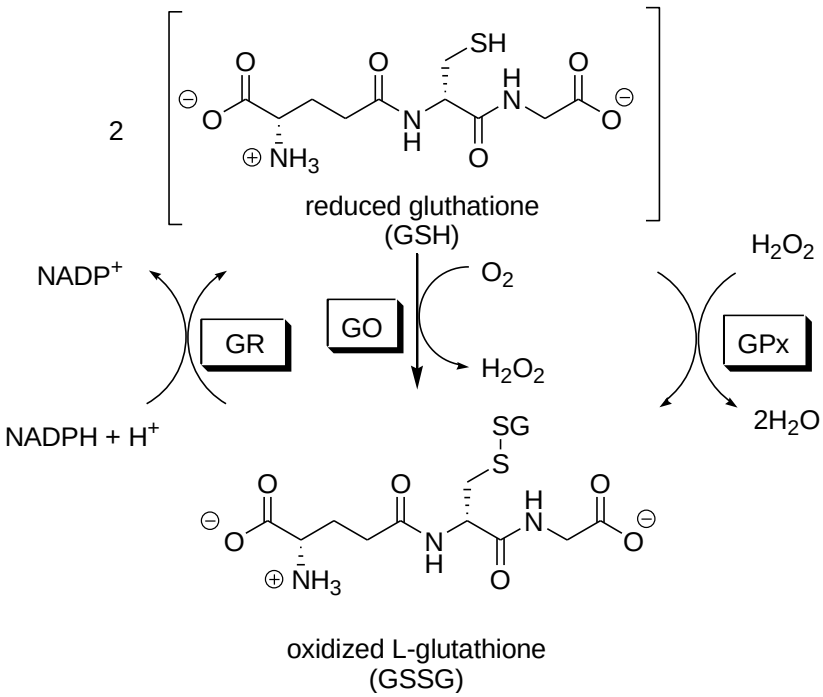

Figure 2. Interconversion of GSH in its reduced and oxidized forms by the action of the GSH peroxidase (GPx), GSH reductase (GSH – GR) and GSH oxidase (GSSG) enzymes.

GSH may be used as an electron donor in reactions involving GSH peroxidase (GPx) or oxidized under the GSH oxidase (GO) catalysis and then reduced by GSH reductase (GR) using nicotinemide adenine dinucleotide phosphate (NADPH) as electron-donor. GSH may also be conjugated with xenobiotic compounds by glutathione transferases (GST), known historically as glutathione S-transferases, a family of multifunctional enzymes that catalyze the nucleophilic attack of GSH on compounds that have an electrophilic carbon, nitrogen or sulfur atom (Figure 2) [21].

Biosynthesized glutathione is distributed in other intracellular compartments, including the mitochondria, endoplasmic reticulum, nucleus, and the extracellular space (e.g., blood plasma and bile), for utilization by other cells and tissues. GSH transport into the mitochondria appears to be mediated in part by the dicarboxylate carrier (DIC, Slc25a10) and

the oxoglutarate carrier (OGC, Slc25a11) [25], whereas transport into the nucleus probably occurs by passive diffusion through the nuclear pores. The mechanism of GSH transport into the endoplasmic reticulum remains undefined, although a role for the ryanodine receptor channel in skeletal muscle sarcoplasmic reticulum membranes has been suggested [26].

2.1. Glutathione and Related Enzymes - Catalytic cycle of GSH

Three groups of enzymes can be identified in the GSH catalytic cycle: glutathione oxidase (GO), glutathione peroxidase (GPx) and glutathione reductase (GR). GO and GPx catalyze the oxidation of GSH to GSH disulfide (GSSG) while GR is responsible for the regeneration of GSH from GSSG in an NADPH-dependent process [21].

Cells can excrete GSSG or convert it to GSH at the expense of NADPH through the action of GSH reductase. However, the *de novo* synthesis of GSH from its amino acid constituents is essential for the elevation of GSH that occurs as an adaptive response to oxidative stress. Both endogenous as well as exogenous oxidants require hours to significantly affect GSH levels in the majority of cells in a given population [4,27]. The presence of the sulfhydryl group in GSH allows it to serve as an antioxidant, protecting against free radicals and other oxidants [17].

Glutathione Oxidase (GO)

GSH is converted to GSSG by the action of GO, an FAD-dependent enzyme that also oxidizes other aminothiols, such as L-cysteine. Disulfides are formed from low molecular weight thiols, polypeptides and proteins. The designation of GSSG as oxidized glutathione is not specific because mixed disulfides of GSH (GSHR), glutathione sulfinic acid (GSO_2H), glutathione sulfonic acid (GSO_3H) and S-sulfoglutathione (GSSO3H) are all oxidized forms of GSH as well [21].

The GOs belong to the flavin-dependent sulfhydryl oxidase class that catalyzes the oxidation of proteins and peptides with the formation of disulfide and hydrogen peroxide:

$$2GSH + O_2 \rightarrow GSSG + H_2O_2$$

The first GO enzyme was characterized by Ostrowski and Kistler as a monomer [28]. Studies have shown that this enzyme has a molecular mass of 66 kDa. This enzyme exhibited no specificity for GSH and could also oxidize other aminothiols. Among the known GOs, only GOT-1, obtained from cultures of Basidiomycetes, is specific for GSH [29]. The main interest in GO is in the development of biosensors based on GSH [30].

GSH Peroxidase (GPx)

The main enzyme defense system against free radicals involves GPx, which is found specifically in the cytoplasm of many mammalian cells and is more abundant in the liver,

kidney and erythrocytes. The active site of GPx is peculiar because it contains a selenocysteine residue [31,32] that uses GSH as an electron donor to reduce hydrogen peroxide (H_2O_2) to water (H_2O). GPx catalyzes the reaction using GSH as a cofactor [6,33-35].

Most cellular glutathione peroxidases are 80 kDa proteins consisting of four identical subunits. Five isoforms of GPx have been well characterized in mammals with a tissue-specific distribution. Changes in the levels of this enzyme are associated with some diseases [9], such as hepatitis [36], HIV [37] and many cancers, including skin [38], kidney [39], bowel [40] and breast cancers [41].

A high level of GSH and/or GPx increases the antioxidant potential. These levels have been observed to increase in many tumor cells by an unknown mechanism [6]. In addition, a decrease in the intracellular concentrations of GSH and/or GPx in experimental systems is followed by an increase in the sensitivity of cancer cells to ionizing radiation and certain chemotherapy drugs [21]. Therefore, the presence of these proteins in neoplastic cells may characterize the low response to cytotoxic treatments [35] and has been exploited for clinical use in cancer therapy [21].

The role of GPx in the control of phospholipidic hydroperoxide levels has been extensively studied since the 60 decade [42] along with the kinetic parameters of the mechanism by which GSH and H_2O_2 participate in the GPx cycle [21,43].

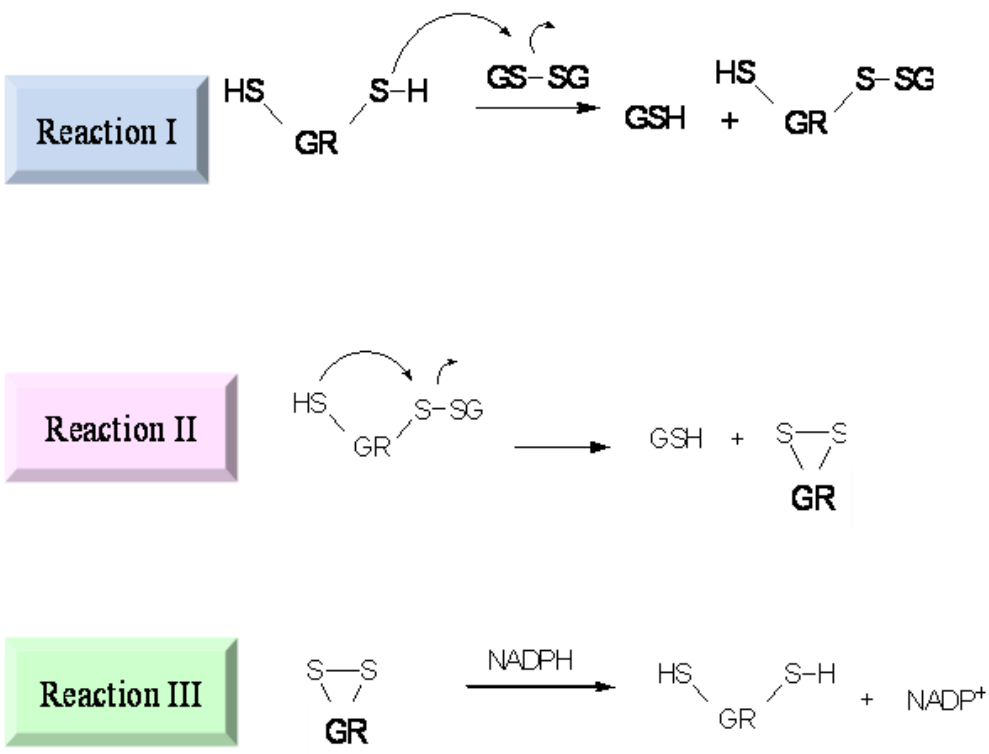

Figure 3. Schematic representation of the GSSG reduction cycle catalyzed by GR. Adapted from Huber et al., 2008.

GSH Reductase (GR)

The enzyme responsible for the reduction of GSSG to GSH is GR. This enzyme is a member of the flavoprotein disulfide-oxidoreductase family and exists as a dimer [44]. The binding sites for GSSG are located at the interface between the two subunits that form the quaternary structure of GR, and each subunit has a distinct domain with a binding site for NADPH.

The steps of the redox mechanism mediated by GR are schematically depicted in Figure 3. The thiol group of a cysteine residue present in the active site of GR attacks GSSG, resulting in a mixed disulfide on GR and the release of a molecule of GSH (reaction I). An intramolecular attack of the thiol group of the second cysteine residue produces the second molecule of GSH, forming the cyclic disulfide GR (reaction II). The conversion of NADPH to $NADP^+$ facilitates the cleavage of the S-S bond of the cyclic disulfide GR (reaction III), restarting the reductive cycle [21].

The active site of GR is characterized by the presence of a disulfide bond formed by Cys58 and Cys63. The site also contains active tyrosine (Tyr197), glutamine (Glu427) and histidine (His467) residues [21]. Figure 4 is a schematic representation of the GR active site containing the alloxazolinic ring of the coenzyme FAD. In the first stage (a), the coenzyme is reduced by hydride transfer from NADPH to the flavin dinucleotide. This step requires a conformational change of the tyrosine residue. The resulting FAD anion transfers a pair of electrons to the disulfide system (Cys58-Cys63) at the Cys63 residue (b). The anionic species generated at the Cys58 residue is protonated to give a thiol group stabilized by His467 (c) that attacks the S-S bond of GSSG, leading to the mixed disulfide enzyme-S-SG. A molecule of GSH is then released. A second molecule of GSH is released when the covalent Cys63-FAD bond is cleaved (d). The regeneration of the Cys58-Cys63 disulfide completes the catalytic cycle [21].

Under conditions of oxidative stress, GR is regulated at the level of transcription as well as by posttranslational modifications. Alterations in GR expression and activity have been implicated in cancer and aging [44-46].

GSH Transferases (GSTs)

The GSH transferases (GSTs), also known historically as GSH S-transferases, comprise a family of multifunctional enzymes that catalyze the nucleophilic attack of reduced GSH on compounds that have an electrophilic carbon, nitrogen or sulfur atom [47,48].

GSTs are generally found in biological systems as homo- or heterodimers, although other complexes may also exist, and contain two active sites per dimer whose activities are independent of one another [49]. Each active site consists of at least two binding regions, one for GSH that is highly specific for this tripeptide and a less specific binding site for electrophilic compounds [21,49]. The most common electrophilic substrates of the GSTs include alkyl halides, epoxides, α,β-unsaturated compounds (such as quinones, iminoquinones, aldehydes, ketones, lactones and esters), aryl halides and nitro aromatics [21,50,51,52].

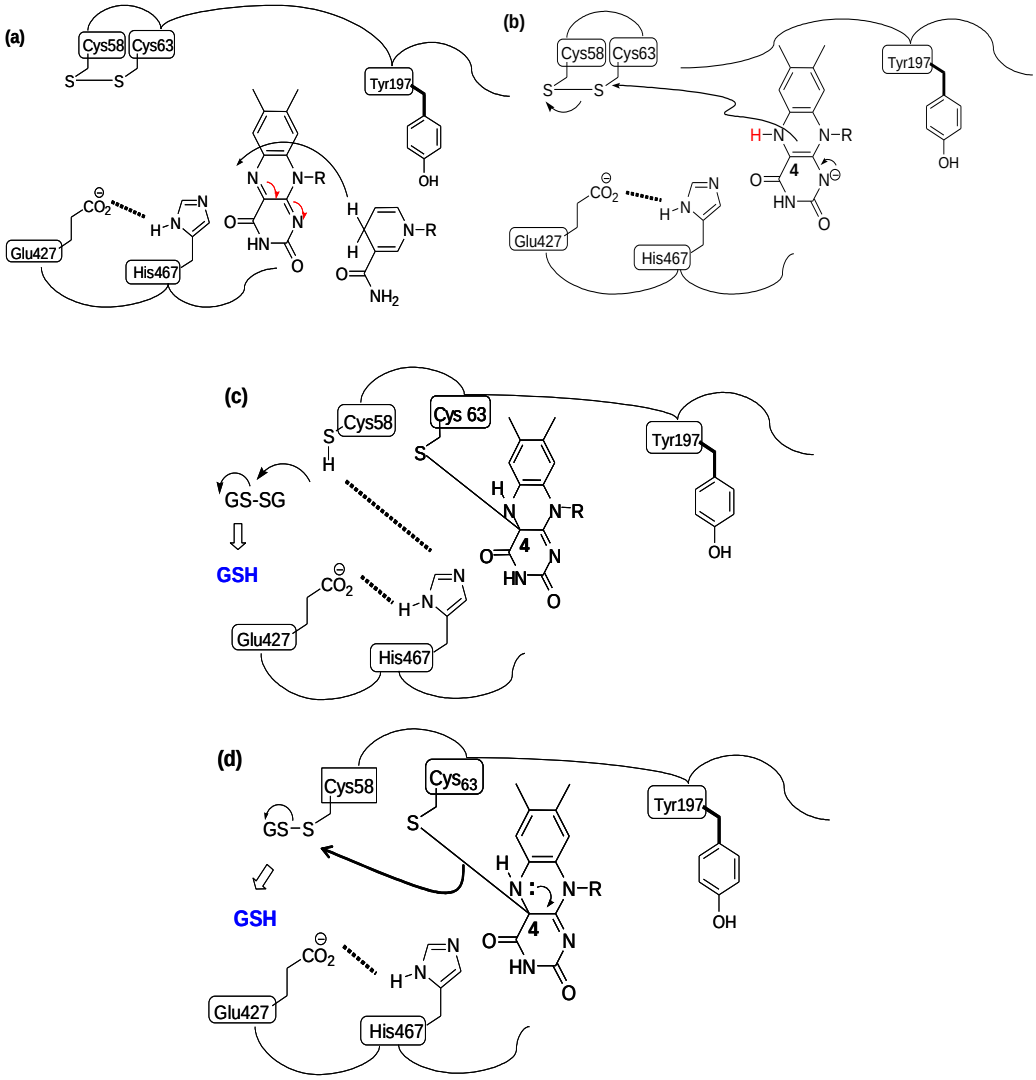

Figure 4. Schematic representation of the steps involved in the formation of GSH. (a) GR active site with the isoalloxazolinic ring of FAD and its reduction by NADPH; (b) hydride transfer from FADH to the Cys58-Cys63 disulfide; (c) nucleophilic attack of the thiol group (Cys63) on GSSG, release of GSH and formation of the mixed disulfide; (d) regeneration of Cys58-Cys63 and FAD and release of a second molecule of GSH (Huber et al., 2008 with permission of the Brazilian Chemical Society).

In recent years, a wide array of GST functions have received increasing attention, including the role of GST in (1) conjugating endogenous electrophilic compounds such as bilirubin, steroids or thyroid hormones via non-covalent GSH-dependent binding to GST [53,54], (2) conjugating with the metabolites of xenobiotics (Figure 5), such as epoxides derived from polycyclic aromatic hydrocarbons (PAHs) and α, β-unsaturated ketones, in phase II of xenobiotic metabolism, (3) maintaining the intracellular redox status, and (4) synthesizing and modifying leukotrienes and prostaglandins [55,56].

The GSTs of mammals can be divided into three large families: cytosolic GST, mitochondrial GST and microsomal GST [51,52,56,58]. The third family is now known as membrane-associated proteins in eicosanoid and GSH metabolism (MAPEG) [59].

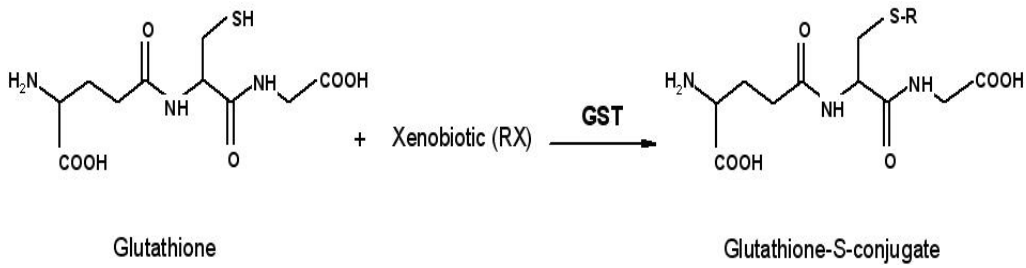

Figure 5. Scheme of the enzymatic reaction catalyzed by GSH S-transferase with the formation of a GSH conjugate (Figure courtesy of Jancova et al., 2010).

The mammalian cytosolic GSTs are all dimers and are divided into nine classes (designated by Greek letters) based on their amino acid sequence similarities. GSTs are divided into soluble cytosolic [cGST: alpha, mu, pi, omega, theta, sigma, zeta, [47,51,52,55,56,60,61] and delta] in microbes, animals and plants [62]; mitochondrial GST; [mGST: a, l, p and kappa] [55]; and the structurally distinct membrane-bound microsomal GST (MGST) [62].

Human *GST* genes are located on several chromosomes: *GSTA* on 6, *GSTM* on 1, *GSTP* on 11, *GSTS* on 4, *GSTT* on 22, *GSTZ* on 14, *GSTO* on 10 and *GSTK* on 7 (see http://www.ncbi.nlm.nih.gov/gene) [56].

GST expression can be induced by many different natural products, such as garlic [63] and plant phenols [64], common therapeutic agents like rifampicin, wich is a steroids regulate phase II xenobiotic metabolizing enzymes [65] or non-steroidal anti-inflammatory drugs (NSAIDs) [66]. Nuclear receptors such as pregnane X receptor (PXR), constitutive androstane receptor (CAR) or nuclear receptor (AhR) have also been suggested to be regulators of GSTs [64,67-69].

3. GSH Functions

GSH is known to have many important cellular functions due to its well-known redox capabilities, such as the maintenance and regulation of the thiol redox status of the cell, defined as the ratio of the concentration of oxidizing equivalents to that of reducing equivalents [2]. GSH plays a major role in protecting the cell against oxidants and electrophiles [16].

GSH has different functions in different cellular compartments. In mitochondria, GSH plays a key role in regulating apoptosis [6,19], so GSH depletion is necessary for apoptosis in lymphoid cells independent of ROS formation [70]. In the nucleus, GSH is a key regulator of cellular division. Although the role of nuclear GSH in the synthesis of DNA and in the protection against oxidative damage or ionizing radiation is well established, little is known about the GSH concentration in the nucleus and its possible regulation. The nucleus changes dramatically during the different phases of the cell cycle. Thus, studies aimed at the determination of the nuclear GSH distribution must take cell cycle physiology into account [15,16].

3.1. Oxidative Stress

Oxidative stress occurs due to a disturbance in the balance between reactive oxygen species (ROS) and in antioxidant defenses and is important in the pathophysiology of several types of disease, including cancer [71].

Oxidative stress is defined as an imbalance between the formation and removal of ROS in the body, due to the reduction of endogenous antioxidants or the increased generation of ROS, that generates a pro-oxidant state favoring the occurrence of oxidative damage to macromolecules and cellular structures, possibly resulting in a loss of cellular functions that ultimately leads to cell death [72]. In addition, oxidative stress is known to cause DNA damage and mutations in the tumor suppressor genes important for the early events of carcinogenesis [73].

An updated and improved definition of oxidative stress has recently been introduced by H. Sies and D. P. Jones in the "Encyclopedia of Stress", 2nd ed., G. Fink, Ed. (2007) as "an imbalance between oxidants and antioxidants for the oxidants, leading to a disruption of redox signaling and control and/or molecular damage" [74,75].

The homeostasis between ROS formation and depletion is maintained by antioxidant molecules and proteins that remove ROS and reconstitute the redox balance [34]. The main antioxidant enzymes in the cell include superoxide dismutase (SOD), which catalyzes the conversion of superoxide to hydrogen peroxide, catalase (CAT), which reduces H_2O_2 to water and oxygen, and glutathione peroxidase 1 (GPX1), which catalyzes the reduction of H_2O_2 by using GSH as a cofactor [6,33-35].

For the antioxidant activity of GSH to be maintained, GSSG must be regenerated by the action of GR. In situations where the redox system is intact, GSH will be regenerated, but in the presence of excess oxidizing agents and/or an impairment of the protective system, an imbalance will occur between the consumption of GSH and GSSG production [35,76,77].

Antioxidants have gone through a gradual transition from "Miracle Molecules" to "Marvelous Molecules" to "Physiological Molecules" [78-83]. Undoubtedly, antioxidants are vital cogs in numerous metabolic reactions and are players in redox homeostasis [84-88], but so are many other cellular molecules of equal importance. Furthermore, many antioxidants perform equally or even more important non-antioxidant functions [83,89,90]. Some examples include the roles of vitamin A in vision [91], vitamin C in collagen synthesis [92-96] and vitamin E in smooth muscle cell proliferation, endothelial dysfunction, platelet aggregation induced by a protein kinase C-dependent mechanism and tumor suppression [90,97-99].

Changes in oxidative metabolism are common in neoplastic cells [77]. These cells usually have high levels of ROS that can be enhanced by cytotoxic treatments. Many drugs can produce ROS as direct or indirect products of their biotransformation [9].

Due to the biradical nature of the oxygen molecule, which has two free electrons in its antibonding p orbitals, molecular oxygen reacts preferentially with molecules of a similar electronic configuration. As most biomolecules are not biradicals, oxygen is blocked by spin restriction from reacting with them, thus preventing major damage in cellular targets [72,100]. However, oxygen can give rise to several reactive species, either by the absorption of energy or electron transfer. The spin restriction is not present for singlet oxygen, which is a more powerful oxidant [72].

Another mechanism of ROS formation, which gives rise to the most important biological oxidants, is the unielectronic reduction of oxygen to water, in which the addition of four electrons to the oxygen molecule promotes the emergence of the superoxide radical (O_2-*), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH-*), partially reduced intermediates of molecular oxygen, according to the scheme [72] shown below:

$$O_2 \longrightarrow O_2\text{-}^* \longrightarrow H_2O_2 \longrightarrow HO\text{-}^* \longrightarrow H_2O$$

The production of ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) is a natural consequence of aerobic respiration in the body [21,101] but can result in oxygen toxicity due to the formation of ROS that can interact with various biomolecules and damage various cellular structures [102].

The OH^- radical is the most harmful to the body and often attacks molecules by hydrogen abstraction and addition to unsaturated hydrocarbons. This radical is primarily formed in the body by two mechanisms: the reaction of H_2O_2 with transition metals and the hemolysis of water (H_2O) by exposure to ionizing radiation. The intense and frequent attacks of this radical can cause DNA mutations and, consequently, lead to the development of cancer in humans within 15 to 20 years ([101,103].

Oxidative stress has been acknowledged to be a pathogenic and/or etiological factor in numerous human diseases, including cardiovascular disease, atherosclerosis, cancer, neurodegenerative diseases, asthma, diabetes mellitus, and ischemia/reperfusion [35,72,86,104-108]. Oxidative/nitrosative stress, along with its resulting damage to all of the major classes of macromolecules in cells, is involved in physiological aging and the degenerative processes that occur in age-related diseases [46,105,109-113].

Mammalian cells generally function in a reduced state, although they can produce low amounts of ROS and reactive nitrogen species (RNS) in a regulated way and use them as signaling molecules. [35,75,114-116]. Under physiological (basal) conditions, ROS/RNS, particularly hydrogen peroxide, are important intracellular signal transducers of growth factors and extracellular matrix receptors. Therefore, the second messengers that activate many downstream signaling molecules and numerous cellular processes, including gene expression, can be regulated by subtle changes in the cellular redox balance/homeostasis [75,116,117].

The excessive production of ROS/RNS and/or impairment of cellular antioxidant defenses or an imbalance between ROS/RNS and antioxidants can lead to a common pathophysiological condition known as oxidative stress, a situation in which the cellular redox homeostasis is altered [75,116].

ROS and RNS also play important roles in the regulation of cell survival. Normally, moderate levels of ROS/RNS may function as signals to promote cell proliferation and survival, whereas the sudden and prolonged presence ROS/RNS can induce cell death [118].

In addition to the antioxidants play a key role in oxidative stress protection, they also assist in the detoxification of drugs. Antioxidants produced by the body act non-enzymatically, as with GSH, or enzymatically in conjunction with the GPx and GST enzymes to assist in the biotransformation and elimination of several xenobiotics and carcinogenic agents [99,119,120]. Thus, ROS can be generated by the action of xenobiotics and as a consequence of metabolic processes [34].

3.2. Metabolism of Xenobiotics

GSTs are a class of GSH-utilizing enzymes that act in one of the phases of xenobiotic metabolism. This process occurs in two phases: the oxidative metabolism, or phase I, mediated by cytochrome P450 (CYPs) enzymes and microsomal epoxide hydrolases (mEHs) that convert many highly reactive metabolites to carcinogenic compounds (Lacko et al., 2009), and the enzyme conjugate, or phase II, reactions that involve GSTs and other enzyme families, including UDP-glucuronyltransferases (UGTs), N-acetyltransferases (NATs), sulfotransferases (SLTs) and thiopurine S-methyltransferases (TPMTs), enzymes that inactivate the products of phase I [57,121-124]. In addition, phase III of biotransformation may activate membrane transporters that function to shuttle drugs and other xenobiotics across cellular membranes [48].

Although several enzyme systems participate in the phase I metabolism of xenobiotics, the most notable pathway in this phase is likely the monooxygenation catalyzed by the CYPs and mEHs. These enzymes detoxify and/or bioactivate a vast number of xenobiotic chemicals and conduct functionalization reactions that include N- and O-dealkylation, aliphatic and aromatic hydroxylation, N- and S-oxidation, and deamination. Examples of the toxins metabolized by this system include nicotine and acetaminophen, as well as procarcinogens like benzene and polyaromatic hydrocarbons [48].

Phase II reactions include glucuronidation, sulfation, methylation, acetylation, GSH conjugation and amino acid conjugation. The products of phase II conjugations are typically more hydrophilic than the parent compounds and, therefore, can usually be more readily excreted. Specific families of phase II xenobiotic metabolizing enzymes include various methyltransferases, such as thiopurine S-methyltransferase and catechol O-methyltransferase, along with GSTs, UGTs, STs, NATs. Of note, the UGTs catalyze glucuronidation reactions, principally with electron-rich nucleophilic heteroatoms, such as O, N, or S, in aliphatic alcohols and phenols [48,125].

Phase III biotransformation is a more recently described phase that refers to active membrane transporters that shuttle drugs and other xenobiotics across cellular membranes. In contrast to the longstanding history of research into phase I and phase II metabolism, the study of phase III/active transport began only in 1976 with the discovery by Juliano and Ling [126] of a 170 kDa carbohydrate complex in the extracellular membranes of Chinese hamster ovary cells that functioned to modulate drug permeability. This complex was named the P-glycoprotein complex. P-glycoprotein was the initial member of what are currently known as the ATP-binding cassette (ABC) family of drug transporters. This research area rapidly gained attention, as P-glycoprotein and subsequently discovered membrane transporters were found to function in the resistance of cancer cells to chemotherapeutics, a phenotype referred to as the multiple drug resistance (MDR) phenotype [127]. The consequences of the MDR phenotype may be severe, as cells can not only develop resistance to the selective agent but can also develop cross-resistance to a broad spectrum of structurally and functionally distinct antibiotics and alkaloids. The MDR genotype-phenotype relationship is complex, with over 18 ABC genes associated with human disease [128]. In addition to the ABC transporters, other important drug/xenobiotic transporters include the organic anion transporters (OATs), which excrete substances with negative charge, the organic cation transporters (OCTs) of the SLC22A superfamily, which excrete substances with positive charge, and the organic anion-transporting polypeptides (OATPs) of the SLCO superfamily [129,130]. Undoubtedly, the

next 50 years of toxicological research will be rich with respect to the further characterization of phase III biotransformation and its defining role in xenobiotic toxicity [48].

The enzymes that metabolize certain xenobiotics, such as alcohol and components of tobaccoand alcohol, may indirectly participate in the mechanism of carcinogenesis. These enzymes strongly influence individual susceptibility to disease because they are responsible for the activation and detoxification of these compounds [124,131-133] (Figure 6). In addition, several studies have shown that the expression of the genes encoding GSTs is usually higher in tumor cells than in non-tumor cells [134,135].

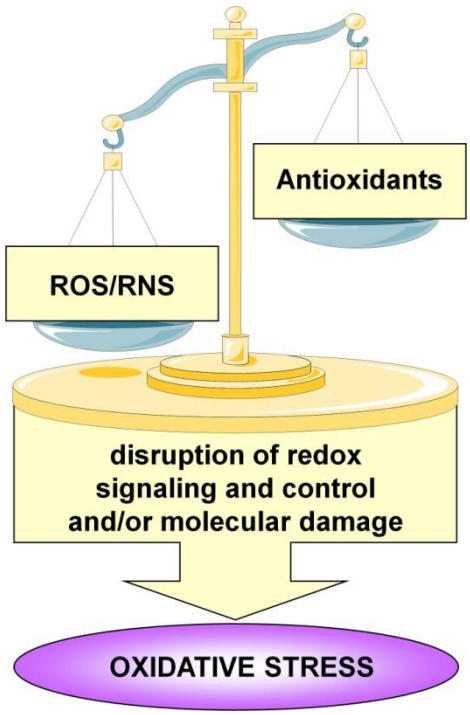

Figure 6. Updated definition of oxidative stress. H. Sies and D. P. Jones (Sies and Jones, 2007) have recently re-defined oxidative stress as “an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage” (Figure courtesy of Dalle-Donne et al., 2008).

4. Polymorphisms and Diseases

Single nucleotide polymorphisms (SNPs) are variations present in at least 1% of the population and are responsible for much of human genetic diversity. Therefore, many studies of genetic variation are focused on characterizing SNPs, which constitute about 78% of human variants. Current estimates suggest that SNPs occur as frequently as every 100-300 bases. This estimation implies that in the entire human genome, there are approximately 10 to 30 million potential SNPs, which are responsible for the majority of human genetic diversity, with approximately 52,266,495 human polymorphisms described. More than 4 million SNPs have been identified. Many of these SNPs have unknown associations [137-140].

The SNPs may be used as prognostic and predictive markers, both separately and in sets of haplotypes. In addition, several SNPs can be used to identify new disease susceptibility genes through population studies and linkage disequilibrium. They can modify translation, the splicing of exons and protein function. The identification of SNPs in noncoding regions is also important, as they may influence the level of transcription or the correct implementation of the splicing mechanism of exons [141].

Several human GSTs are polymorphic, containing variant alleles with altered enzyme function. The ability of these enzymes to conjugate electrophiles makes them critical for the detoxification of a wide range of epoxides and certain other agents of environmental concern, including pesticides, therapeutic drugs such as chemotherapeutic agents, or dietary components [142-144].

Because GST is often referred to as an antioxidant enzyme, it and its polymorphisms are associated with diseases to which oxidative stress contributes. One such disease may be diabetes. The study of patients with diabetic nephropathy (DN) revealed that patients with reduced levels of GSH and lower GST activity were double null genotypes for *GSTM1* and *GSTT1* [145]. Thus, the double null genotype population has a greater risk of DN development. Another study associated a polymorphism in these two genes with an increased risk of human male infertility [146]. In addition to these two examples, there are many other diseases associated with GSTs and their polymorphisms. Some data have shown that GST genetic polymorphisms are also closely correlated with drug effects [147], several cancers, and the outcomes of therapy for asthma, coronary heart disease, and atherosclerosis [148-153].

Polymorphisms in genes that encode enzymes involved in xenobiotic metabolism can modify the functions of these enzymes, resulting in the incorrect activation or detoxification of carcinogens, such as smoke and alcohol metabolites. Therefore, these polymorphisms can influence the development of several types of cancer [133,154-163], including bladder, testis, prostate [164,165], lung [166], breast [71,167,168], esophageal [60,169], liver [170], colorectal [171], and head and neck squamous cell carcinoma (HNSCC) [142-144,172-175].

The data found in the literature on *GSTT1* and *GSTM1* deletion polymorphisms in HNSCC, the type of cancer studied by our group, are contradictory. Some studies have shown the association of HNSCC with *GSTT1* and *GSTM1* nulls [124,142,161,176-178]. However, studies by other labs [179-183] and our research group [172,173] have not shown this association.

The association between *GSTP1* polymorphisms and cancer risk is also contradictory. The *GSTP1* gene is located on chromosomal region 11q18 and encodes an enzyme expressed in placental, spleen, heart and lung tissues. The *GSTP1* A313G and *GSTP1* C341T polymorphisms result in the Ile105Val and Ala114Val variations, respectively, and can induce significant differences in enzyme activity that lead to altered levels of DNA adducts and the increased risk of esophageal cancer and HNSCC [60,183] respectively.

The homozygous polymorphic genotypes have been associated with the increased risk for several other cancer types such as bladder, testis, prostate [165,184], lung [166], breast [167], esophageal [60,169] and HNSCC [142-144]. In addition, the variant allele associated with a smoking habit increases the risk of cancer of the esophagus [60] and oral cavity [185].

However, an increased frequency of the wild-type allele of the *GSTP1* A313G polymorphism was observed by Jain et al. [60] in patients with esophageal cancer. Therefore, the *GSTP1* 313GG genotype has been found to protect against lung cancer [186].

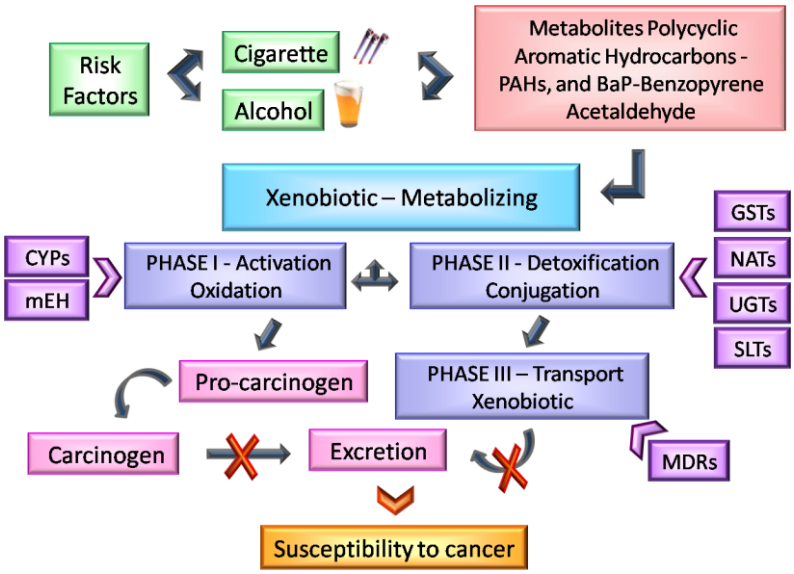

Figure 7. Representative scheme of xenobiotic metabolism and its principal enzymes – CYPs: cytochromes P450; mEH: microsomal epoxide hydrolase; NATs: N-acetyltransferases; GSTs: glutathione S-transferases; UGTs: UDP-glucuronosyltransferase; SLTs: sulfotransferases; MDRs: multiple drug resistances. Adapted from Russo et al., 2011.

With respect to HNSCC, the presence of at least one polymorphic allele of *GSTP1* A313G is associated with decreased risk of this disease, according to Singh et al. [124]. Moreover, these authors showed that the combination of the wild-type genotype 313AA *GSTP1* with the *GSTT1* and *GSTM1* null genotypes is associated with a 4-fold increase in the risk for HNSCC [124]. This result was different from the study by Soya et al. [142] that observed a 7.8-fold increase in the risk of upper aerodigestive tract cancers in individuals carrying *GSTM1* null and *GSTT1* null genotypes and 313GG *GSTP1* polymorphic variants.

Different combinations in a single individual biometabolism of *GST*, *CYP* and *mEH* polymorphisms associated with smoking and alcohol consumption may increase the risk for HNSCC. In addition, these polymorphisms also may influence the metastasis and aggressiveness of the cancer [123,125, 132,133,187,188].

5. Clinical Implications

Disturbances in GSH homeostasis, relationships between changes in GSH levels or redox state have been implicated in the etiology and/or progression of a number of human diseases, including cancer, diseases of aging, cystic fibrosis, and cardiovascular, inflammatory, immune, metabolic, and neurodegenerative diseases. It is known that GSH deficiency manifests itself largely through an increased susceptibility to oxidative stress and the resulting damage. However, elevated GSH levels generally increase the antioxidant capacity and resistance to oxidative stress, which is observed in many types of cancer cells [6,9,35,123,189-192].

A deficiency in the first and rate-limiting enzyme in GSH synthesis, namely γ-glutamylcysteine synthetase, also known as glutamate cysteine ligase, is a rare autosomal

recessive disease characterized by hemolytic anemia and, in some cases, by neurological symptoms. Some patients exhibit spinocerebellar degeneration, peripheral neuropathy, myopathy, and aminoaciduria. The second step of GSH biosynthesis is catalyzed by GSH synthetase, the deficiency of which is the most frequently recognized disorder of GSH metabolism [193,194].

Patients with a mild GSH synthetase deficiency often have mutations that affect the stability of the enzyme and display a compensated hemolytic anemia. Moderate GSH synthetase deficiency leads to hemolytic anemia and metabolic acidosis, whereas patients with a more severe deficiency also develop 5-oxoprolinuria, recurrent bacterial infections, and progressive dysfunction of the central nervous system, including mental retardation and motor functional disturbances [6]. The reason for this sensitivity is unknown. The high susceptibility of the brain to oxidative stress due to its high oxygen consumption and the possibility that GSH may be a neuromodulator or neurotransmitter and may thus be essential for central nervous system activities [6,195] have, however, been suggested to account for the effects of GSH synthetase deficiency.

The third enzyme of the GSH biosynthetic pathway is the ectoenzyme γ-glutamyl transpeptidase. Patients with a deficiency in this enzyme have increased GSH concentrations in their urine and blood plasma (glutathionuria) and display central nervous system abnormalities. The glutathionuria is due to their inability to break down the GSH that is filtered and secreted in the renal tubular fluid [6,196,197].

In addition, GSH is strongly associated with cardiovascular diseases, pulmonary diseases and inflammatory and immune system diseases [191,198,199]. Regarding cardiovascular diseases, it has been speculated that oxidative events can lead to the release of atherosclerotic plaques, thus increasing the risk of myocardial infarction and stroke [198]. Moreover, individuals of advanced age may exhibit a gradual lowering of GSH levels and of general antioxidant defenses [46], and this decline is associated with a higher incidence of age-related chronic illnesses [45]. In relation to pulmonary diseases, an imbalance in GSH homeostasis and in the thiol redox state may contribute to the etiology and/or progression of both chronic obstructive pulmonary diseases (COPD), such as emphysema and asthma, and more acute lung diseases, such as acute respiratory distress syndrome (ARDS) [191,199]. In the immune system, many correlations exist between low GSH levels and autoimmune and inflammatory diseases, including rheumatoid arthritis, systemic lupus erythematosus, Crohn’s disease, multiple sclerosis and contact dermatitis [200-204].

Although the exact mechanisms for the events caused by alterations in GSH levels are unknown, the decreased activities of γ-glutamylcysteine ligase, GSH synthetase, γ-cystathionase and glutathione reductase are thought to be contributing factors for the development of several diseases [205-207].

GSH also plays an important role in cancer development. It is now well established that ROS and electrophilic chemicals can damage DNA and that GSH can protect against this type of damage [35].

GSH can also directly detoxify carcinogens through phase II metabolism and the subsequent export of these chemicals from the cell. However, elevated GSH levels have been observed in various types of cancerous cells and solid tumors, which tends to make these cells and tissues more resistant to chemotherapy [189,208].

Conclusion

The Glutathione (GSH) is the major antioxidant agent of the body responsible for the inactivation of various metabolites, and it has a central role in the biotransformation and elimination of xenobiotics and the protection of cells against oxidative stress. In addition, GSH is essential for cell proliferation and maintaining the thiol redox potential in cells. Disturbances in GSH homeostasis, relationships between changes in GSH levels or redox state have been implicated in the etiology and/or progression of a number of human diseases, including cancer, diseases of aging, cystic fibrosis, and cardiovascular, inflammatory, immune, metabolic, and neurodegenerative diseases. Therefore, the identification of genetic variations can provide insight and new information regarding prevention or treatments decision. Furthermore, the prognostic factors with greater sensitivity and specificity may contribute to new therapeutic strategies.

Acknowledgments

Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-CAPES, Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq and FAMERP/FUNFARME.

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In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter II

Central Functions of Glutathione, and Peptides and Amino Acids Related to Glutathione Synthesis and Metabolism, on Stress Responses

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Abstract

Glutathione reductase irreversibly catalyzes the reduction from oxidized glutathione to reduced glutathione.Both oxidized glutathione and reduced glutathionedose-dependently decreased distress vocalizations and induced sleep-like behavior in chicks under acute stressful conditions. Energy expenditure was rapidly decreased by intracerebroventricular injection of reduced glutathione, but thenincreased gradually.This change in energy expenditure was correlated with behavioral changes in whichreduced glutathione induced hypnotic and sedative effects shortly after injection, followed by a period where activity increased. There are three types of dipeptides related to reduced glutathione, i.e., glutamyl-cysteine, cysteinyl-glycine and glutamyl-glycine that are theoretically present in tissues.These peptides also attenuated distress vocalizations and spontaneous activity induced by social isolationand did not vary in their action from each other.Reduced glutathione is a tripeptide which consists of L-glutamate, L-cysteine and glycine. All three amino acids induced sedative and hypnotic effects.In conclusion, glutathione and its constituents are helpful to ameliorating an acute stressful condition.

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Abbreviations

ACTH: adrenocorticotropic hormone
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
CNS:central nervous system
CO_2 production: carbon dioxide production
CRF:corticotropin-releasing factor
Cys-Gly: cysteinyl-glycine
D-Cys: D-cysteine
DHPG: (S)-3, 5-dehydroxyphenylglycine
EAAs: essential amino acids
EE: energy expenditure
GABA: γ-aminobutytic acid
γGlu-Cys:γ-glutamyl-cysteine
γGlu-Gly:γ-glutamylglycine
γGT:γ-glutamyl transpeptidase
GLP-1: glucagon-like peptide-1
Gly: glycine
GSH: reduced glutathione
GSSG: oxidized glutathione
H_2O_2: hydrogen peroxide
HPA axis: hypothalamic-pituitary-adrenal axis
iGluRs: ionotropic glutamate receptors
L-Cys: L-cysteine
L-Glu:L-glutamate
L-Ser: L-serine
mGluRs: metabotropic glutamate receptors
NMDA: *N*-methyl-*D*-aspartate
NREMS: non-REMS
O_2 consumption: oxygen consumption
REMS: rapid-eye-movement sleep
RQ: respiratory quotient
SAA: sulfur-containing amino acids
SMG: s-methylglutathione
SPS: sleep-promoting substance

1. Introduction

1.1. The Principle of “Stress”

Stress is a distortion or the cause of the distortions brought by the outside.Hans Selye (1907 - 1982) characterized the stress resonse and used the term stress to describe both medicinal and physiological changes.He proposed "the stress theory" in reference to earlier studies in which stress caused increases in adrenal and medulla hormones through an increase

in adrenocorticotropic hormone (ACTH) in blood.This response is mediated through the hypothalamic-pituitary-adrenal (HPA) axis that is activated during the stress response [79].

1.2. Daily Stress

Modern society encounters an increasing amount of stress. Excessive daily stress causes diseases affecting both the body and the mind.Mental diseases including depression, schizophrenia and post-traumatic stress disorder are attributable to stress generated from working and daily life.However, we can decrease the development of mental diseases by relieving stress through actions such as enjoying a relaxing meal, spending relaxing time with friends, etc.

Each person must develop strategies to handle stress.While our daily meals have important significance with respect to providing essential nutrieints and energy, recent research also suggests that several nutrients may function to alleviate or reduce the stress response.

Growth and reproduction are affected by stress.The adaptive stress response is characterized by both behavioral and physiological changes.The behavioral changes include increased arousal and alertness, heightened attention, and suppression of sexual and feeding behavior.

The stress response leads to redirection of energy (i.e., oxygen and nutrients) to the affected body sites as well as the central nervous system (CNS).The CNS needs relatively more oxygen and nutrients than the remainder of the body.Stress is classically defined as a nonspecific response to any demand upon the body, and is accompanied by physiological changes including activation of the pituitary-adrenal axis.This activation is dependent on corticotropin-releasing factor (CRF). CRF regulates the secretion of ACTH and is a part of the HPA axis.

Domestic chickens are precocial, as opposed to altricial, meaning they are relatively mature and mobile at hatch and can spontaneously look for food.Domestic chickens, therefore, have relatively well developed mechanisms of food intake control at hatch.As a result of genetic selection for growth in meat-type chickens, the performance of modern broiler chickens has been significantly improved.

This improvement in growth rate is largely explained by increased food intake and/or enhancement in food efficiency.As mentioned above, growth is also directly linked to the stress system.Since stress negatively affects performance, understanding the mechanisms of stress at the neonatal stage will help in developing strategies to attenuate the stress response and improve animal production.

Multiple factors are involved with the stress response, even in neonatal chicks.To reduce the stress response, the expression of stress-induced hormones and neurotransmitters should be reduced while those that reduce stress should be enhanced by genetic selection, environmental control, and dietary treatment.Further understanding of stress responses and the development of techniques for reducing stress should be required for animal health and welfare.

2. The Possibility of Glutathione to Reduce Stress Responses

The stress response has been investigated focusing on stress related peptides in neonatal chicks [20].Hovatta et al. [28] reported anxiety regulation by glyoxalase 1 and glutathione reductase 1 in mice.

As shown in Figure 1, glyoxalase 1 detoxifies dicarbonyl metabolites, and then uses glutathione as a cofactor [85].Glutathione reductase irreversibly catalyzes the reduction from oxidized glutathione (GSSG) to reduced glutathione (GSH) [15].

GSH is a tripeptide which consists of L-glutamate (L-Glu), L-cysteine (L-Cys) and glycine (Gly), and GSSG is a dimer of GSH.GSH serves to protect the brain against oxidative stress by interacting directly with reactive oxygen species or by participating in enzyme-catalyzed redox cycling reactions [12].

Glutathione acts not only as an antioxidant or disulfide-reducing agent, but also as a neurotransmitter and neuromodulator in the CNS [31].GSH and GSSG are endogenous ligands of the *N*-methyl-*D*-aspartate (NMDA) receptor, binding preferably to the Glu recognition site via their γglutamyl moiety [55].

These facts imply that central glutathione or its associated metabolites may be involved in the regulation of stress response. In this chapter, we discuss the effect of glutathione and related substances on the stress behavior in neonatal chicks.

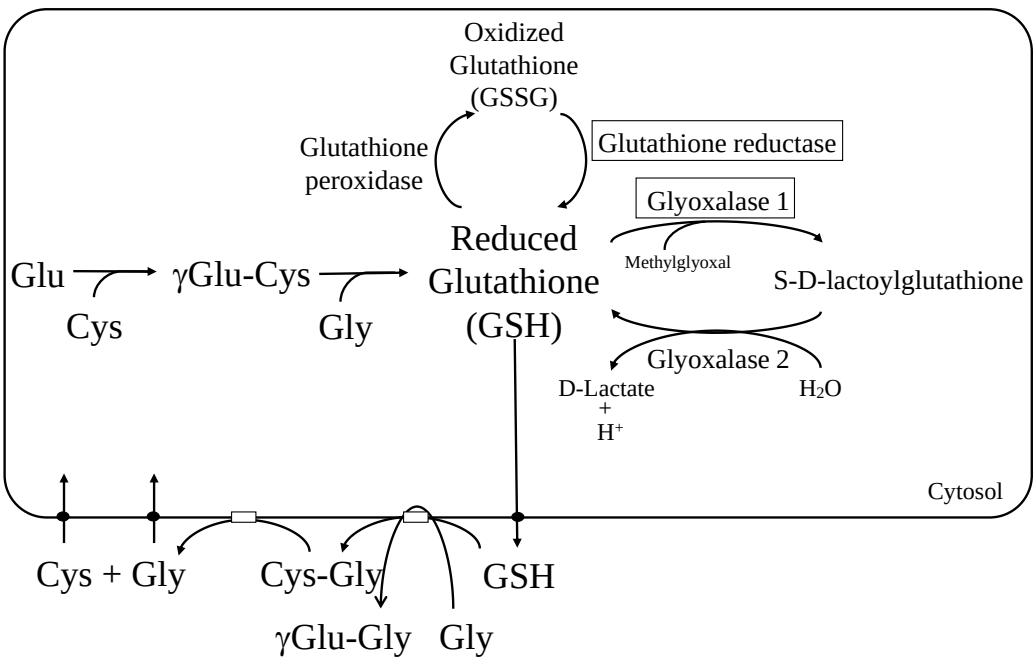

Figure 1.The pathway for the synthesis and catabolism of glatathione. GSH released to extracellular degrades γglutamy moiety and Cys-Gly via glutamyl transpeptidase. Then γglutamy moiety can combine with amino acids. Gly is described as a representation in this figure.

2.1. Intracerebroventricular (i.c.v.) Injection of Glutathione and Its Derivative Induces Sedative and Hypnotic Effects under an Acute Stress in Neonatal Chicks

Greater than 99.5% of tissue "total glutathione" (i.e., GSH and GSSG, in GSH equivalents) is in the form of GSH [1].GSH is a major protectant in the brain against oxidative stress by interacting directly with reactive oxygen species or by participating in enzyme-catalyzed redox cycling reactions [12].In the reaction of the latter, glutathione peroxidase catalyzes the reduction of potentially toxic hydrogen peroxide (H_2O_2) (or lipid peroxides; ROOH) to H_2O (or ROH) with the concomitant conversion of GSH to GSSG.

The NMDA receptor has an ion channel domain permeable to Ca^{2+} and Na^+, K^+ [50].Additionally, there are many reports about interactions of glutathione with neuronal Ca^{2+} influx.GSH caused a dose-dependent increase in Glu release from synaptosomes.GSH also induced a dose-dependent increase in Ca^{2+}, while GSSG failed to induce a Ca^{2+} increase in rat cultured hippocampal neurons [11].

In past studies, the behavior of chicks was investigated exposing chicks to stressful conditions [19, 62, 63, 68].Chicks exhibit less stress-related behavior when they are in crowds whereas isolation induces acute stress.When chicks are isolated, they express characteristic behaviors including an increase in distress vocalizations and spontaneous activity [62, 63, 81].Additionally, this model has a high utility in that chicks are relatively inexpensive to purchase and maintain.Therefore, this social separation-stress paradigm has been used for anxiolytic drug screening with vocalization and spontaneous activity as the index of the behaviors induced by the stressor.

Yamane et al. [91] investigated whether i.c.v. injection of glutathione influenced isolation-induced stress responses in neonatal chicks.First, behavioral responses after i.c.v. injection of GSH and GSSG were monitored.The response to s-methylglutathione (SMG) was also investigated.SMG is found in the brain extracellular space, and is released upon hypoxia and depolarization [35].SMG has a methylated SH group on the Cys moiety of GSH, and is not able to form a dimer, i.e., GSSG.Accordingly, SMG was used to clarify the effect of GSH without the conversion to GSSG. Behavioral changes were characterized according to the method of van Luijtelaar et al. [88]. Chick behaviors were classified into four categories: (1) active wakefulness; (2) standing/sitting motionless with eyes open; (3) standing motionless with eyes closed; (4) sitting motionless with head drooped (sleeping posture).Central GSH dose-dependently ameliorated distress vocalizations induced by isolation.The time for active wakefulness was reduced with increasing GSH.Additionally, the time spent in sleeping posture dose-dependently increased.GSSG also dose-dependently suppressed vocalizations.The time for active wakefulness was reduced and sleeping posture dose-dependently increased with increasing doses of GSSG. Similarly, SMG dose-dependently suppressed vocalization.The time for active wakefulness was reduced with increasing SMG.When behavioral categories were divided into active wakefulness and sedation (standing/sitting motionless with eyes opened, standing motionless with eyes closed and sleeping posture), the effect of SMG on the latter was dose-dependent.

A sleep-promoting substance (SPS) was isolated from the brains of 24 h sleep-deprived rats, and one component of the SPS was GSSG [40].When infused i.c.v. for 10-h during the nocturnal period, GSSG enhanced rapid-eye-movement sleep (REMS) and non-REMS

(NREMS) in unrestrained rats [26].This result indicated that GSSG had the function of hypnosis.Honda et al. [27] reported that GSSG was actually responsible for the GSH-induced enhancement of sleep.However, GSH had the function of hypnosis through GSH itself and/or through GSSG produced from GSH in chicks.Since GSH is structurally only half of GSSG, it is reasonable that a 2-fold greater dose is required for the somnogenicity of GSH equivalent to that of GSSG [30].Thus, Yamane et al. [91] applied the 2-fold dose in the GSH treatment.The results obtained were consistent with the previous report.

Anxiety regulation by glyoxalase 1 and glutathione reductase 1 in mice reported by Hovatta et al. [28] may be inconsistent withthe function of glutathione.Their report indicated that an increase in enzymes induced anxiety-like behavior.Since glutathione reductase catalyzes the reduction from GSSG to GSH [15], the increase of glutathione reductase induces GSH generation together with anxiety.However, the injection of GSH caused sedation and sleep [91].Therefore, the role of glyoxalase 1 and glutathione reductase 1 in anxiety is not yet clear.Since species and age were different, the results of Hovatta et al. [28] may not be directly explained by Yamane et al. [91].However, GSSG is a sleep-promoting substance and enhanced REMS and NREMS in unrestrained rats [26, 40].GSSG also induced sleep in neonatal chicks.Thus, since the similar effect was confirmed among species, the action of glutathione on stress may be conserved.It is possible; however, that GSH has an important role as an antianxiety agent, since GSH is a major form in the tissue [1].

The effect of glutathione (GSH and GSSG) and SMG on behavior varied.Although there were significant differences, the injection of SMG induced sedative and hypnotic effects, and active wakefulness was rarely confirmed.Instead of the posture for active behavior confirmed in GSH and GSSG, the posture for standing/sitting motionless with eyes open increased.Considering these points, it is suggested that the functions of glutathione and SMG may be different.The effect of glutathione might depend on the ratio of GSH and GSSG.Therefore, sedation and sleep might be regulated by an interaction among GSH, GSSG and glutathione derivatives.

There are a number of reports about the relationship of glutathione and the NMDA receptor, and its associated Ca^{2+} influx.GSH and GSSG induce the inhibition of Glu binding in rat brain synaptic membranes [54] and increased Ca^{2+} influx at the NMDA receptor into rat brain cells [44].GSSG inhibits the Ca^{2+} influx evoked by Glu or Glu agonists in mouse brain synaptic membranes [33].GSH caused a dose-dependent increase, while GSSG failed to induce a Ca^{2+} increase, in rat cultured hippocampal neurons [11].On the other hand, SMG is able to act as a ligand of NMDA receptors, and inhibits Ca^{2+} influx induced by NMDA [33].Thus, since SMG might be an NMDA receptor antagonist, it is thought that SMG inhibits the binding of Glu, or inhibits NMDA receptor activation.Accordingly, it is difficult to explain the mechanism by which GSH, GSSG and SMG induced sleep at NMDA receptors since GSH acts as an agonist, and GSSG and SMG as antagonists of the NMDA receptor.A separate GSH receptor is speculated as one of the binding sites for GSH [31, 80].GSSG and SMG are able to displace [^{3}H]GSH binding in rat brain synaptic membranes *in vitro* [54].Therefore, it is suggested that each substance binds not only to the NMDA receptor, but also to its own GSH receptor.

In conclusion, the i.c.v. injection of GSH, GSSG, and SMG attenuates stress-induced behaviors and triggers sleeping-like behavior in neonatal chicks.There is the possibility that glutathione might be effective in the improvement of stressful insomnia induced by psychological stressors.

2.2. I.c.v. Injection of Glutathione Suppresses Food Intake of Neonatal Chicks

Food intake is reduced by an excess or deficiency of sulfur-containing amino acids (SAA) in growing chicks [82, 86]. Dietary SAA content is a major determinant of glutathione concentration in some tissues since GSH is a tripeptide which consists of L-Glu, L-Cys and Gly.Paterson et al. [65] reported that an SAA deficiency was confirmed by a reduction in liver Cys and GSH concentrations, decreased food intake, and weight loss.Furthermore, the GSH concentration was significantly depressed in the neocortex and thalamus of deficient rats.

GSH acts as an antioxidant and performs detoxification of xenobiotics [15].In addition, GSH also acts as a neurotransmitter and neuromodulator [31]. Hovatta et al. [28] reported that glyoxalase 1 and glutathione reductase 1 regulates anxiety in mice. Glyoxalase 1 is related to detoxification and uses GSH as a cofactor [85]. Glutathione reductase is related to the antioxidation reaction due to glutathione, and catalyses the conversion from GSSG to GSH [15].Therefore, since both enzymes are related to the metabolism of GSH, there may be a relationship between brain GSH and stress responses such as anxiety and fear.

I.c.v. injection of GSH dose-dependently induced sleep-like behavior in chicks under acute stressful conditions [91].Similarly, central administration of glucagon-like peptide-1 (GLP-1) stimulated sleep in neonatal chicks.Food intake was strongly inhibited by GLP-1 [8], an effect that may be due to induction of sleep.

Taken together, GSH may be related to the regulation of food intake.To investigate this possibility, Yamane et al. [92] investigated the effect of i.c.v. GSH on food intake in fasted chicks. GSH dose-dependently suppressed food intake at all time periods after the i.c.v. injection of 1.0 and 2.0 μmol GSH.A similar experiment with lower doses of GSH (0, 0.025, 0.05, 0.2 μmol) found no effect.GSSG is identified as a sleep promoting substance [40].I.c.v. injection of GSH, as well as GSSG, induced sleep-like behavior in neonatal chicks as mentioned above.It was suggested that GSH or GSSG, which was generated from injected GSH, induced sleep-like behavior.The i.c.v. injection of L-Cys and Gly, which are the constituent amino acids of GSH, induced a sedative effect in chicks exposed to isolation stress [3].In addition, L-pipecolic acid, a major metabolic intermediate of L-lysine in the brain, functions not only in the induction of sleep, but also inhibition of food intake in previously fasted chicks [83].Therefore, it is believed that the inhibition of food intake observed byYamane et al. [92]was due to the induction of sleep.

In conclusion, GSH has an important role for the regulation of feeding behaviors in neonatal chicks.Particularly, GSH may contribute to the reduction of food intake in chicks given a diet high in SAA.

2.3. GSH Decreases Energy Expenditure in Chicks Exposed to Separation Stress

Glutathione (γ-L-glutamyl-L-cysteinylglycine) resides in all plant and animal cells.Glutathione exists in two forms.GSH is a monomer, while GSSG is a dimer of GSH.Glutathione functions in antioxidation and detoxification, as a metabolic regulator and

coenzyme, and also has a role in immune function [48].In addition, glutathione functions as a neurotransmitter and neuromodulator [31].I.c.v. injection of GSH induced hypnotic and sedative effects in chicks exposed to an acute stress [91].

Energy homeostasis is essential for life, and its regulation is related to various factors; e.g. a function of body size, body composition, behavior including food intake and physical activity, and health status.

Energy is obtained from food while energy expenditure (EE) occurs during metabolism.EE includes not only heat production but also physical activity [45, 90]. Therefore, the behavioral changes induced by central GSH may influence EE.To clarify this relationship, an isolation stress model similar to the previous study was used [91].

Yamane et al. [94] reported that the oxygen (O_2) consumption, carbon dioxide (CO_2) production, EE and respiratory quotient (RQ) in the GSH injected group were lower than in the control within 10 min after injection.However, both O_2 consumption and CO_2 production in the GSH treated chicks was higher than the controls after 27 min.A similar trend was seen in both EE and RQ.

The time of active wakefulness was longer in the control group immediately after injection whereas the GSH injected chicks were generally observed in the sleeping posture within 10 min after injection.However, at 20 min post-injection, chicks injected with GSH spent more time in active wakefulness and less time in a sleeping posture.

These shifts were similar to those of O_2 consumption, CO_2 production and EE, although the changes in posture occurred a little more rapidly.Significant regression equations were obtained between EE (J/min/g) and behavior (control, active wakefulness (sec/3 min) = $-174.128 + 1.047 \times X$, $P < 0.0001$, $R^2 = 0.493$; GSH, active wakefulness (sec/3 min) = $-126.081 + 0.881 \times X$, $P < 0.0001$, $R^2 = 0.257$).

It is suggested that the change of EE was due to the behavioral changes, an effect that is similar to that observed with L-alanine [42].The i.c.v. GSH quickly and strongly induced sedative and hypnotic effect over 10 min post-injection [91].On the other hand, the sedative effect of GSH gradually disappeared around 20 min post-injection [94].These facts suggest that the effect of GSH for sedation is rapid and short.At 33 and 36 min post-injection, hyperactivity was observed in the GSH group.Chicks may have experienced more stress as the effect of GSH disappeared.

With respect to the action as a neurotransmitter, GSH appears to bind to Glu receptors [31].While the metabotropic Glureceptors (mGluRs) 5 mediates appetite and energy balance in rodents [6], GSH does not induce activation of mGluRs [89].

Accordingly, the changes in EE may not be the result of direct activation of mGluRs by GSH. Furthermore, dipeptides, e.g. cysteinyl-glycine (Cys-Gly) and γ-glutamyl-cysteine (γGlu-Cys), are generated when GSH is catalyzed by γ-glutamyl transpeptidase (γGT).

Subsequently, Cys-Gly is hydrolyzed by ectopeptidases to L-Cys and Gly [15].GSH-related dipeptides including γGlu-Cys, Cys-Gly and γ-glutamylglycine (γGlu-Gly) have a sedative effect, and all amino acids which constitute GSH have a sedative effect, in chicks as discussed below.

These dipeptides and amino acids, generated from exogenous GSH in the brain, might influence EE as observed with GSH.

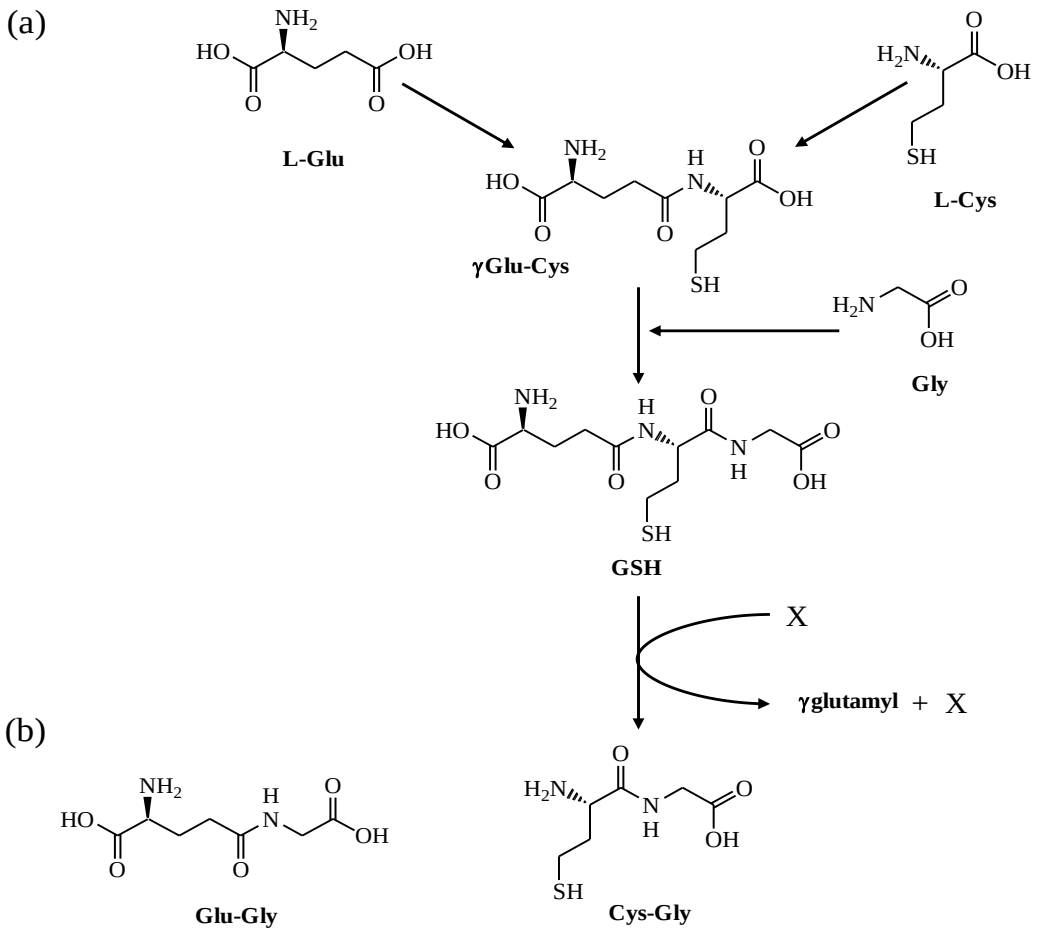

Figure 2. (a) The synthesis and metbolisn of reduced glutathione (GSH). Abbreivations were: γGlu-Cys, γglutamyl-cysteine; Cys-Gly, cysteinyl-glycine; L-Glu, L-glutamate; L-Cys, L-cysteine; and Gly,glycine. The γglutamyl moiety in γglutamyl transpeptidase (γGT) reaction. 'X' represents an acceptor of the γglutamyl moiety transferred by γGT from GSH. (b) The structural formula of glutamyl-glycine (Glu-Cys). It is not confirmed in the metabolism of glutathione, but exists in the brain.

In conclusion, the i.c.v. injection of GSH in chicks induced a sedative effect under stress condition, which correlated with a decrease in EE for a short period, followed by a rebound.

2.4. I.c.v. Injection of Glutathione-Related Dipeptides Induces Sedative and Hypnotic Effects during Acute Stress in Neonatal Chicks

GSH is a tripeptide which consists of L-Glu, L-Cys and Gly. There are three types of dipeptides, i.e., Glu-Cys, Cys-Gly and Glu-Gly that are theoretically present in tissues (Figure 2). The combination of Glu-Cys is called γGlu-Cys, which is a GSH precursor. γGlu-Cyssynthetase uses Glu and Cys as substrates to generate γGlu-Cys.This dipeptide is combined with Gly to synthesize GSH in a reaction catalyzed by GSH synthetase [48].Cys-Gly is generated from GSH by theγGT reaction in the body [17].The γGT-product Cys-Gly is efficiently utilized in micromolar concentrations as a precursor for neuronal GSH [18], and as a supply of L-Cys and Gly for neurons [16].Glu-Gly is present in the cortex of the rat brain, at

1.1 ± 0.1 nmol/g wet tissue [72].The combination of Glu-Gly might be synthesized in the metabolism of glutathione via the γglutamyl cycle, since a wide variety of amino acids serves as an accepter of γglutamyl group generated by the degradation of glutathione [49].

I.c.v. injection of GSH, GSSG, or SMG induced sedation and hypnosis in neonatal chicks [91].Glutathione binds to the NMDA receptor which not only has a Glu recognition site but also a Gly recognition site localized on the NMDA receptor [31, 38].Accordingly, all three dipeptides mentioned above have the possibility to bind to the NMDA receptor since they have a Glu or Gly moiety.These peptides may thus have sedative and hypnotic effects on stress behavior similar to that observed with glutathione.

To confirm which parts of glutathione are required for sedative and hypnotic effects, the effects of these three peptides, i.e., Glu-Cys, Cys-Gly and Glu-Gly were compared following injection into the brain of neonatal chicks exposed to an isolation-induced stress condition.Yamane et al. [93] reported that Glu-Cys and Glu-Gly significantly suppressed vocalization, while Cys-Gly had no effect.Glu-Gly and Cys-Gly significantly suppressed spontaneous activity when compared to the control.While the effect of Glu-Cys was not significant, it tended to decrease spontaneous activity. I.c.v. injection of Glu-Cys and Glu-Gly decreased the time of active wakefulness.No significant effect was found on the time for sleeping posture.If behavioral categories were divided into active wakefulness and sedation (standing/sitting motionless with eyes opened, standing motionless with eyes closed and sleeping posture), all three dipeptides caused sedative effects.

In the brain, Glu-Cys serves as the precursor of GSH.GSSG, a dimer of GSH, is a sleep promoting substance in rats [40].I.c.v. injection of GSSG induced sleep-like behavior in chicks [91], as observed in rats.It is suggested that the Glu-Cys moiety of GSSG may play an important role in hypnotic effects.However, the injection of Glu-Cys exhibited not only hypnotic but sedative effects.The dipeptide Glu-Cys may also have a partly different function or a different mechanism from the action of GSSG in the brain.In most parameters examined, the effect of Cys-Gly was weakest.Taken together, glutamyl rather than the SH group may be important in inducing sedative effects.Additionally, a combination of the glutamyl and SH groups in the molecule may have a strong effect.

Glu-Gly is recognized as an effective antagonist of NMDA and kainate-induced sodium efflux from rat striatum slices [72].Glu-Gly inhibited Ca^{2+} influx activated by Glu in a dose-dependent manner [87].Therefore, Glu-Gly might be an antagonist of Glu receptors.Neither Glu-Cys nor Cys-Gly induced an intercellular Ca^{2+} increase [11].Glu-Cys, therefore, might bind to a glutamyl recognition site on the NMDA receptor and prevent activation of the NMDA receptor.The three dipeptides, as well as GSH, may be able to work at the NMDA receptor and induce sleep-like behavior.Glu through NMDA and kainate receptors activates the HPA axis at the level of the paraventricular nucleus [99].Therefore, the NMDA receptor may be involved in the stress response pathway.

There was a possibility that the dipeptides studied bound to not only Glu, but also the Gly, recognition sites on the NMDA receptor.The binding of an agonist to the Gly recognition site modulated the NMDA receptor-mediated excitatory response [71].Dipeptides having a Gly moiety might act as an antagonist, and inhibit the NMDA receptor activation.However, Cys-Gly dose not function as a Gly recognition site agonist at the NMDA receptor [51].Accordingly, it is thought that Cys-Gly did not bind to the Gly site on the NMDA receptor in this experiment.Since Glu-Gly has both a Glu and Gly moiety, it may

be able to bind to both recognition sites.It is probable that activation of the NMDA receptor was affected by binding of Glu-Gly at both sites.

It was also considered that the three dipeptides bound to a GSH receptor.Although Glu-Cys and Cys-Gly were effective displacers in the rat cortex, Glu-Gly was unable to displace [^{3}H] GSH binding [31].It is suggested that the site of action of Cys-Gly might be the GSH, but not NMDA, receptor.

Although no significant effect of plasma corticosterone concentration was found following injection of the three dipeptides, Glu-Cys and Glu-Gly attenuated corticosterone release induced by social isolation [93].Both peptides might partially affect the HPA axis.However, the effect of GSH and its dipeptide on plasma corticosterone concentration was different, since no direct effect of GSH was found (unpublished data).The mechanism for different responses between glutathione and dipeptides was unclear.However, since sleep-like behavior was observed in Glu-Cys and Glu-Gly groups, the induction of sleep may occur by the glutamyl moiety.It is suggested that the glutamyl moiety is involved in the induction of sedation.However, Cys-Gly without the glutamyl moiety also induced a sedative effect [93].It has also been confirmed that i.c.v. injection of L-Cys and Gly induced sedative effects in chicks [3].

In conclusion, sedative and hypnotic effects can be induced by all combinations of amino acids which constitute the GSH structure, but their functions were somewhat different from each other, and from GSH.

3. The Contribution of Amino Acids which Constituents Glutathione

3.1. Glu is Involved in the Induction of Sedative Effects under an Acute Stress in Neonatal Chicks

Essential amino acids (EAAs), including Glu and aspartate, act as neurotransmitters in the CNS.They can induce neuronal activity with powerful stimulatory effects [50].Glu receptors are divided into two groups: ionotropic Glu receptors (iGluRs) and mGluRs.The iGluRs, which form ion channels, include three main classes: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA),kainate receptors and NMDA.The NMDA receptor is usually blocked by Mg^{2+}.When the synaptic membrane is slightly depolarized, e.g. by previous activation of AMPA and kainate receptors, the Mg^{2+} block of the NMDA receptor is removed.The NMDA receptor is activated after binding of an agonist [5].On the other hand, the mGluRs are coupled to GTP-binding proteins, and regulate the production of intracellular messengers.The mGluRs have eight members (mGluR1-8), categorized into three groups based on sequence homology, second messenger coupling and pharmacology.Group I includes mGluR1 and 5, group II is mGluR2 and 3 and group III is mGluR4, 6, 7 and 8.Group I mGluRs are localized postsynaptically and predominantly activate phospholipase C.Group II and III mGluRs are localized in presynaptic densities and inhibit adenylyl cyclase activity.They contribute to the regulation of synaptic plasticity and transmission [13, 39].

It was shown that GSH, which is a tripeptide consisting of L-Glu, L-Cys and Gly, induced hypnosis during a stressful condition in neonatal chicks, and this effect may involve

the NMDA receptor [91].Furthermore, glutathione appears to play a role as a neurotransmitter or neuromodulator, as it can bind to the NMDA receptor and other Glu receptors via its γ-glutamyl moiety and thereby induce the movement of intracellular Ca^{2+} [31].Thus, it is hypothesized that Glu receptors are involved in sleep regulation in neonatal chicks.

The distribution of iGluRs ligand binding sites is markedly different in the chick brain as shown using autoradiography [24].Although [^{3}H]L-Glu, [^{3}H]AMPA and [^{3}H]kainate strongly label the molecular layer of the cerebellum, the density of [^{3}H]kainate is particularly intense.[^{3}H]L-Glu densely labels the telencephalon, particularly the neostriatum.[^{3}H]AMPA binding sites are densely located in the hippocampus, and are also extensively distributed in the telencephalon.These regions correspond with brain structures involved in the regulation of stress, including anxiety and fear [9, 43, 47].In general, the NMDA receptor usually coexists with the AMPA receptor in the postsynaptic membrane [52].In addition, the structure and function of mGluRs has been demonstrated in several experiments in chicks [29, 70, 101].

Yamane et al. [95] reported that distress vocalizations were dose-dependently decreased by i.c.v. Glu.The time of active wakefulness decreased dose-dependently.On the other hand, the time of sitting motionless with head drooped increased gradually.NMDA also dose-dependently affected distress vocalizations.The time of active wakefulness decreased dose-dependently.In contrast, the time of standing/sitting motionless with eyes open increased dose-dependently.AMPA tended to dose-dependently decrease distress vocalizationsbut this effect was not significant.The time of active wakefulness decreased dose-dependently.The behavior of standing motionless with eyes closed and sleeping posture were found only in the 150 pmol AMPA group, and the time of sleeping posture exhibited a significant increase.On the other hand, no significant effects were found by kainite in total distress vocalizations.This was the case for behavioral changes.I.c.v. kinate also did not influence behavioral observations.Yamane et al. [95] also investigated the contribution of group I mGluRs, since group I mGluRs have an excitatory neurotransmission similar to iGluRs. For this objective, the group I selective mGluRs agonist (S)-3, 5-dehydroxyphenylglycine (DHPG) was applied.I.c.v. DHPG did not influence distress vocalizations and chicks receiving DHPG spent the most time in active wakefulness, and they were comparable to the control group.Standing motionless with eyes closed and sleeping posture were not found in these groups.

The i.c.v. injection of Glu, NMDA and AMPA attenuated total distress vocalizations and induced sedation [95].In contrast, previous studies showed that localized infusion of Glu induced vocalizations in cats [4] and squirrel monkeys [34].Additionally, the injection of NMDA, AMPA and kainate into the substantia innominata/lateral preoptic area of rats dose-dependentlystimulated locomotion [75].Lateral hypothalamic injection of kainate also increased locomotor activity in rats [25, 77].Further, when injected intracranially to restricted brainstem regions, NMDA elicited locomotion in decerebrate geese and ducks [76].On the other hand, injection of Glu into the lateral hypothalamus increases sleeping time [77].The difference between these studies and Yamane et al. [95] may be due to species differences or the site of injection.

The i.c.v. injection of Glu dose-dependently decreased distress vocalizations in isolated chicks similarly to that observed by Panksepp et al. [64] who injected Glu at doses from 25 to 500 μg.In contrast to the results of NMDA,Panksepp et al. [64] reported the administration of NMDA (0.1, 0.25, 0.5 and 1.0 μg) had no effect on the number of vocalizations.Differences

in the time for behavioral observation, experiment conditions, or genetic line of chicks may contribute to the contrasting results for vocalizations.For example, the stress response differs between meat- and layer-type neonatal chicks, being relatively higher in the layer-type [69].

Although i.c.v. injection of the excitatory amino acidGlu, and the Glu analogue NMDA, induced sedation in chicks, the behavioral results for the two compounds were somewhat different [95].NMDA induced an increase in the time spent standing/sitting motionless with eyes open while Glu increased the time in sleeping posture.AMPA had a tendency to decrease wakefulness activity and increase sleep-like behavior.The central administration of kainate (0.05, 0.1, 0.25 and 0.5 μg) had no effect under stressful conditions in chicks [64].No significant effect of kainate was also observed compared with the control [95].

Henley et al. [24] demonstrated the distribution of iGluRs ligand binding sites in the chick brain using autoradiography.The binding sites of NMDA and AMPA are related to the brain regions of stress response.However, kainite binding sites are mainly in the molecular layer of the cerebellum which is not generally considered a stress-related region in the brain.In general, the NMDA receptor usually coexists with the AMPA receptor in the postsynaptic membrane [52].Therefore, kainate may not be related to the stress response.In any case, kainate, in the doses used here, appears to have no sedative effect against isolation stress in chicks.It is suggested that the hypnotic effects of Glu involved not only the NMDA receptor, but also the AMPA receptor.The group I selective mGluRs agonist DHPG did not affect sleeping posture [95].Although the effect of group II and III mGluRs has not been investigated, the data supports the theory that the iGluRs NMDA and AMPA, but not kaiante, are related to sleep-induction in neonatal chicks.

γ-Aminobutytic acid (GABA) and taurine are representative inhibitory amino acids.Both the GABAergic system and taurine exert anti-anxiety actions [98, 100].Several experiments have shown that NMDA and Glu can cause the release of GABA from cultured neurons in mice and rats [14, 22, 67].In addition, an increase of taurine is induced when NMDA is delivered into the medio-rostral neostriatum/hyperstriatum ventral using microdialysis in chicks [21].Therefore, the sedative effects seen in this experiment may be due to the Glu triggered release of GABA.Indeed, it has been confirmed that the i.c.v. injection of GABA induced a sedative effect under acute stress in chicks [74].

Glu, which exhibited the same effect as GSH, might act via a different mechanism than GSH.Since Glu can bind to both AMPA and kainate receptors, its action is probably mediated via NMDA, AMPA and kainate receptors.The results obtained supported that both NMDA and AMPA had sedative effects.The NMDA receptor usually coexists with the AMPA receptor in the postsynaptic membrane [52].On the other hand, DHPG had no effect [95], and GSH has no action on the mGluRs [89].Thus, it appears likely that the hypnotic and sedative effects of Glu are induced by interaction with NMDA and AMPA receptors.However, the activity of group II mGluRs regulates the HPA axis [73], and group III mGluRs agonists have anxiolytic- and antidepressant-like effects in rats [61].

In conclusion, the central administration of Glu attenuates stress-induced behaviors and triggers sleep-like behavior in neonatal chicks.Furthermore, it was demonstrated that NMDA and AMPA, but not kainate and DHPG, also have a sedative effect.It is suggested that Glu-induced hypnosis and sedation may require an interaction between NMDA and AMPA receptors.Therefore, further investigation, for example co-administration with NMDA or AMPA antagonists, is needed for a better understanding of the mechanism of Glu.In addition,

whether or not NMDA and AMPA which have a sedative effect also have an antianxiety effect should be confirmed in future.

3.2. L-Cys involved in the Induction of Sedative Effects under an Acute Stress in Neonatal Chicks

L-Cys is a non-essential and glycogenic amino acid, and D-cysteine (D-Cys) is its optical isomer.In the synthesis and metabolism of L-Cys, L-homocysteine is generated from L-methionine which is converted to L-cystathione in a condensation reaction with L-serine (L-Ser).Subsequently, L-cystathione is degraded to homoserine and L-Cys by hydrolysis.L-Cys is oxidized to L-cysteine sulfinic acid by cysteine dioxygenase, and is transaminated to 3-sulphenylpyruvic acid.Finally, L-Cys is metabolized to pyruvic acid [78].Since L-Cys is a precursor of taurine, an inhibitory amino acid [7], L-Cys is important in the maintenance of brain function.L-Cys is a rate-limiting precursor for glutathione synthesis in neurons [48].L-Cys itself provides inorganic sulfate for detoxification reactions [23], as well as glutathione.In addition, L-Cys also acts as a neuromodulator since it is released from brain slices upon depolarization in a Ca^{2+}-dependent manner [37, 97] and excites neurons [58].

Asechi et al. [3] reported that i.c.v. injection of L-Ser and L-Cys induced sedative effects under social-isolation stress in neonatal chicks.However, D-Ser had no effect on chick behavior during an acute stress.According to Yamane et al. [96], both L- and D-Cys significantly decreased distress vocalizations compared to the control, and no vocalizations were observed in the D-Cys treated group.Both L-Cys and D-Cys significantly decreased spontaneous activitycompared to the control.The time for active wakefulness was significantly decreased in both the L- and D-Cys groups compared with the control.No active wakefulness was found in the D-Cys group including distress vocalization.On the other hand, the time for sleeping posture increased in the L- and D-Cys injected groups.However, the time for sleeping posture in the D-Cys was shorter than in the L-Cys, and abnormal behavior was observed in the D-Cys treated group.This abnormal behavior was categorized as syncope as confirmed by the chick falling down rather than having the head droop.

Both L- and D-Cys induced sedative and hypnotic effects under social isolation stress in neonatal chicks.L-Cys is considered a potent excitotoxin comparable in potency to other excitatory amino acids such as Glu and aspartate [36, 58, 66], but it lacks the omega carboxyl group required for excitotoxic actions via excitatory amino acid receptors.For instance, L-Cys destroys neurons when given orally in high doses (3.0 mg/g body weight) to infant mice [56], while subcutaneous injection of L-Cys in lower doses (0.8 mg/g) induces a more devastating neurotoxic syndrome [57].In addition, L-Cys appears to act as a neuromodulator and neurotoxin [32].

L-Cys enhances neuronal influx of Ca^{2+} induced by Glu and NMDA.This effect is inhibited by a competitive NMDA receptor antagonist [58].Thus, L-Cys can selectively activate the NMDA receptor, but the mechanism of this effect, such as its binding site, is unclear [59].The i.c.v. injection of NMDA induces a sedative effect during stressful conditions in neonatal chicks [93].Additionally, GSH also causes the induction of hypnosis under an acute stressful condition in chicks [91].Since GSH appears to bind to the NMDA receptor [31], the influence of GSH may also be mediated via the NMDA receptor.Therefore, it is suggested that L-Cys evoked sedative and hypnotic effects via the NMDA

receptor.However, there was also a report that L-Cys may be a very weak NMDA agonist [60], and further study is necessary to elucidate its mechanism.

L-Cys is metabolized to L-cysteine sulfinic acid, 3-sulphenylpyruvic acid and finally pyruvic acid.Asechi et al. [2] administrated equimolal pyruvic acid i.c.v. to chicks exposed to stressful conditions.However, pyruvic acid had no effect on behavior and plasma corticosterone concentration compared with the control.Therefore, it was suggested that sedation was not induced by pyruvic acid.Additionally, taurine is generated from L-Cys via hypotaurine [84].Taurine has anxiolytic activity in rodents [10, 41].Since it was a probability that taurine has a similar effect in chicks as well as rodents, taurine may be involved in the effects observed here.

Asechi et al. [3] reported that, unlike L-Ser, D-Ser has no sedative effect.However, both L- and D-Cys exhibited sedative and hypnotic effects [96].Thus, an optical isomer does not always have different effects.However, Yamane et al. [96] reported that an equimolar dose of D-Cys with L-Cys induced abnormal behavior.Therefore, it is suggested that D-Cys may be harmful compared with L-Cys.In conclusion, the central administration of L- and D-Cys attenuates stress-induced behaviors.However, D-Cys may have a harmful effect compared to L-Cys.

3.3. Contribution of Gly on Sedative and Hypnotic Effects

Asechi et al. [3] conducted a series of experiments to investigate the relationship between L-Ser in the CNS and stress-related behavior of animals using the chick separation stress paradigm.I.c.v. injection of L-Ser and its derivatives, including Gly and L-Cys, induced sedative and hypnotic effects under isolation-induced stress in neonatal chicks.

Shigemi et al. [74] investigated the contribution of the Gly receptor for the sedative and hypnotic effect of Gly by using the Gly receptor antagonist strychnine. Gly significantly decreased total spontaneous activity, while strychnine had no effect and the interaction between Gly and strychnine was not significant.On the other hand, in chick vocalization, an interaction between Gly and strychnine was significant suggesting that strychnine inhibited the suppressive effect of Gly.The sedative effect of Gly on chick behavior was also inhibited by co-administration with strychnine.

The Gly receptor is a pentameric transmembrane protein complex which forms an anion-selective channel.Presently, five different subunits have been cloned in mammals including one β-subunit and four α-subunits (α_1-α_4).Fetal Gly receptors are predominantly α_2-homomers, whereas adult receptors are predominantly $\alpha_1\beta$-heteromers.In the neonatal rat, α_1-, α_2-, and β-subunits exist in abundance, implying a mixture of receptor isoforms, but the switch towards the adult isoform is complete by around postnatal day 20 [46].Although there are few findings regarding avian Gly receptor subunit composition, such a switch (α_2-homomers to $\alpha_1\beta$-heteromers) may occur in the course of avian CNS development. Strychnine inhibited the sedative and hypnotic effects of Gly suggesting that activation of the Gly receptor also induced sedative and hypnotic effects, and these effects were suppressed by blocking the Gly receptors with strychnine

Conclusion

In conclusion, glutathione and its constituents are helpful to ameliorating an acute stressful condition.

Acknowledgments

A part of studies described here was supported by grant-in-aid for scientific research from Japan Society for the Promotion of Science (Nos. 17208023, 18208023 and 23248046)to MF.

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In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter III

Glutathione: Disease-related Changes and Biomarkers of Oxidative Stress

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Abstract

Free radicals are normally generated in many metabolic pathways. These radicals may interact with different cellular components and induce cell injury. However, there is an efficient antioxidant system like Glutathione (GSH) that protects the organism against free radical-induced cell injury. Oxidative stress results when there is a serious imbalance between generation of free radicals and antioxidant capacity, or disturbance in glutathione homeostasis in the body. When free radicals exceed antioxidant capacity, an induction of cell injury takes place which leads to diverse physiologic and pathologic changes, including clinical diseases. This chapter reviews the possible mechanisms of GSH that are involved in usual clinical disorders and their studies in animal models. Moreover, a description of the analytical techniques and biochemical markers used to monitor GSH and oxidative stress in the same disorders is made in this recompilation.

1. Introduction

Organisms are endowed with a protective antioxidant (AO) system that limits damage caused by free radicals (FRs). This protective system includes enzymes and pharmacological

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therapies that, apart from preventing FR generation and trapping those that have already been generated, removes or repairs damaged biomolecules. AO system and FR generation coexist in a balanced way, and when this equilibrium is altered, the result is oxidative stress, which not only provokes cell injury, and triggers physiologic disorders but also promotes pathologic processes [1].

FR represents any chemical species that exists independently and has one or more unmatched electrons rotating in its external atomic orbits [2]. This highly unstable configuration causes chemical species to be very aggressive and to have a short life span. There is an inverse correlation between the magnitude of this reactivity and its life span and ability to diffuse through cells. Chemically, a FR can be generated through different pathways, but the most frequent in a living organism is the addition of an electron to a stable molecule (figure 1). Most of the molecules of an organism are non-radicals, which mean they only have even electrons in their atomic orbits.

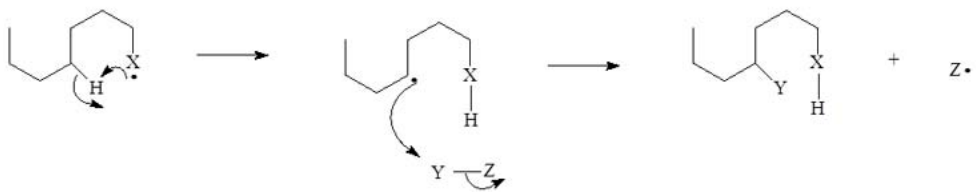

Figure 1.Unstable molecules for production of free radicals.

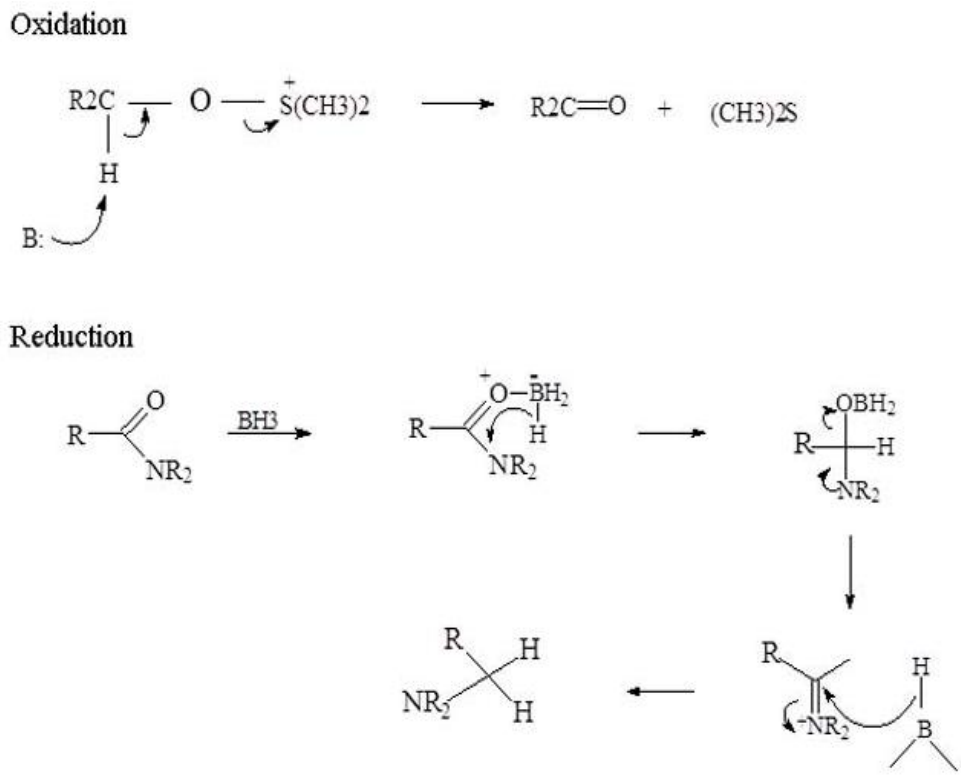

Figure 2. Oxidation or reduction reactions in molecules.

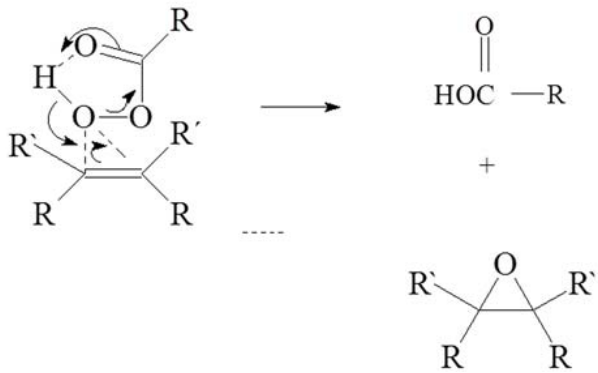

Figure 3. Oxygen free radicals, in which oxygen is the functional center.

Once generated, the FR interacts with other molecules through redox reactions to obtain a stable electronic configuration. In a redox reaction, the transference of electron between the participating chemical species takes place. One loses free electrons (oxidation process) and the other gains them (reduction process). The oxidation of one chemical species implies the reduction of another (figure 2). The molecule losing electrons is a reducing agent, while the molecule gaining electrons is an oxidant agent. When a FR reacts with a non-radical molecule, it may lose or gain electrons, or simply join the molecule. In any case, the non-radical molecule turns into a FR and a chain reaction is triggered: one FR generates another FR. The reaction will stop only when two FR meet [3].

Several authors have classified FR according to the functional group in their molecule [4]. The most frequent is an oxygen FR, in which oxygen is the functional center (See figure 3). Thiol radical group contain sulfur (S) while others contain carbon (C), phosphorous (P) or nitrogen (N) in their reactive groups. Free radicals are generated from normal metabolic reactions, and exogenous factors can increase them [5]. These groups are formed by one superoxide anion, one hydroxyl radical, and a FR that comes from organic compounds: alcoxyl, peroxyl, hydrogen peroxide**,** and singlet oxygen [6].

The denomination reactive oxygen species (ROS) is used nowadays to include chemical species that act like oxidants but which are not FR (hydrogen peroxide, hypochlorous acid, hydroperoxides and epoxide metabolites) (Table 1).

Table 1. Main reactive species
(Table adapted from Juárez and Calderón 2009 [7])

Name	Formula	Formation
Superoxide	0_2-	Intermediate in 0_2 reduction to H_2O
Hydroxyl	HO.	Powerful oxidant in biological systems
Peroxyl	ROO.	Low oxidant ability, but high diffusibility
Alkoxyl	RO.	Medium oxidant ability with lipids
Hydrogen peroxide	H_20_2	Originated from 0_2
Hypochlorous acid	HClO	Formed through myeloperoxidase action
Singlet oxygen	lO_2	Molecularly excited oxygen through sunlight and radiation

The major site of production of superoxide is considered to be the respiratory chain in the mitochondria, but the exact mechanism and the precise location of the physiologically relevant ROS generation within the respiratory chain have not been disclosed yet. Its mechanism could be relevant, because evidence indicates that oxidative stress is a crucial factor in the pathogenesis of clinical disorders [8].

Reactive nitrogen species (RNS) refers to various nitrogenous products, such as NO, nitroxyl (HNO), nitrosonium cation (NO'), higher oxides of nitrogen, S-nitrosothiols (RSNOs), $ONOO^-$, and dinitrosyl iron complexes, excluding NO_3^- (nitrates) (Table 2).

RNS have been recognized as playing a crucial role in the physiologic regulation of many, if not all, living cells, such as smooth muscle cells, cardiomyocytes, platelets, and nervous and juxtaglomerular cells.

All these possess pleiotropic properties on cellular targets after both posttranslational modifications and interactions with reactive oxygen species. Each of these compounds has distinctive properties in terms of reactivity, a half-life, lipid solubility, and biologic activity [9].

ROS have defined roles in cell signaling events as well as in human disease pathologies, an imbalance in expression of GSH and associated enzymes that has been implicated in a variety of circumstances [10]. ROS induce reactions between GSH through chemical reaction or functional interaction or both. This might be possible due to rearrangement to a more stable S-nitrous glutathione (GNSO), depending upon the presence of endogenous oxygen [11].

Such interaction can be explained since NO requires the presence of GSH to diffuse across the cells. GSH (figure 4), is an antioxidant that prevents damage by reactive oxygen species such as free radicals to important cellular components [12].

Table 2. Main reactive nitrogen species
(Adapted from Juárez and Calderón 2009 [7])

Name	Formula	Formation
Dinitrogen trioxide	N_2O_3	From NO- and O_2
Nitric oxide or nitrogen monoxide	NO-	From NOS
Nitrite	NO_2-	From NO-
Nitrogen dioxide	NO_2	From ONOO-decomposition
Nitronium cation	$N0_2^+$	From $ON00CO_2$-decomposition
Nitrosonium cation	NO^+	From NO-
Nitrosoperoxycarbonate anion	$ONOOCO_2$-	ONOO- and CO_2
Nitroxyl.	HNO	From one-electron reduction of NO-
Nitryl. chloride	Cl-NO_2	From NO_2- and HOCl
Peroxynitrite	ONOO-	From NO- and 0_2-
S-Nitrosothiols	RSNOs	From covalent addition of an NO- group to a cysteine thiol/sulfhydryl

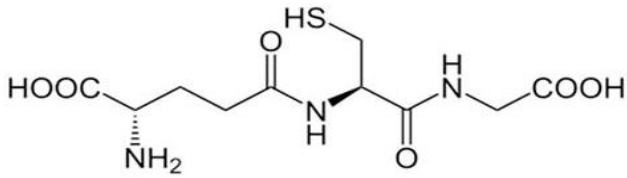

Figure 4. GSH (2*S*)-2-amino-4-{[(1*R*)-1-[(carboxymethyl)carbamoyl]-2-sulfanylethyl] carbamoyl} butanoic acid.

This is a tripeptide that contains an unusual peptide linkage between the amine group of cysteine, (which is attached by normal peptide linkage to a glycine) and the carboxyl group of glutamate side-chain [13].

Glutathione is found almost exclusively in its reduced form, since the enzyme that revert it from its oxidized form (GSSG), glutathione reductase (GSSG-R), is constitutively active and inducible upon oxidative stress. In fact, the ratio of reduced glutathione to oxidized glutathione within cells is often used as a measure of cellular toxicity [14]. Antioxidant enzymes like copper/zinc superoxide dismutase (SOD), and glutathione peroxidase (GSHPx) with Selenium as cofactor, are part of intracellular protection mechanisms to overcome oxidative stress [15].

Glutathione is not an essential nutrient (this means that the source is not via food intake), since it can be synthesized in the body from the amino acids L-cysteine, L-glutamic acid, and glycine. The sulphydryl (thiol) group (SH) of cysteine acts as a proton donor and it is responsible for the biological activity of glutathione. Provision of this amino acid is the rate-limiting factor in glutathione synthesis by the cells, since cysteine is relatively rare in foodstuffs. Furthermore, if released as free amino acid, cysteine is toxic and spontaneously catabolized in the gastrointestinal tract and blood plasma [16].

Animal glutamate cysteine ligase (GCL) is a heterodimeric enzyme composed of a catalytic (GCLC) and modulatory (GCLM) subunit**s**. GCLC constitutes all the enzymatic activity, whereas GCLM increases the catalytic efficiency of GCLC. Mice lacking GCLC (i.e., all de novo GSH synthesis) die before birth [17]. Mice lacking GCLM demonstrate no outward phenotype, but exhibit marked decrease in GSH and increased sensitivity to toxic insults [18]. While all cells in the human body are capable of synthesizing glutathione, liver glutathione synthesis has been shown to be essential. Mice with genetically-induced loss of GCLC (i.e., GSH synthesis) only in the liver die within 1 month of birth [19].

GSH is the most abundant non-protein thiol that buffers ROS in the brain tissue. It eliminates H_2O_2 and organic peroxides by glutathione peroxides (GSH-Px). GSH also transports amino acids across the cellular membrane by the γ-glutamyl cycle and detoxifies foreign agents by glutathione *S*-transferase (GST), which plays a vital role in phase 2 of biotransformation of many substances. Normal levels of glutathione are maintained by de novo glutathione synthesis and the salvage pathway. GSH is comprised of three amino acids, glutamate, cysteine, and glycine.

Cysteine thiol acts as a nucleophile in reactions with both exogenous and endogenous electrophilic species. As a consequence, reactive oxygen species (ROS) are frequently targeted by GSH in both spontaneous and catalytic reactions. The first enzyme in de novo synthesis is γ-glutamylcysteine synthetase (GCS) that catalyzes glutamate and cysteine to form γ-glutamylcysteine. γ- Glutamylcysteine and glycine form GSH by second enzyme, the

glutathione synthetase (GS). During detoxification, ROS are reduced by GSH-Px at the expense of GSH to form glutathione disulfide (GSSG). GSH is regenerated by redox recycling, in which GSSG is reduced to GSH by glutathione reductase (GR) with a consumption of one NADPH. During transportation of amino acids into mammalian cells, one GSH is lost for each amino acid transported, but it can be restored by the salvage pathways including recovery of cysteine and de novo GSH synthesis. These salvage pathways maintain homeostasis of glutathione system (figure 5) [20].

Glutathione exists in reduced (GSH) and oxidized (GSSG) states. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to other unstable molecules, such as reactive oxygen species. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form glutathione disulfide (GSSG). Such a reaction is possible due to the relatively high concentration of glutathione in cells (up to 5 mM in the liver). GSH can be regenerated from GSSG by the enzyme glutathione reductase. In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the disulfide form (GSSG). An increased GSSG-to-GSH ratio is considered indicative of oxidative stress.

The oxidation-reduction pathways of GSH are summarized in figure 6. Glutathione is an essential cofactor for antioxidant enzymes, namely the GSH peroxidases (both Se-dependent and non-Se-dependent forms exist) and the more recently described phospholipid hydroperoxide GSH peroxidases. The GSH peroxidases serve to detoxify peroxides (hydrogen peroxide, and other peroxides) in the water-phase, by making them react with GSH; the latter enzymes use GSH to detoxify peroxides generated in the cell membranes and other lipophilic cell phases. This is one instance of the water-soluble GSH providing electrons to help reduce oxidized biomolecules located away from the water phase.

Enzymes collectively known as GSH transhydrogenases use GSH as a cofactor to reconvert dehydroascorbate to ascorbate, ribo-nucleotides to deoxyribonucleotides, and for a variety of -S-S- <--> -SH inter- conversions. After GSH has been oxidized to GSSG, the recycling of GSSG to GSH is accomplished mainly by the enzyme glutathione reductase. This enzyme uses as its source of electrons the coenzyme NADPH (nicotinamide adenine dinucleotide phosphate, reduced). Therefore NADPH, coming mainly from the pentose phosphate shunt, is the predominant source of GSH reducing power. Cathcart et al, [21], used this to explain why subjects unable to make adequate NADPH may be at increased risk of oxidative damage from GSH insufficiency.

2. Glutathione Functions

1. Glutathione is the major endogenous antioxidant produced by cells directly participating in the neutralization of free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms [22].

2. GSH participates in the regulation of nitric oxide cycle, which is critical for life but can be problematic if unregulated [23].

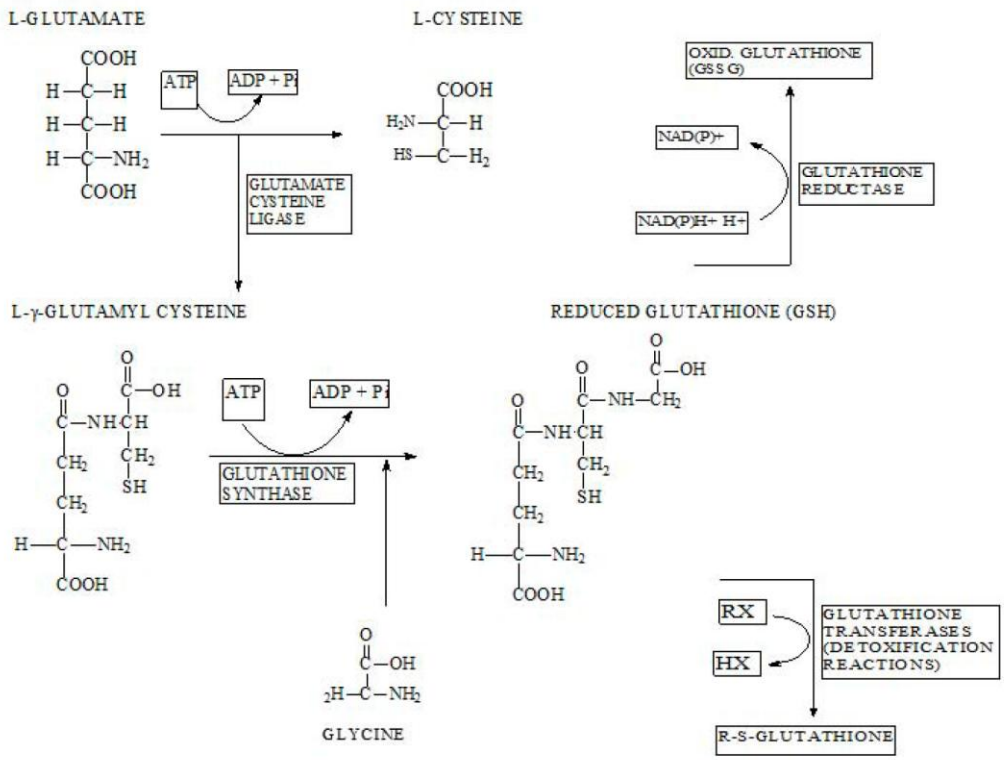

Figure 5. Molecule with GSH Biochemical Pathways.

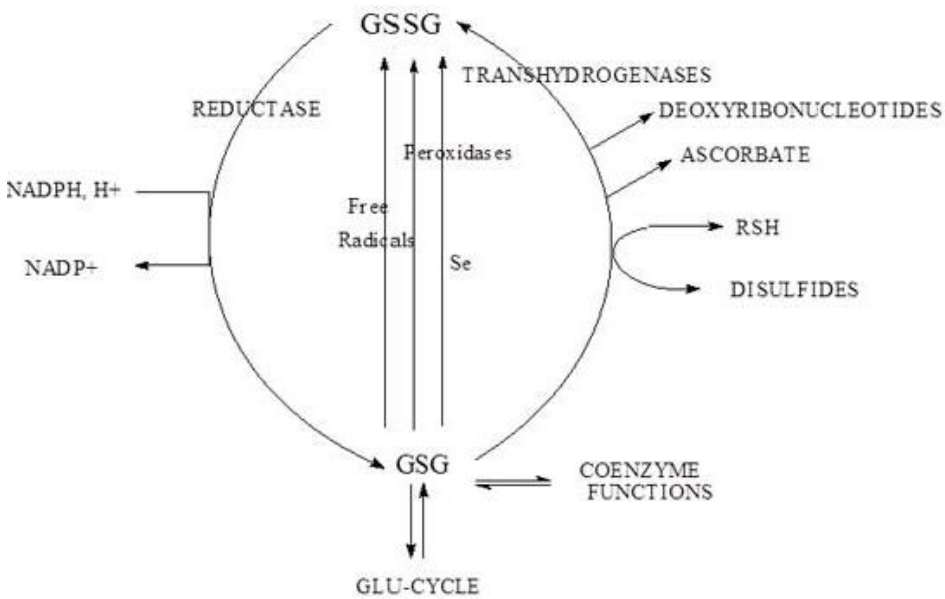

Figure 6. The Oxidation-Reduction pathways of GSH.

3. GSH is used in metabolic and biochemical reactions such as DNA synthesis and repair, protein synthesis, prostaglandin synthesis, and amino acid transport. Thus, every system in the body can be affected by the state of the glutathione system, especially the immune system, the nervous system, and the gastrointestinal system [24]. Likewise, variation in the expression of enzymes of their metabolism suggests individual differences for the degree of antioxidant protection and geographical differences in the distribution of these groups. It is known the designation for comprehensively explore the role of these genetic polymorphisms in the etiology of human diseases which may help to prevent inconsistent genotype-phenotype associations in pharmacogenetic studies [25]. Indeed, Glutathione S-transferases (GSTs) are polymorphic enzymes that catalyze glutathione conjugation of alkylating agents, platinum compounds, and free radicals formed by radiation used to treat medulloblastoma. GSTM1 and GSTT1 polymorphisms may predict adverse events, including cognitive impairment after therapy, in patients with medulloblastoma [26].

2.1. Toxic Effects

GSH is known as a substrate in both conjugation reactions and reduction reactions, catalyzed by glutathione S-transferase enzymes in cytosol, microsomes, and mitochondria. However, it is also capable of participating in non-enzymatic conjugation with some chemicals. GSH is essential for mammalian development, and GSH synthesis via GS (glutathione synthetase) is an indispensable pathway for survival [27].

In the case of *N*-acetyl-*p*-benzoquinone imine (NAPQI), the reactive cytochrome P450-reactive metabolite formed by paracetamol (or acetaminophen as it is known in the US), becomes toxic when GSH is depleted by an overdose of acetaminophen [28]. GSH is an essential antidote to overdose. It conjugates NAPQI and helps to detoxify it. In this capacity, it protects cellular protein from thiol groups, which would otherwise become covalently modified. When all GSH have been spent, NAPQI begins to react with the cellular proteins, killing the cells in the process. The preferred treatment for an overdose of this painkiller is the administration (usually in atomized form) of *N*-acetyl-L-cysteine, which is processed by cells to L-cysteine and used in the de novo synthesis of GSH.

GSH participates in synthesis of leucotrienes and it is a cofactor for the enzyme glutathione peroxidase. It is also important as a hydrophilic molecule that is added to lipophilic toxins and waste in the liver during biotransformation before they can become part of the bile. Glutathione is also needed for detoxification of methylglyoxal, a toxin produced as a by-product of metabolism. GSH has recently been used as an inhibitor of melanin in the cosmetic industries. In countries like Japan and Philippine, this product is sold as a whitening soap. Glutathione competitively inhibits melanin synthesis in the reaction of tyrosinase and L-DOPA by interrupting L-DOPA's ability to bind to tyrosinase during melanin synthesis (figure 7).

The inhibition of melanin synthesis was reversed by increasing the concentration of L-DOPA, but not by increasing tyrosinase. Although the synthesized melanin was added within 1 h, this was inhibited by the addition of glutathione. These results indicate that glutathione inhibits the synthesis and agglutination of melanin by interrupting the function of L-DOPA. [29].

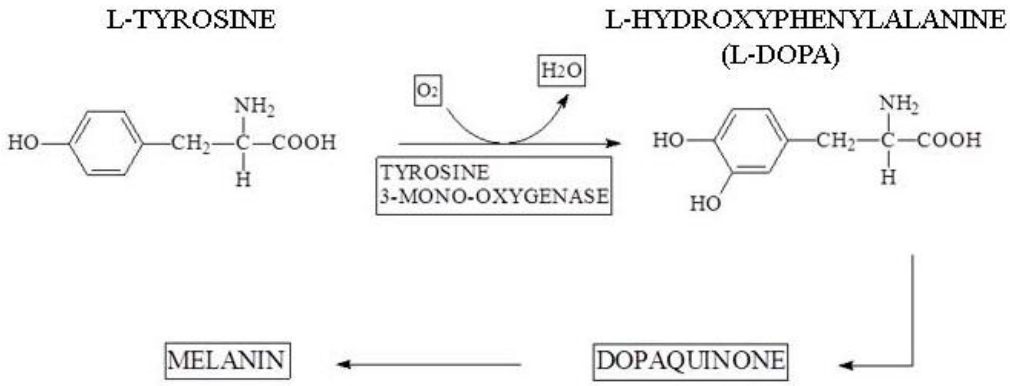

Figure 7. Melanin synthesis in the reaction of tyrosinase and L-DOPA.

Animal and human studies demonstrate that adequate protein nutrition is crucial for the maintenance of GSH homeostasis [30]. In addition, enteral or parenteral cysteine, methionine, N-acetyl-cysteine, and L-2-oxothiazolidine-4-carboxylate are effective precursors of cysteine for tissue GSH synthesis [31]. Raising GSH levels through direct supplementation of glutathione is difficult. Research suggests that glutathione taken orally is not well absorbed across the gastrointestinal tract. In a study of acute oral administration of a very large dose (3g) of oral glutathione, Witschi and coworkers found that it is not possible to increase circulating glutathione to a clinically beneficial extent by the oral administration of a single dose of 3g of glutathione [32]. Figure 8 shows the role of GSH in homeostasis. [33].

Vitamin D increases glutathione levels in the brain and appears to be a catalyst for glutathione production [34]. The amount of activated vitamin D in the brain is tied to how much vitamin D_3 (Cholecalciferol) one has either ingested through supplements or created in the skin via sun exposure. This suggests that taking vitamin D_3 (Figure 9) supplements and/or getting adequate sun exposure boosts glutathione production.

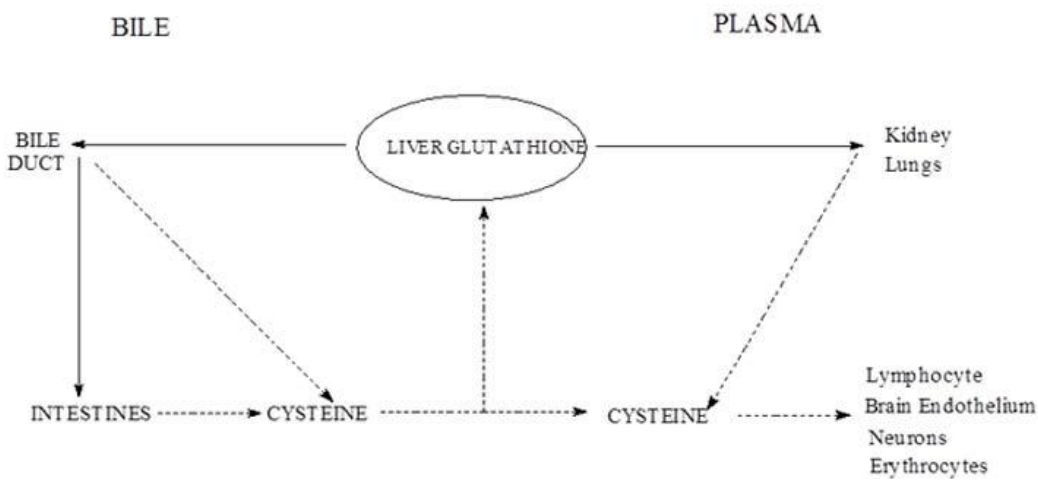

Figure 8. Homeostasis of GSH and Cysteine.

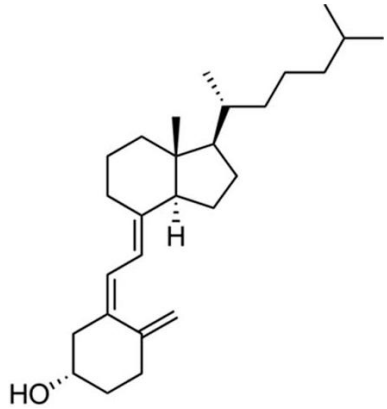

Figure 9. Cholecalciferol (Vitamin D_3).

In addition, plasma and liver GSH concentrations can be raised by administration of certain supplements that serve as GSH precursors. N-acetylcysteine, commonly referred to as NAC (figure 10), is the most bio-available precursor of glutathione [35].

Other supplements, including *S*-adenosylmethionine (SAMe) (figure 11) [36], and whey protein [37], have also been shown to increase glutathione content within the cell.

NAC is available both as a drug and as a generic supplement. Alpha lipoic acid has also been shown to restore intracellular glutathione [38]. Melatonin has been shown to stimulate a related enzyme, glutathione peroxidase [39].

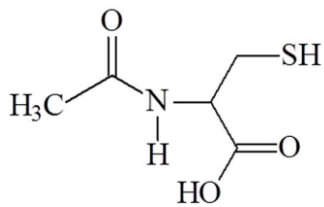

Figure 10. N-Acetylcysteine.

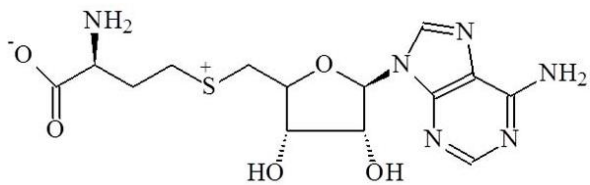

Figure 11. S-adenosylmethionine.

Glutathione (gamma-glutamyl-cysteinyl-glycine; GSH) is the most abundant low-molecular-weight thiol, and GSH/glutathione disulfide is the major redox couple in animal cells. The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes, gamma-glutamylcysteine synthetase and GSH synthetase. Compelling evidence shows that GSH synthesis is regulated primarily by gamma-glutamylcysteine synthetase activity, cysteine availability, and GSH feedback inhibition [11]. Its production is limited by negative feedback inhibition generated by its own synthesis

through the enzyme gamma-glutamylcysteine synthetase, thus greatly minimizing any possibility of over dosage. Glutathione augmentation using precursors of glutathione synthesis or intravenous glutathione is a strategy developed to address states of glutathione deficiency, high oxidative stress, immune deficiency, and xenobiotic overload, in which glutathione plays a part in detoxification of the xenobiotic in question (especially through the hepatic route). Glutathione deficiency states include, but are not limited to, HIV/AIDS, chemical and infectious hepatitis, myalgic encephalomyelitis chronic fatigue syndrome ME/CFS [40], prostate and other cancers, cataracts, Alzheimer's disease, Parkinson's disease, chronic obstructive pulmonary disease, asthma, radiation poisoning, malnutritional states, arduous physical stress, and aging, and has been associated with suboptimal immune response [41]. Indeed, many clinical disorders have been associated with GSH metabolisms and some oxidative stress biomarkers as shown in Table 3.

Low glutathione is also strongly implicated in wasting and negative nitrogen balance as seen in cancer, AIDS, sepsis, trauma, burns and even athletic overtraining [42]. Glutathione supplementation can oppose this process, and in AIDS, for example, results in improved survival rates [43]. However, studies in many of these conditions have not been able to differentiate between low glutathione as a result of acutely (as in septic patients) or chronically (as in HIV) increased oxidative stress, and increased pathology as a result of preexisting deficiencies.

Schizophrenia and bipolar disorder are associated with lower glutathione. Accruing data suggest that oxidative stress may be a factor underlying the pathophysiology of bipolar disorder, major depressive disorder (MDD), and schizophrenia. GSH is the major free radical scavenger in the brain [44].

Diminished GSH levels elevate cellular vulnerability towards oxidative stress, characterized by accumulating reactive oxygen species [45]. Preliminary results indicate that changes in the level of reactive oxygen species by glutathione in isolated cells grown in a laboratory may reduce cancer development [46].

Glutathione (GSH), glutathione S-transferases (GSTs), and the multidrug resistance-associated protein 1 (MRP1) have been independently studied for their contributions to drug resistance. O´Brien and cols. [47], assembled these proteins together, because they can act as a concerted coordinated pathway, and increased their expression through single cDNA transfection of GSTpi, gamma-glutamylcysteine synthetase (gamma-GCS) (regulatory plus catalytic subunits), or MRP1 enhanced resistance to a number of anticancer drugs. When all three were transfected, significantly higher levels of resistance were found for doxorubicin and etoposide.

Huang et al. [48], suggest that GSTP1 contributes to doxorubicin and cisplatin resistance in osteosarcoma, which may be mediated in part by the activation of extracellular signal-regulated kinase 1/2. Targeting of GSTP1 combined with chemotherapy may have synergistic therapeutic effects on osteosarcoma.

Excess glutamate at synapses, which may be released in conditions such as traumatic brain injury, can prevent the uptake of cysteine, a necessary building-block of glutathione. Without the protection from oxidative injury afforded by glutathione, cells may be damaged, because GSH plays a critical role in cellular defense against electrophiles, oxidative stress and nitrosating species.

Table 3. Clinical disorders on GSH system and some biomarkers of oxidative stress, Under (↓), Up (↑)

No	Disease	Effect on GSH or Inherited biomarkers	Tissue	References
1	Parkinson´s disease	GSH-Px ↓, GSH ↓, Lipid peroxidation ↑	Substantia nigra	Del Hoyo et al, 2010[67].
2	Poisoning by polychlorobiphenyls	GSH-Px ↓, GSH-S-transferase ↓	Liver	Agletdinov et al, 2009[68].
3	Poisoning by cadmium	NO ↓, Lipid peroxidation ↑	serum	Martynowiccz et al, 2004[69]
4	Acute stroke	GSH ↑, GSH-Px ↑	serum	Zimmermann et al, 2004[15]
5	Erythema migrans	Lipid peroxidation ↑	Plasma	Pancewicz et al, 2001[70]
6	Alzheimer´s disease	GSH ↓	Lymphoblasts	Cecchi et al, 1999[71]
7	Axonal neuropathy	GSH	Axon	Hilkens and ven den Bent 1997[72]
8	Severe neurologic disorders	GS deficiency	Multiple tissues	Winkler et al 2011[27]
9	Traumatic brain injury	Gamma-glutamylcysteine ethyl ester ↑	Brain endothelium	Lok et al, 2011[73]
10	Severe bronchiolitis	GSH- S-transferase, GSH-Px, SOD ↓	Lungs and airways	Hosakote et al, 2011[74]
11	Resuscitated 100% oxygen	GSH ↓	Hippocampus	Walson et al, 2011[75]
12	Hypoxia/Reoxygenation	GSH and GSH-Px ↓	Intestinal samples	Ozdemir et al, 2010[76]
13	Sickle cell disease	GSH Reductase, NOx ↑	Erythrocytes	Rusanova et al, 2010[77]
14	Migraine	GSH-Px, Catalase ↓	Erythrocytes	Erol et al, 2010[78]
15	Osteosarcoma	GST polymorphisms	Bone tumor	Salinas et al, 2010[79]
16	Cholestatic chronic liver	GSH-Px, Catalase ↑	fresh liver	Ismail et al, 2010[80]
17	Acute rheumatic fever	Malonyldialdehyde ↑, GSH ↓	Blood	Uner et al, 2010[81]
18	Neuroinflammatory demyelinating	GSH ↓	white matter	Khan et al, 2010[82]
19	Glycogen storage disease Ia and III	GSH-Px ↓	Blood	Kalkan et al, 2010[83]
20	Antiasthmatic therapy	DNA, GSH ↓	Blood	Hasbal et al, 2010[84]
21	Bronchopulmonary dysplasia	Glutathione disulfide ↑	Lung	Rogers et al, 2010[85]
22	Necrotizing enterocolitis	GSH-Px ↓	Intestinal	Guven et al, 2009[86]
23	Type 1 Diabetes mellitus	GSH-Px ↓	Blood	Codoñer et al,

No	Disease	Effect on GSH or Inherited biomarkers	Tissue	References
				2010[87]
24	Hyperoxia-induced	Malonyldialdehyde ↓	Lung	Pan et al, 2009[88]
25	Down Syndrome	SOD, GSH-Px ↑	Neutrophils	Akinci et al, 2010[89]
26	Baby response to hyperoxia	SOD, GSH-Px ↓	Lung	Patel et al, 2009[90]
27	Autism	GSH/GSSG ratio ↓	Mitochondria of Lymphoblastoid cells	James et al, 2009[91]
28	Migraine	GSSG-R, GSH-Px ↑	Serum,erythrocytes	Bockowski et al, 2008[92]
29	Severe malnutrition	GSH ↓	Blood	Jahhor et al, 2008[93]
30	Epilepsy	SOD, GSH-Px ↓	Serum, saliva	Sobaniec et al, 2007[94]
31	Gastroenteritis in Children	GSH-Px ↑,	Blood	Bayiroglu et al, 2009[95]

Administration of L-cysteine precursors and other strategies allow GSH levels to be maintained under conditions that would otherwise result in GSH depletion and cytotoxicity. Conversely, inhibitors of gamma-GCS have been used to deplete GSH as a strategy for increasing the sensitivity of tumors and parasites to certain therapeutic interventions [49].

However, once a cancer has already developed, by conferring resistance to a number of chemotherapeutic drugs, elevated levels of glutathione in tumor cells are able to protect cancerous cells in bone marrow, breast, colon, larynx, and lung cancers [50], because selenium is a cofactor of GSH-Px [15], and distinctive phenotype can only be explained by the combined deficiency of functionally important protection of normal cell from selenoproteins [51].

Some recent studies show the effect of antioxidant enzyme cofactors-zinc and selenium-supplementation on clinical disorders (Table 4).

This trace element supplementation may prevent free radical damage and sometimes shorten treatment time in patients using long-term drug treatments [52]. Further studies are needed to assess whether antioxidant supplementation will be beneficial as an adjunct to conventional relevant therapy of any disease.

When patients are admitted in the Intensive Care Unit in hospital due to health complications as consequence of any disease, it is common that the clinical conditions of nutrition be found in deficiency in many cases even when the minimum daily requirements had been met [53].

Deficient ingestion of nutrients affects all body cells in varying degrees, with repercussions in different organs and systems, thereby modifying the capacity of the organism to respond to aggressors of diverse nature.

Nutritional oxidative stress describes an imbalance between the pro-oxidant load and the antioxidant defense as a consequence of excess oxidative load or of inadequate supply of the organism with nutrients [54].

Table 4. Studies of trace elements (Zn=Zinc and Se=Selenium) in recent years

Symbol	Biological effects	References
Zn	The zinc and micronutrients treatment increased the lymphocyte ratios of CD4(+) to CD8(+) and of CD4(+)CD45RA(+) to CD4(+)CD45RO(+).	Sandstead et al, 2008[96]
Zn	Vitamin A plus zinc reduces Giardia lamblia incidence, whereas zinc supplementation increases Ascaris lumbricoides incidence but decreases Entamoeba histolytica-associated diarrhea.	Long et al, 2007[97]
Se	The concentrations of minerals and trace elements in infant formulas for full-term infants are generally higher than in human milk, and all appear to be more than adequate, with the possible exception of selenium, which may need to be increased in some formulas.	Flynn 1992[98]
Zn	The incidence rate ratio (IRR) of developing a cold (IRR 0.64; 95% CI 0.47 to 0.88) (P = 0.006), school absence (P = 0.0003) and prescription of antibiotics (P < 0.00001) was lower in the zinc	Singh and Das 2011[99]
Se, Zn	Elevated levels of serum lead and mercury and low levels of zinc and selenium may suggest some disturbances in the antioxidant system in children with recurrent wheezing.	Vernacchio et al, 2011 [100]
Zn	Zinc reduces the frequency and improves recovery rates of diarrhea in any form and has better compliance and outcomes with the use in suspension form.	Van Santen et al, 2011[101]
Fe, Zn	The Micronutrients powder is likely to enhance absorption of the high native iron content of complementary foods based on cereals and/or legumes.	Habib et al, 2010[102]
Zn	Aspects of intellectual functioning including working memory, inhibitory control, and fine motor functioning among offspring were positively associated with prenatal iron/folic acid supplementation in an area where iron deficiency is prevalent.	Troesch et al, 2011[103]
Zn	Zinc is safe in HIV-infected adults and children. It may have similar benefits in HIV-infected children and adults, and uninfected children with diarrhoea, as it does in HIV-uninfected children	Christian et al, 2010[104]
Zn	Receiving zinc the mean weight of body mass index decreased significantly. Exploring the direct clinical application of zinc supplementation in childhood obesity in future studies.	Kelishadi et al, 2010[105]
Se, Fe	May be alterations in the serum levels of trace elements in obese children and these alterations may have a role in the pathogenesis of obesity.	Tascilar et al, 2010[106]
Se	The serum selenium level in the children who had simple febrile seizures was significantly lower than in the nonseizure control group. It seems that there is an association between serum selenium deficiency and simple febrile seizures.	Mahyar et al, 2010[107]
Zn	Profound changes in the thymus can also be seen in deficiencies of vitamins and trace elements. Taking Zn deficiency as an example, there is a substantial thymic atrophy.	Savino et al, 2010[108]
Se	Se status is depressed among pediatric patients with burns and that	Dylewski et

Symbol	Biological effects	References
	recommended Se intake for healthy children is likely insufficient for this population.	al, 2010[109]
Zn	Zinc supplementation is effective in hepatic encephalopathy and consequently improves patient health-related quality of life.	Takuma et al, 2010[110]
Zn	Concentrations of Zn, Ca, K, and Mg were lower in the scalp hair and blood but higher in the urine samples of night blindness children.	Afridi et al, 2010[111]
Se	Studies of populations exposed to Methyl-Mercury (MeHg) by eating Se-rich ocean fish observe improved child IQs instead of harm.	Ralson and Raymond 2010[112]
Se	Cerebrum Spinal Fluid Se values were 32 times lower when compared with those for plasma and there was an association observed between CSF Se and GSH-Px activity.	Tondo et al, 2010[113]

Thus, when malnutrition is present, it is expected that the imbalance between production of free radicals and their safe disposal through the activation of antioxidant enzymes or antioxidant nutrients, promotes increased oxidative stress [55]. Nutritional recovery is a complex process which is not free from complications, because not only the amount of nutrients but also the type and form of administration, are determinants [56]. Experimental studies have shown that the selective administration of nutrients, and the addition of some supplements, tends to show positive results in animals that have been subjected to malnutrition [57].

With the recognition that nutritional recovery is not just a feed related procedure, the clinical management of malnutrition, has incorporated focused strategies to deal with specific aspects of malnutrition that could affect the rehabilitation of the whole organism. The development of clinical strategies targeted to the reduction of the risk of suffering additional damage by oxidative stress during nutritional recovery has become very important issue. The incorporation of cysteine prodrugs or zinc in the diet of animals that have suffered from malnutrition has proven to be useful in regulating glutathione levels [58]. L-cysteine (figure 12), is thought to be a conditionally essential (i.e., essential under certain conditions) amino acid for neonates. It is a precursor of glutathione, an antioxidant that may reduce oxidation injury and potentially improve bone mineralization. However, there is insufficient evidence to assess the risks of cysteine supplementation in neonates, especially regarding metabolic acidosis, which has been reported during the first two weeks of cysteine chloride administration [59].

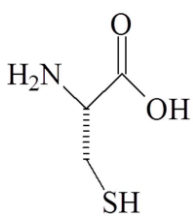

Figure 12. L-Cysteine.

Studies carried out in recent years, strongly suggest that a therapy focused on restoring the antioxidant capacity by applying cysteine equivalents in the form of glutathione is beneficial for biochemical and clinical recovery.

2.2. Methods to Determine Glutathione and some Biomarkers Related to Oxidative Stress

The most common techniques reported are as follows: Radioimmunological methods of markers such as glutathione S-transferase [60].

Concentrations of malondialdehyde (MDA) may be examined by means of Bioxytech LPO-586 kit in plasma. The total sulphydryl groups (-SH) according to Ellman and reduced glutathione (GSH) may be measured using a Bioxytech GSH-400 test in plasma (Bioxytech Co).

Reduced glutathione (GSH), may be visualized using Ellman's reagent or bimane derivates such as monobromobimane. The monobromobimane method is more sensitive. In this procedure, cells are lysed and thiols extracted using HCl buffer. The thiols are then reduced with dithiothreitol (DTT) and labeled by monobromobimane. Monobromobimane becomes fluorescent after binding to GSH. The thiols are then separated by HPLC and the fluorescence quantified with a fluorescence detector. The quantification is done by confocal laser scanning microscope after application of the dye to living cells [61].

Another approach, which allows measuring the glutathione redox potential at a high spatial and temporal resolution in living cells is based on redox imaging using the redox-sensitive green fluorescent protein (roGFP) or redox sensitive yellow fluorescent protein (rxYFP) [62].

Assessment of GSH and redox state by tandem mass spectrometry [63]. ER stress and apoptotic signaling examined through RT-PCR [64].

Glutathione carried out according to a modified method from Hissin y Hilf [65], $HClO_4$-homogenised tissue was centrifuged at 5000 rpm for 5 minutes (Mikro 12-42, Germany), and reduced glutathione levels were measured in the supernatant. 1.8 mL phosphate buffer pH 8.0/0.2% EDTA were poured in a test tube, then 1 mL aliquot of the supernatant and 100 µL of 1 mg/mL ortho-phthaldehyde dissolved in methanol (w/v), were added. The entire mixture was incubated at room temperature for 15 minutes, totally protected from light, and then the samples were read in a Perkin Elmer LS 55 spectrofluorometer at 350 nm/420 nm, excitation/emission.

Thiobarbituric acid reactive substances (TBARS), carried out according to the method of Gutteridge and co-workers [66], as follows: Each tissue sample was homogenised in 5 mL of phosphate buffer pH 7.4, from which, a 1 mL aliquot was taken and added to 2 mL of a thiobarbituric acid (TBA) solution containing TBA (1.25 g), trichloroacetic acid (40g), concentrated HCl (6.25 mL), dissolved in 250 mL deionised water. The whole mixture was heated to boiling point of water for 30 minutes (Thermomix 1420). Samples were then put in an ice bath for 5 minutes and then centrifuged at 3000 g for 15 minutes (Sorvall RC-5B Dupont). Supernatant absorbances were read in a three-set scheme at 532 nm spectrophotometrically (Heλios-α, UNICAM).

Conclusion

The incorporation of antioxidants in the diet during nutritional recovery should be considered of great importance in the clinical management of malnutrition and any disease, because when GSH is employed in conjunction with antioxidants and nutritional factor, it becomes a powerful orthomolecular tool for quality of life.

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Part II: Glutathione in Mammalian Cell and Clinical Implications

In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter IV

Glutathione Studies and its Applicability in Animal Reproduction: An Alternative Therapy against Infertility?

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Abstract

Glutathione (L-gammaglutamyl-L-cysteinylglycine; GSH) is a tri-peptide of the amino acids cysteine, glycine, and glutamic acid, is the most abundant non-protein thiol in mammalian cells, being present in concentrations of 0.5 – 10 mmol/L. GSH participates in various cellular processes such as cell proliferation, amino acids transport and cysteine reserve. However, the most important function is the cellular protection against oxidation, since the sulfhydryl group (SH) is a strong nucleophile, which protects against damage from oxidants, neutrophils and free radicals. GSH is a critical intracellular antioxidant due to its relative abundance and ability to quickly interchange between the reduced and oxidized forms (glutathione disulfide - GSSG). During periods of oxidative stress, GSH eliminates intracellular free radicals excess and by oxidation of the thiol group and its cysteine residue, it is oxidized to GSSG by the action of glutathione peroxidase (GPx), which then converts back to GSH via GSSG reductase. Thus, several studies on the biological evolution of GSH indicate that there is a close correlation between GSH and aerobic eukaryotic metabolism, showing that this

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molecule evolved to protect cells against the toxicity caused by oxygen. Sperm cells possess a plasma membrane as a coating, rich in polyunsaturated fatty acids, which provide the fluidity needed to participate in the sperm membrane fusion events necessary for the fertilization. However, sperm suffers peroxidation that alters its fluidity, reducing the ability of fertilization. Furthermore, they reduced their cytoplasm during spermatocytogenesis, this highly specialized cell features small amount of antioxidant stored, becoming more susceptible to damage caused by oxidative stress. The action of reactive oxygen species (ROS) in semen has been identified in the mid-'50s, when sperm cells from human exposure to high concentrations of oxygen resulted in toxicity, and consequently,cause rapid loss of motility. Excessive production of ROS in sperm determines dysfunction and decreased their fertilizing capacity. Under natural conditions, the sperm is exposed to a nearly anaerobic environment, thereby reducing the potential damage caused by ROS. *In vitro*, the oxygen metabolites, such as hydrogen peroxide (H_2O_2), are continuously produced. Moreover, spermatozoa manipulated for in vitro fertilization or cryopreservation undergo exposure to oxygen in various stages of processing, that lead to ROS production and reduction of natural defense antioxidants due its previous dilution. Thus, the antioxidants addition, only or in combination, in procedures for handling semen has been performed in several species of wild and domestic animals. Due to the ability of GSH, several studies have been conducted to evaluate the effect of adding different concentrations of this antioxidant in semen diluents for refrigeration and freezing. However, the results still differ as to the benefits of supplementation. This chapter will contemplate the action of GSH and the results obtained from its use as antioxidant agent.

Abbreviations

GGT= γ-glutamiltransferase
GPx= glutathione peroxidase
GR= glutathione reductase
GSH= glutathione reduced
GSSG= glutathione disulfide
Gt= glutathione transferase
LPO= lipoperoxidation
MDA= malonaldehyde
RNS= reactive nitrogen species
ROS= reactive oxygen species
SOD= superoxide dismutase

1. Introduction

The advance of medicine and concern for the welfare enable the search for alternatives to increase the prospect of human life. Throughout several researches, it has been observed that appearance of premature aging and disease are related to changes in the diet, lifestyle, rest time reduced and, consequently, an increase of stress conditions [1].

Moreover, the easy communication between different levels of society by the media and possibility of new treatments, made enable to search for solutions to problems stemming from

post-modern life, such as hair loss, insomnia, digestive disorders, difficulty in children generation, among others [2]. Currently, available medical treatments were based on oxidants attack caused by free radicals formed by exposure of individuals to stressful conditions. Due to this, the knowledge of the mechanism which initiates the formation of free radicals is extremely important.

Oxygen is one of essential molecules to sustain life in aerobic organisms. However, oxidative metabolism as main source for cell energy is capable to generate active oxygen metabolic called reactive oxygen species (ROS). Among them may be cited superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) [3], which are involved in hundreds of disease, as arthritis and other tissue connective diseases, cancer, aging, physical injuries, infections and acquired immunodeficiency syndrome (AIDS) [4].

Reactive oxygen speciesare molecules which contain one or more unpaired electrons, become highly energetic and able to react with cells and modify several biomolecules structure, including protein, lipids and nucleic acids. As most of molecules belonging to biological systems have only paired electrons, ROS are often involved in chain reactions, where new “free radicals” are formed [5] due to their inherent characteristic highly reactive oxidant [6].

It is known that to avoid deleterious ROS effects, cells developed a mechanism that work together against their excess production, establishing the antioxidant control. Antioxidant term means “to prevent oxidation of other chemical substances”, and are a heterogeneous substances group consisting of vitamins, minerals, enzymes, pigments and other natural compounds that blocks free radicals action or their deleterious effect [7]. According Halliwell [8], antioxidant substances, when present in low concentrations compared to that oxidizable substrate, retards or prevents substrate oxidation.

Cells present a defense system that can operate in two lines, first as a detoxifying oxirreductor agent before it causes cell damage, consisting by reduced glutathione (GSH), superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and vitamin E. The other line of antioxidants has the function of lesion repair and it is formed by ascorbic acid (vitamin C), GPx, glutathione reductase (GR), glutathione transferase (Gt), among others. Antioxidants are located in intracellular environment, except Vitamin E (α-tocopherol) that is a structural antioxidant of the membrane [9].

As above mentioned, this chapter will discuss about glutathione, redox cycle and its applications in reproduction due to its important antioxidant function.

2. Glutathione Biochemistry

Glutatione (γ-glutamilcysteinilglycin) is a water soluble antioxidant, recognized as non-protein thiol of greater biological significance in living systems. It is classified as a linear tripeptide, three amino acids composed: glutamic acid, cysteine and glycine, with cystein thiol group responsible for its biochemical properties.

GSH is glutathione reduced form and its sulfhidryl group (SH) is responsible for cell protection against oxidative stress [10].

The GSH molecular formula is $C_{10}H_{17}N_3O_6S$, with 307.325 molecular weight and fusion point between 185 to 195 ºC, water soluble and ethanol insoluble. GSH is presented in

colorless crystals or white crystalline powder. GSH is present in most cells at 1 to 8 mM concentrations and usually in large quantities in liver, its production site. GSH synthesis occurs in two steps (Fig. 1), intracellular environment, primarily catalyzed by γ-glutamilcystein synthetase, which acts in peptide bond formation between cysteine and glutamate amino acids, resulting in glutamilcystein substrate. Glutathione synthetase acts in current glycine catchment and performs a connection of glycine with γ-glutamilcystein, resulting in reduced glutathione production. These reactions occur with adenosine triphosphate (ATP) consumption and magnesium (Mg^{+2}). The γ-glutamilcystein synthetase undergoes GSH regulation by negative feedback, which prevents excessive production or γ-glutamilcystein accumulation [11].

Once formed, GSH is distributed by blood stream to all tissues. In extracellular region, glutathione concentration is among 5 to 50 mM [12] and can be found in reduced form (GSH) or oxidized (GSSG), its dimerized GSH, also known as glutathione disulfide [13]. GSH/GSSG ratio is normally used to estimate redox state (spontaneous or chemical tendency in electrons to acquire and thereby be reduced) of biological systems. In normal situations, GSSG represents only small fraction (less than 10%) of total glutathione [14].

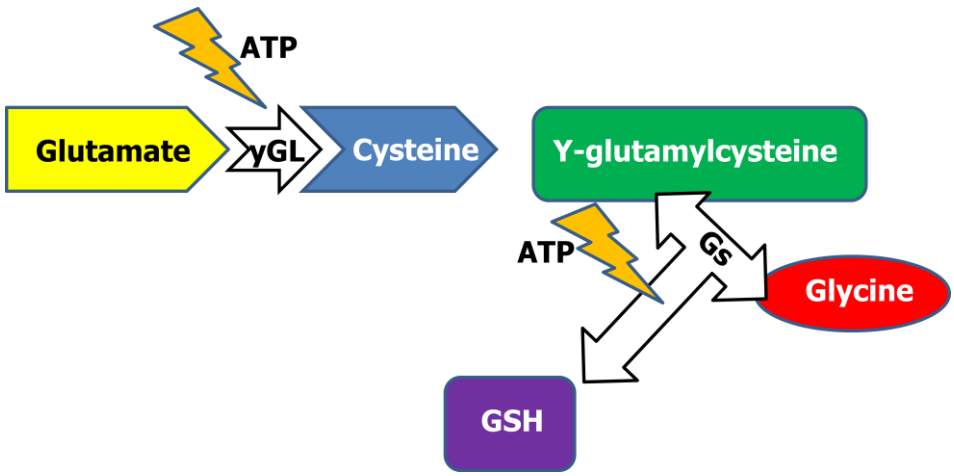

Figure 1. GSH biosynthesis. yGL: gamaglutamylcysteine ligase; Gs: Glutathione synthetase. Enzyme yGL acts binding glutamate and cysteine to form y-glutamylcysteine, as well as in Gs activity binding glycine and y-glutamylcysteine to GSH synthesis. These reactions need ATP consumption. Source: Li [13] adaptation.

The GSH and GSSG degradation occurs notably in kidneys, through an extracellular catalyzed reaction by γ-glutamiltransferase (GGT) and cysteinil-glycin-dipeptidase. GGT acts by breaking bonds between amino acids that constitute GSH, which are absorbed and utilized in a variety of intracellular reactions, which subsequently undergo rearrangement. In renal excretion, GSH produced inside cells by connection with cysteine is transported by renal tubule and glutathione is oxidized, being degraded by GGT and dipeptidase, where cystine is released into renal tubule, reabsorbed and reduced in into cells to cysteine [15].

GGT may also be involved in GSH synthesis, when attached to plasma membrane due its ability on amino acids recruitment or acts on γ-glutamil group transfer of GSH extracellular located in extracellular environmental for into cell.

2. Action Mechanism of Glutathione

According Meister and Anderson [16], GSH has ability to act at the body functions as cell proliferation, transport of amino acids, DNA and protein synthesis, disulphide and other chemical components reduction, cysteine reserve and antioxidant function. In mechanism related to cell proliferation, there are reports that amino acids released in GSH degradation are responsible for cell growth stimulation, such as glutamic acid [17].

As antioxidant action, GSH is known to be involved in a protection mechanism that promotes ROS inactivation. These toxic species can be detoxified by reducing glutathione peroxidase action, which converge GSH to GSSG. The GPx uses either two GSH molecules as electron donor in hydrogen peroxide conversion in water, but also uses it as substrate in catalytic reaction for organic hydroperoxides (LOOH) decomposition, from plasma membrane lipid peroxidation (LPO), to form water and your alcohols correspondents (LOH) [18].

Glutathione transferase, another enzyme present in GSH redox cycle, constitutes about 10% of cytosol protein in some mammals and serves to catalyze GSH conjugate (via sulfhydryl group) to electrophilic centers of a substrates variety. Therefore, this enzyme also has biological function of detoxify endogenous compounds such as lipid peroxides, as well as decrease xenobiotics toxicity, turning them into xenobiotics GSH-conjugated [19]. Glutathione transferase has binding capacity with proteins, thus, serves like a protein transport function. As antioxidant activity, glutathione transferase uses GSH as electron donor and converts LOOH to LOH and water. Thus, glutathione acts preventing both plasma membrane degradation, avoiding H_2O_2 deleterious action, as metabolization of damaged membrane products, making them less aggressive structures [13].

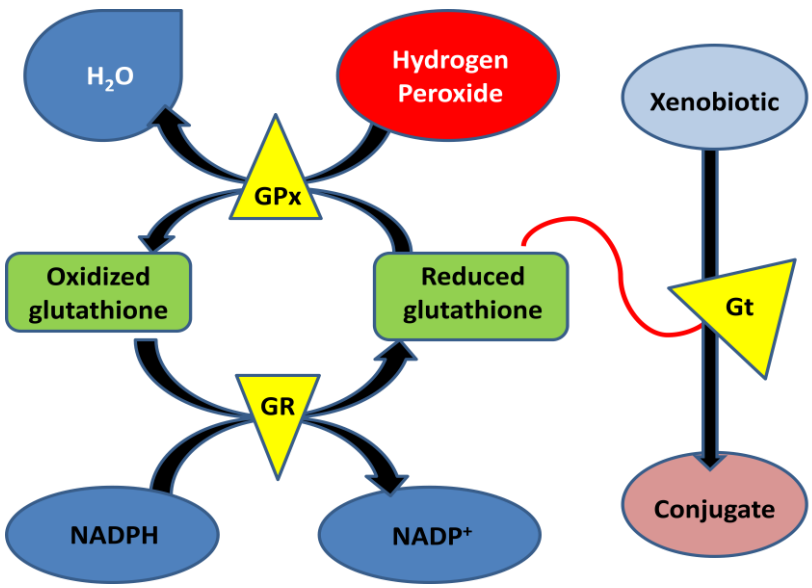

Figure 2. Glutathione cycle. GPx: glutathione peroxidase; GR: glutathione reductase; Gt: glutathione transferase. Source: Li [13] adaptation.

It should be noted that GSH interacts with other non-protein antioxidants, such vitamin E (α-tocopherol) and vitamin C (ascorbate). It is known that α-tocopherol function is to reduce lipid peroxyl radical to hydroperoxide lipid, consequently, result in its oxidized form, α-tocopherol radical. The ascorbate, on the other hand, converts α-tocopherol radical again to α-tocopherol oxidizing the dehydroascorbate. Finally, dehydroascorbate reductase enzyme reduced the dehydroascorbate to its form ascorbate, using GSH as a substrate. Thus, notes how is important maintain biological GSH concentrations for homeostasis and vitamins regulation in body, ensuring antioxidant defense action in biological system [20].

GSSG is reduced by action of glutathione reductase in NADPH presence, which acts as an electron donor, from a pentose, where one GSH molecule results in two GSSG molecules (Fig. 2).

3. Glutathione and Clinical Implications

Glutathione was first studied in mid-twentieth century and soon his presence was observed in most biological activities. After GSH functions knowledge, it was noted that GSH absence causes serious damages to body as failure in their development. Thereafter, experimental conditions simulated enzymes inactivation responsible for GSH regeneration and negative results were obtained, as cellular sensitization by radiation and ROS action, lower vitamin C concentration and visual changes such as cataracts [21].

Apoptosis or programmed cell death is an essential process to develop maintaining of living beings, it is important to eliminate redundant or defective cells. During apoptosis, cell undergoes morphological characteristics changes of this cellular death type. Such changes include cell shrinkage, adhesion loss between extracellular matrix and neighboring cells, chromatin condensation, DNA internucleossomic fragmentation and apoptotic bodies formation [22]. Apoptotic cell releases intracellular GSH by changes expression in plasma membrane. However, in GSH release inhibition by experimental condition was observed that cells remains viable and no triggers apoptosis process, indicating the substrate importance in cell viability maintenance [23].

In immunosuppressed patients, GSH concentrations are reduced drastically compared to patients with stable immune state. It is known that GSH in lower concentration may contribute to apoptosis induction in TCD4+ lymphocytes, resulting in higher lymphocytes DNA fragmentation, indicative of apoptosis [24].

Imbalance between oxidant/antioxidant activities and consequent increase in free radical concentrations at patients with chronic renal failure showed higher lipid peroxidation in erythrocytes and other blood cells, with important implications in patient’s morbidity, mainly caused by cardiovascular disease. Enzymatic antioxidants concentrations such as glutathione peroxidase, superoxide dismutase and catalase are reduced, as well as selenium and zinc elements. Thus, enzymes responsible for GSH regeneration are in low amounts, conferring lower protection to blood cells, which are exposed to oxidative damage and can to alter erythrocytes membrane fluidity, contributing for hemolysis intensification and anemia in these patients.

4. Glutathione in Male Reproduction

Previously, due to cultural factors and reproductive physiologic ignorance, it was believed that infertility or inability to reproduction was destined only to female. After reproduction science development was observed that male may be directly involved in infertility.

Infertility term is defined as a reproduction failure due to dysfunctions of reproductive organ, as well as gametes defective or abnormal fetuses. A previously fertile male can become infertile by diseases or problems that occur throughout his life. In addition, aging itself causes changes that reduce sperm and seminal fluid production in men, just as occur with oocytes in female, in the period known as reproductive senescence. Other factors such as illicit drugs, tobacco, alcohol, excessive exercise, anxiety, stress, depression and/or use of drugs interferes with this process, significantly altering sexual performance [26]. Current lifestyle also may influence exacerbated ROS production and organic antioxidant system cannot neutralize effectively oxygen byproduct deleterious effects, causing imbalance between antioxidants and oxidants [27].

Based on interest to find explanations to sperm physiology knowledge, since its formation, the interaction between seminal fluid and antioxidant are important for integrity cell preservation so there is fertilization.

4.1. Spermatogenesis and ROS

In reproduction, along post-pubertal reproductive life, spermatozoa are formed from spermatogonial stem cells through a highly controlled process, called spermatogenesis. The entire process consists in proliferation and differentiation sequential phases [28], resulting in a spermatozoon, where cellular changes of velocity may vary according to cellular sub-region [29].

During various spermatogenesis stages there are oxidative stress susceptibility in different levels. In early differentiation stages, where cell division process occurs in active form, deoxyribonucleic acid (DNA) is the main mutation target induced by ROS and an active cytoplasm presence plays a protective role, with different evaluation points for any faults detection and possible repair. Later, spermatozoa cytoplasm is absorbed on epididymal maturation became these cells more susceptible at ROS action. Finally, in DNA condensation phase, sperm plasma membrane became enriched with phospholipids, where approximately 50% of them are highly unsaturated and therefore more sensitive for LPO [30].

During spermatogenesis, there is an active lipid metabolism process that induces changes in constitution of fatty acids presents in plasma membrane. Polyunsaturated fatty acids (PUFAs), found in high concentration on sperm cells membrane are main targets LPO ROS-induced, with this process intensively related to cellular homeostasis regulation [31]. The most common LPO types are no-enzymatic and enzymatic, which is NADPH and ADP dependent, by ADP-Fe3+ O2- complex. In spermatozoon, there are indications that malonaldehyde (MDA) is LPO end product induced by iron ions. In general, the most LPO significant effect in cells is a structural disorganization such as cellular membrane and loss of organelles functions [6].

When a cell presents absence of cytoplasm, this cell isn't able to perform transcript and, consequently, there isn't enzyme activity in cytoplasmatic area, thus, there is low a defense capability against lesions ROS induced by sperm cell, a compounded factor by its PUFAs in high concentration at plasma membrane. On the other hand, ROS production inherent by sperm cells is key factors in training process [3].

In lower concentrations, ROS participates in various pathways signaling [5] and normal sperm functions such as capacitation, hyperactivation, acrosome reaction and spermatozoon-oocyte fusion [32]. When ROS imbalance becomes pro-oxidant, either by over-production or by antioxidants activity decreasing, it can be stated that system is in oxidative stress condition [6] associated with increase cell damage rate [33]. At this time, male fertility rate is compromised and negative results begin to be noticeable, leading many couples to seek expert help to resolve their reproduction inability.

4.2. Behavior, Reproduction and Fertility

Several studies have been developed about possible causes in male infertility and, consequently, treatment protocols establishing. Although the literature is very rich in researches based in drugs consumption patients and its toxic effects, such as tobacco and alcohol, their results are very diverse, with negative influence or no [34]. Cigarette smoking appears to have an adverse effect on reproductive processes, as well as gametes production and function, ovulation, cyclicity, fertilization, cleavage, embryo transport and implantation. Other studies demonstrated a significant increased risk of spontaneous fetus abortion with normal chromosomes, as well as birth of premature babies in smokers [35]. In men, there is clear evidence that smoking results in low sperm concentration, as well as sperm motility loss when compared to men do not smoke [36].

Smoking not only increases endogenous ROS production as well as negatively interferes in antioxidant activity. This statement is grounded in studies that showed superoxide dismutase, catalase, GPx and Gt enzymes activity reduced after cigarettes agents' exposure [37]. Thus, there is increased in ROS production and decreased in enzymes responsible for oxidation and oxidizing agent reduction, cellular damage is intensified, resulting in membrane sperm LPO and fertility reduction [38]. Through discussions about causative agents to cell deleterious effects, was initiated to investigate antioxidants defenses, as well as its role on spermatozoon.

Concurrently to treatments where reproductive cells are exposed to aggression, reproductive medicine also used in cryobiological conservation knowledge for adverse effects prevention, as existing in treatment for several diseases, for example, the cancer. It's known that cancer medication therapy usually attack multiply cells and may adversely affect production gametes. Thus, its suggested sperm collection and storage in patients exposed to chemotherapy and/or radiotherapy [39].

Unlike the purpose of human medicine, veterinary medicine uses cryopreservation applied to animals with genetic superiority, in order to genetic material quality maximize as well as to preserve sperm in life endangered animals, like captivity animals who have difficult mating, thus, sperm can be stored and used for artificial insemination in the future.

Cryopreservation process requires sperm samples submitted to controlled and moderate cooling phase in order to preserve its function, which oocyte fertilization [40]. Temperature

reduction control decreases possibility in cell viability loss by reducing in large ice crystal formation inside sperm cell [41]. Several studies suggest that sperm from different species have specific cryobiological properties and sensitivity degrees varieties to experimental manipulation, as heat shock (lipid transitions phases), freezing and osmolarity tolerance [42].

According Ladha [43], sperm plasma membrane is rich in phospholipids and physiologically asymmetric, with phosphatidylcholine and sphingomyelin located in outer leaflet in lipid bi-layer, while phosphatidylserine and phosphatidylethanolamine are located in inner leaflet. During cryopreservation, plasma membrane undergoes modifications to suit temperature changes, as phospholipids translocation movement, with phosphatidylserine externalization.

Oxygen toxic effects are resulting from cellular components oxidation such thiols, cofactors, proteins, nucleotides and lipids, especially polyunsatured fatty acids, mediated by ROS and reactive nitrogen species (RNS) action [44]. ROS and RSN reactions with polyunsatured fatty acids presents in membrane cellular and spermatozoa lipoproteins initiates LPO, which can be evaluated and used as an oxidative stress indicator in sperm cell [45]. This plasma membrane dislocation can to alter spermatozoon selective capacity, leading at intracellular contents loss, including GSH concentrations.

In addition, decreasing temperature and its deleterious effects promotes reduction on antioxidants present naturally in ejaculate, associated cryopreservation steps as prior dilution and centrifugation that interfere to initial ejaculate volume, which cause imbalance between oxidants and antioxidants quantities, resulting cellular stress [46]. Antioxidant enzyme concentrations such as superoxide dismutase and catalase decrease considerably in dilution sperm, as well as substrates that electrons provider, like GSH [47].

Reduced glutathione has to be studied in detail by directly participation in ROS metabolization and to suffer direct interference in enzymes glutathione biodisponibility. Still, for to solve possible imbalance, antioxidants addition to preparation, maintenance and freezing extenders [48] began to be studied in different species and cryopreservation process.

4.3. GSH Biological Activity in Reproduction

Initially, developed studies seek for information about quantities of substrate and enzymes presents in ejaculate, as well as glutathione distribution in male reproductive system, according several species. Later, knowing about GSH presence both cytosolic and extracellular regions, Martensson et al. [49] evaluated glutathione quantities in spermatozoon and seminal plasma. A highest glutathione concentration was found in sperm cells, with small amount this antioxidant in seminal plasma. However, there are species that have this inverse proportion (Table 1).

Human sperm contains an enzymatic system comprising GPx, GR and its substrates, GSH and GSSG [55]. This defense system acts against sperm LPO by lipid hydroperoxides formation reduction, which are highly reactive and causes plasma membrane damage, transforming them into hydroxilipids, which are inert and not confer plasma membrane destruction. Enzymatic activity, measured by time reaction, determined that GPx and GR in humans are constant in healthy donors groups, in contrast to other enzymes such SOD, which presents variable concentrations and demonstrates no correlation between fertile and infertile men [56].

Studies had shown that glutathione transferase enzyme (Gt) is located on the sperm surface and is classified sperm-binding protein, responsible for binding between spermatozoon and oocyte [57]. In animals, goats specifically, Hemachand e Shaha [58] found that Gt uses extracellular GSH to inhibit sperm loss fertilization. In the same experiment, the authors used Gt inhibitors and observed that this enzyme was unable to perform its biological function, decreasing fertility rates.

Table 1. Glutathione concentrations in spermatozoa and seminal plasma of mammalian different species

Species	Spermatozoon	References	Seminal Plasma	References
Human	6.2±0.6nmol/10^8 sptz	Griveau et al. [50]	0.19±0.11µM	Daunter et al. [51]
Boar	0.03nmol/10^8 sptz	Li [52]	185.8±46.7µM	Strzezek et al. [53]
Bovine	566±72pmol/MG Protein	Bilodeau et al. [46]	17±7 pmol/mg protein	Bilodeau et al. [46]
Canine	0.53±0.11nmol/10^8	Li [52]	-	-
Ovine	0.45±0.14nmol/10^8	-	-	-
Equine	-	-	77.27±48.0mg/100 cm^3	Strzezek et al. [53]
Cuniculi	0.01nmol/10^8	Li [52]	-	-
Cricetidae	30-40nmol/mg protein	Den Boer et al. [54]	-	-
M. musculus	90nmol/10^8	Alvarez and Storey [55]	-	-

Source: Luberda [10] adaptation.

According Raijmakers et al. [59], glutathione concentrations in seminal plasma is essential for fertility, since lower concentrations of this thiol are associated with subfertility or infertility in men. This hypothesis is based in glutathione redox cycle function, a time when lower antioxidants concentrations, sperm cells undergo major ROS action, determined LPO process. In sperm cell, LPO results in cell inability to undergo capacitation, because prior plasma membrane destabilization and, thus, there is no fusion between acrosomal outer and plasma membranes, making it impossible for sperm fertilization. When antioxidants concentrations are very low, most cells undergo LPO action and hence the fertilizing ability of ejaculate also reduced drastically, becoming infertile or subfertile man [60].

In comparison with human species, yet it does know that glutathione enzymes concentrations expressed in large amounts in both seminal plasma and sperm cells in animals. Marti et al. [61] quantified enzymes present in ejaculated ram submitted for cryopreservation, and observed that GPx and GR presents high quantity and activity, even in after cryopreservation sperm, compared to fresh sperm. On the other hands, Bilodeau et al. [46] identified up to 78% reduction in GSH substrate concentrations at bovine sperm post-freezing. In boar sperm cryopreservation also observed GSH concentration reduction [62], precluding oxidants formed reduction, independent of enzymes GPx and GR concentrations. Thus, become interesting to GSH addition in sperm cells diluent cryopreservation.

Bilodeau et al. [63] evaluated GSH, GPx and GSSG addition to bovine sperm diluent freezing, which concluded that only GSSH addition failed efficiently on GSH regenerate, as well as GSH micromolar concentrations were no efficient in bovine sperm preservation. Bilodeau and coworkers suggested GSH addition to be made in milimolar concentrations and GPx in small quantities (5 U/mL). Gadea et al. [64] added 1 or 5 mM GSH in bovine diluent freezing and found a higher number of viable sperm, reduced ROS formation reduced DNA fragmentation and increased oocytes penetration rate *in vitro*, as well as higher embryo production rate, compared to sperm that didn't receive GSH substrate supplementation. These authors declared that GSH addition enabled better quality to sperm cells after thawing.

Sinha et al. [65] tested GSH supplementation (2 and 5 mM) in goat's sperm cryopreservation, and identified satisfactory results in fertilization rate at 5 mM GSH addition, compared to control group. Soares et al. [66] tested GSH addition (2, 5 and 7 mM) in cryopreservation goat semen and found better results with 2 mM concentration on motility and integrity sperm cells. Probably, these differences in results between animals of the same species may be related to factor such as breed, individuality, as different breeds can express changes in seminal plasma composition, according to *habitat*, just as between individuals and ejaculates from a same individual, since conditions of sperm subpopulations [67].

Even with goat sperm, Khalifa and El-Saidy [68] tested GSH addition at 0.61 mg/mL concentration at diluent freezing and observed that both diluted sperm before freezing and immediately after thawing, there were no difference between in the supplemented and control group. However, after one hour incubation was observed that sperm without GSH addition significantly reduced motility and increased MDA production, a LPO byproduct, whereas sperm with GSH addition kept motility percentage, while MDA production remained low. These findings suggest that antioxidant action may be not verified in the first moment after thawing, but maintains sperm cells longevity, which can be useful for sperm passage in female reproductive tract.

Ram's sperm cell, when submitted to crypreservation, either by refrigeration or freezing, significantly reduces its fertility capacity. Bucak and Tekin [69] evaluated GSH addition in 5 to 10 mM concentrations in stored sperm cells at 5 ºC and observed an improvement in motility and integrity sperm parameters. Uysal et al. [70] have increased motility and integrity sperm after GSH addition (5 mM) in freezing diluents of ram's sperm. This research group worked with GSSG substrate addition (5 mM) in ram diluents cryopreservation and obtained better of motility sperm parameter post-thaw, differently in ram's sperm without antioxidant addition [71].

Silva et al. [72] found that GSH addition in 2 and 5 mM concentrations acted to protect acrosome ram sperm structure submitted to freezing. However, these same authors found deleterious effects when added GSH concentrations higher 5 mM, indicating that antioxidant excess may become pro-oxidant, by disrupting balance between oxidants and antioxidants. Câmara et al. [73] tested GSH addition at different concentrations (0.5; 1.0 e 2.mM) in ram spermatozoa cryopreservation and observed that GSH concentrations addition increased, and also increased the antioxidant total capacity. However, there was evident improvement in sperm kinematics in the group that received antioxidant addition comparison. These authors also pointed out that probably these effects weren't obtained due to egg yolk properties, which have antioxidant activity of difficult estimate because not know their total components.

After verifying the reduction organic levels of GSH during boar sperm cryopreservation, Gadea et al. [62] decided to add GSH substrate at two different times. A group of ejaculated

received a GSH addition in freezing diluents and other group received GSH in thawing. In the first group was observed that sperm cells were not benefited, or even GSH had no biological effect at this time. In group that received GSH post-thaw, it was observed motility increased and more capacity of fertilization in vitro. These authors affirmed that GSH substrate was rapidly consumed in freezing process or temperature reduction and the sperm cells became unable for GSH use. The first hypothesis is most widely accepted, whereas if GSH would be available for non-use sperm cells, GSH would be used in the post-thaw moment.

Foote et al. [74] evaluated different GSH concentrations (0.1; 0.5; 1.0 e 2.0 mM) in bovine sperm refrigeration and identified that casein micelles presents in diluent, based on skim milk, interfered in protect ability of added substrate, in other words, casein micelles were also effective in sperm cell protection. Bovine sperm stored at 5 ºC for five days in medium containing egg yolk and 0.5 mM GSH preserved acrosome structure, in comparison with untreated antioxidant group cell. [75]. Stradaioli et al. [76] tested two commercial diluents and observed that GSH addition significantly reduced oxidative stress and preserved bovine sperm subjected to freezing process. Thus, GSH addition has been studied and identified as an interesting feature in reducing oxidative stress that sperm cell of several species can undergo and more researches is needed on the optimal concentrations of this antioxidant substrate, as well as their interactions with different cryoprotectants.

Conclusion

Glutathione plays an important role in protecting body cells and its mechanism of action differs with type, location and bioavailability. Current lifestyle changes negatively interfere in expression of GSH antioxidant, reducing its biological activities and, consequently, its yield organic. Therefore, infertility may be directly related to glutathione lower concentrations and antioxidant therapy can be an interesting alternative to rescue satisfactory fertility rates, as happens in other bodily functions.

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In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter V

The Significance of Astrocytic Glutathione System in Neuroprotection: A Potential Role for Curcumin

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Abstract

Decreased brain content of thiol antioxidant glutathione (GSH) is regarded as an indicator of excessive oxidative stress, which, in turn, is recognized as a contributing factor to the development and progression of several age-related neurodegenerative diseases. Brain GSH is mainly produced by astroglia and their metabolic coupling with neurons allows the latter cells to maintain a sufficient antioxidant defense under physiological conditions. Neurons, however, are very sensitive to GSH depletion; hence any impairment in astrocyte-neuron GSH metabolic interactions, as it may occur in brain pathologies, would render neurons more susceptible to oxidative injuries. This also implies that agents capable of elevating brain GSH level could prove useful as neuroprotectants. In this chapter, we review data that supports these suppositions, generated using human NT2/N neurons and NT2/A astrocytes obtained by differentiation of NT2/D1 cells. Indeed, the results showed that astrocytes rendered neurons more resistant to toxic injuries and supported neuronal survival even in the presence of low GSH levels. They could not, however, support the neurons under chronic GSH depletion.

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We also tested curcumin, a naturally occurring polyphenol with anti-inflammatory and antioxidant properties, for its ability to alter the GSH levels in NT2/N and NT2/A cells. Since curcumin is insoluble in aqueous media it is difficult to establish convincingly its efficacy. Here we have applied a solubilizing method developed in our laboratory (US patent No. 6,045,826 and 7,645,816; Ubisol technology licensed to Zymes LLC) to produce a water-soluble nano-micellar complex between PSS (polyoxyethanyl β-sitosteryl sebacate) and curcumin (WS-Cur) which can be readily tested under any experimental conditions. The data showed that a 24 h treatment with WS-Cur increased GSH levels in both cell types and that this treatment was neuroprotective. Our results add to the growing body of evidence indicating that agents capable of modulating GSH, such as WS-Cur, might prove highly beneficial in the management of oxidative stress-induced neurodegeneration. Furthermore, strategies aimed at modulating and restoring astrocyte function, for example, by increasing GSH, might present new opportunities for repairing and restoring brain function.

Abbreviations

AD, Alzheimer's disease;
BSO, L-buthionine-(*S*,*R*)-sulfoximine;
CNS, central nervous system;
GCEE, γ-glutamylcysteine ethyl ester;
γ-GCS, γ-glutamylcysteine synthase;
GFAP, glial fibrillary acidic protein;
GSH, glutathione;
6-OHDA, 6-hydroxy dopamine;
iNOS, inducible nitric oxide synthase;
IL-6, interleukin-6;
MAP2, microtubule associated protein 2;
NAC, *N*-acetyl-L-cystine;
NT2/A, NT2-derived astrocytes;
NT2/D1, human NTera-2 teratocarcinoma cells;
NT2/N, NT2-derived neurons;
NT2-N/A, NT2-derived mixed cultures of neurons and astrocytes;
PD, Parkinson's disease;
PSS, polyoxyethanyl β-sitosteryl sebacate;
TNF-α, tumor necrosis factor-alpha;
WS-Cur, water-soluble curcumin

1. Introduction

1.1. Role of Astrocytes in the CNS

Astrocytes are the most abundant glia in the central nervous system (CNS) and are key mediators of brain development, function and plasticity [1-5]. A particularly important

astrocytic function is the maintenance and regulation of extracellular microenvironment. Such actions include buffering of K^+, H^+ and Ca^{2+} ions to maintain ionic homeostasis, neurotransmitter uptake and release, and regulation of synaptic transmission and neuronal excitability [6]. Astrocytes also contribute to CNS homeostasis by provision of energy substrates and nutrients, detoxification of ammonia, metal sequestration, free radical scavenging and regulation of the blood-brain barrier [7]. In addition, they secrete several growth factors (e.g., TGF-β, FGF-2) and neurotrophic factors (e.g., GDNF, BDNF, NT-3, NGF) as well as several pro-inflammatory cytokines, which are crucial for the survival of neurons [8].

Another fundamental function of astroglia is the uptake of excitatory neurotransmitter glutamate and the regulation of glutamatergic neurotransmission to avoid excitotoxic injury and neuronal death [9-11]. Synaptic homeostasis of glutamate involves its removal from the synaptic cleft via high-affinity glutamate transporters, GLT-1, GLAST and EAAC1 and glutamate-catabolizing enzyme, glutamine synthase [12,13]. In addition, astrocytes are the main cells in the brain synthesizing the antioxidant glutathione (GSH) and have higher GSH content than neurons. The reported brain concentration of GSH is ~2 mM [14] and at this concentration, GSH acts as an important intracellular antioxidant and protects cells by removing toxic molecules. Its *de novo* synthesis ensures the maintenance of stable intracellular redox environment and thiol status.

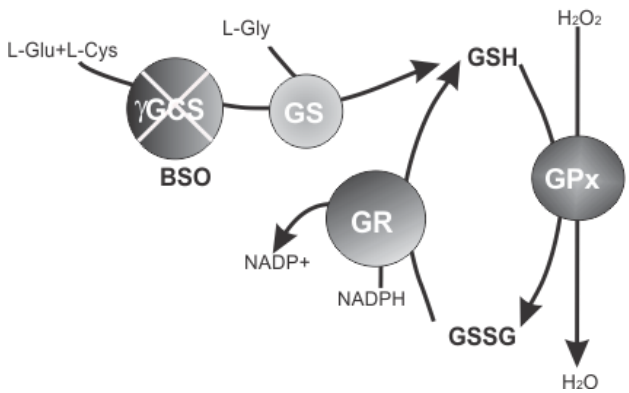

Figure 1. Schematic representation of glutathione biosynthesis. GSH is synthesized in two consecutive ATP-dependent reactions: (i) condensation of glutamate (Glu) and cysteine (Cys) to form the dipeptide γ-glutamylcysteine by γ-glutamylcysteine synthase (γ-GCS), and (ii) synthesis of GSH from γ-glutamylcysteine and glycine (Gly) by GSH synthase (GS). GSH acts as a substrate for GSH peroxidase (GPx), the predominant mechanism to reduce H_2O_2 leading to the generation of GSH disulfide (GSSG, or oxidized GSH). Because this process results in the consumption of GSH, it can be regenerated from GSSG *via* the action of GSH reductase (GR) that uses NADPH from the pentose phosphate shunt. L-buthionine-(*S,R*)-sulfoximine (BSO) is a selective inhibitor of γ-GCS. Once inside a cell, the sulfoximine site is phosphorylated by ATP and binds irreversibly and specifically to the active site on γ-GCS, preventing the *de novo* synthesis of GSH and reducing the cytosolic pool of GSH.

1.2. Astrocyte Activation

Astrocytes become activated in response to CNS injuries such as stroke, trauma or neurodegenerative diseases [15]. This reaction is commonly referred to as reactive gliosis and

is characterized by astrocytic hypertrophy, proliferation and altered gene expression, such as upregulation of glial fibrillary acidic protein (GFAP) [16]. Published reports suggest that activated astrocytes play a role in epilepsy, AD and even in schizophrenia, depression and other psychiatric disorders [17]. Indeed, numerous studies point to the harmful role of activated astrocytes in the CNS [18]. Astrocytes have been shown to release glutamate and/or aspartate in response to hypoxia/ischemia or inhibition of glycolysis. Astrocyte-derived proteoglycans inhibit neurite outgrowth and contribute to the development of neuritic plaques in Alzheimer's disease (AD) [19,20]. In addition, astrocytic glycoproteins such as tenascin, hyaluronate-binding protein and others exert a repulsive action on neurite outgrowth and are believed to contribute to aberrant synaptogenesis.

Astrocytes are the major producers of nitric oxide via the induction of inducible nitric oxide synthase (iNOS). They also express functional NADPH oxidase, which produces superoxide anions [21-23]. The nitric oxide and superoxide released by these enzymes reacts spontaneously to yield peroxynitrite, a potent oxidizing and nitrating chemical [24]. More recent studies show that astrocytes harboring a mutant form of superoxide dismutase 1 (SOD1G93A) induce specific degeneration of spinal motor neurons in rodent models of amyotrophic lateral sclerosis [25-27]. In addition, several cytokines, i.e., IL-1β, TNF-α, IL-6 and IL-3 produced by activated astrocytes can also contribute to tissue injury in neurodegeneration [28]. Similarly, chemokines capable of recruiting the inflammatory cells to the sites of injury, e.g., MCP-1 and MIP-1 are also synthesized by astrocytes [29,30].

2. GSH Antioxidant System in the CNS

2.1. Biosynthesis of GSH

As in other cells, GSH synthesis in neurons and astrocytes occurs in the cytosol in two consecutive ATP-dependent reactions. The first is a condensation reaction where γ-glutamylcysteine synthase (γ-GCS) uses glutamate (Glu) and cysteine (Cys) as substrates to form the dipeptide γ-glutamylcysteine, which is the rate-limiting step in the pathway (Figure 1). The second reaction is catalyzed by GSH synthase (GS) and results in synthesis of GSH from γ-glutamylcysteine and glycine (Gly). Intracellular GSH is compartmentalized between cytosol (85-90 %) and mitochondria (10-15 %) [31]. Because mitochondria lack the enzymes needed for GSH synthesis, mitochondrial GSH originates from cytosol *via* energy-dependent transport. Mitochondrial GSH pool appears to be important for astrocytic cell survival under oxidative and nitrative stress conditions [32,33].

2.2. Neuron-astrocyte Metabolic Coupling in GSH Metabolism

The metabolic coupling between neurons and astrocytes is crucial for survival and function of neurons. Astrocytes provide metabolic support for neurons in the form of lactate, glutamate uptake and conversion into glutamine, and synthesis of glutathione and its precursors. Although both neurons and astrocytes can synthesize GSH, it has been shown that astrocytes produce much more GSH than neurons [14,34] and that astrocytes actually provide

neurons with most of the GSH precursors necessary for its synthesis [35,36]. Interestingly, neuronal GSH content increases significantly when neurons are cultured with astrocytes, compared to being cultured alone [37]. A model presented in Figure 2 represents the likely interactions between astrocytes and neurons regarding GSH metabolism. Astrocytes synthesize GSH via the 2-step reaction (Figure 1) and also provide to neurons the three precursor amino acids (Gln, Cys and Gly) required for GSH synthesis. Within astrocytes, glutamate (Glu) is amidated to Gln through the action of glutamine synthetase. This enzyme is present in astrocytes, but not neurons [38]. Astrocytes release Gln which is rapidly taken up by neurons where it is hydrolyzed by phosphorylated glutaminase to regenerate Glu.

While astrocytes can use both cysteine and cystine for GSH synthesis, neurons can only use cysteine which limits neuronal GSH synthesis [35,39]. To provide Cys and Gly for neuronal GSH synthesis, GSH itself is released from astrocytes into the extracellular space where γ-GT, found on the extracellular membrane of the astrocytes, hydrolyzes GSH into the precursor CysGly *via* an acceptor of the γ-glutamyl moiety (X). The CysGly dipeptide is subsequently cleaved into Cys and Gly by an aminopeptidase ecto-enzyme on the neuronal extracellular membrane [40]. The amino acids are transported into neurons for GSH synthesis [36,41]. Consequently, the three individual precursors required for GSH synthesis in neurons are provided by astrocytes. The same or similar relationship between these two cell types most likely exists in the living brain; hence, the integrity of their interactions is crucial for brain homeostasis. Furthermore, a compromised astrocyte GSH system might render both the astrocytes and neurons more susceptible to oxidative damage and ultimately to cell death.

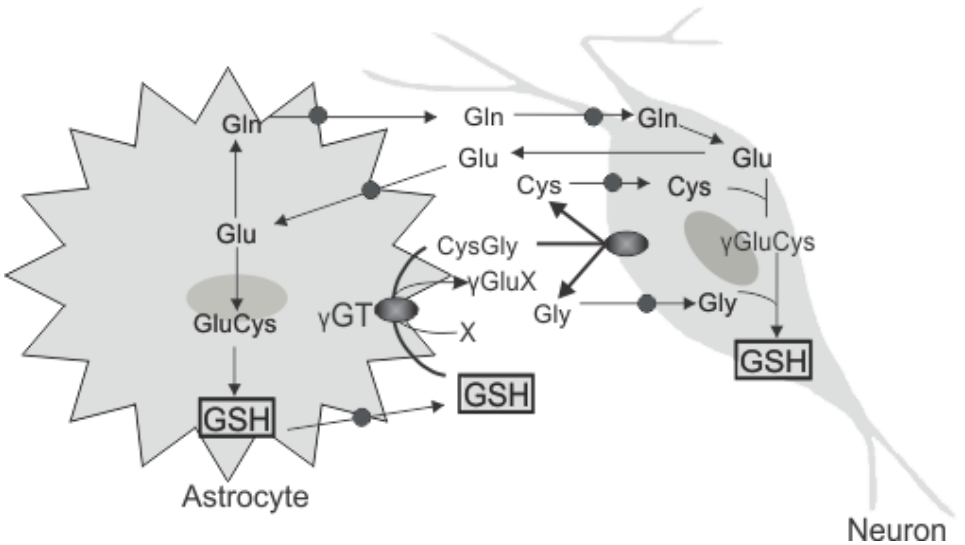

Figure 2. A proposed model of metabolic interactions between astrocytes and neurons in GSH metabolism in the CNS. Both astrocytes and neurons can synthesize GSH; however, astrocytes have a major role in supplying GSH substrates to neurons. GSH is synthesized in astrocytes and subsequently released from the astrocytes and acts as a substrate for the astroglial ectoenzyme γ-glutamyl transpeptidase (γ-GT), which transfers the γ-glutamyl moiety of GSH to an acceptor amino acid (X). This generates CysGly, which acts as a precursor for neuronal GSH, and γ-glutamyl-X (γGluX). The CysGly is further hydrolyzed into the individual amino acids, cysteine (Cys) and glycine (Gly) extracellularly by dipeptidases on the glial and neuronal membranes. In astrocytes, Glu is converted to glutamine (Gln) and released, which can be taken up by neurons and converted to Glu. All the three amino acids can then be readily taken up by neurons to be used for GSH biosynthesis. This figure is adapted from [24,27].

2.3. Effects of Astrocytic GSH Depletion on Neurons

We have extensively studied the effects of GSH depletion on neuronal vulnerability under oxidative stress using a well characterized cellular model of human NTera-2 (NT2/D1) [10,13,37,42-44]. This human teratocarcinoma cell line readily undergoes terminal differentiation into neurons and astrocytes in response to retinoic acid (RA) (Figure 3A). Therefore, both brain cell types are generated from the same source and can be propagated separately or as mixed cultures (Figure 3B). We used these cells to examine the relationship between neurons and astrocytes in GSH metabolism [37]. Here we present data obtained from cells propagated as mixed cultures of neurons and glia, which were treated with L-buthionine-(*S*,*R*)-sulfoximine (BSO) for up to 72 h to inhibit *de novo* GSH synthesis [45]. Subsequently the cells were separated into individual populations and analyzed for the GSH content (Figure 4). Thus, NT2/A have a significantly higher (~10-fold) basal GSH content than NT2/N (Figure 4A), suggesting that their free radical scavenging capability is greater than neurons. Both cell types respond to BSO treatment by ≥95% reduction in the GSH levels, the astrocytic GSH content dropped from 17.8±2.8 to 0.77±0.2 (Figure 4B) and neuronal from 2.8±0.7 to 0.05±0.10 (Figure 4C) by 3 days. Despite such significant depletion of GSH, astrocytes remain alive in culture for at least 2 weeks (solid bar, Figure 4D) and neurons for only 1 week (50% of neuronal loss was measured on day 6 in the GSH-depleted mixed cultures of neurons and astrocytes). Significantly, the GSH-depleted NT2/N neurons maintained in the presence of astrocytes remained viable for much longer periods of time than neurons propagated alone, i.e., 6 days instead of 24 h , as shown previously [37]. These differences in neuronal sensitivity to GSH depletion clearly reflect the importance of astrocytic support and their contribution to the neuronal GSH metabolism. For example, the basal GSH content in neurons maintained in the presence of astrocytes is significantly higher (2.8±0.7 pmoles/µg protein) than in neurons propagated alone (1.27±0.21 pmoles/µg protein, Figure 4A, [37]). Thus, astrocytes and neurons represent a functional unit in relation to GSH metabolism and their unaltered interactions are important for survival of neurons under the conditions of oxidative stress.

2.4. GSH Depletion and Neurodegeneration

Reductions in GSH content are found in various age-related neurodegenerative disorders, including Alzheimer's, Parkinson’s, Huntington’s and amyotrophic lateral sclerosis [46-48]. The relationship between perturbed GSH levels and neurodegeneration is perhaps best exemplified in Parkinson’s disease (PD). For example, a 40-50% decrease in GSH appears before neuronal loss in presymptomatic PD [49] and similar loss of GSH is associated with incidental Lewy body disease, an asymptomatic form of PD [50]. In addition, alterations in the specific activities of enzymes involved in GSH metabolism and the defense against free radical damage have been reported [51-53]. Although the exact mechanism responsible for GSH depletion in the substantia nigra of PD patients remains unknown, the demise of dopamine neurons might be a consequence of these changes. A critical function of cellular GSH is to maintain the redox status of the cell (GSSG:2GSH). Under excessive oxidative

stress, the increased usage of GSH results in the altered redox potential and contributes to cells' vulnerability [54-56].

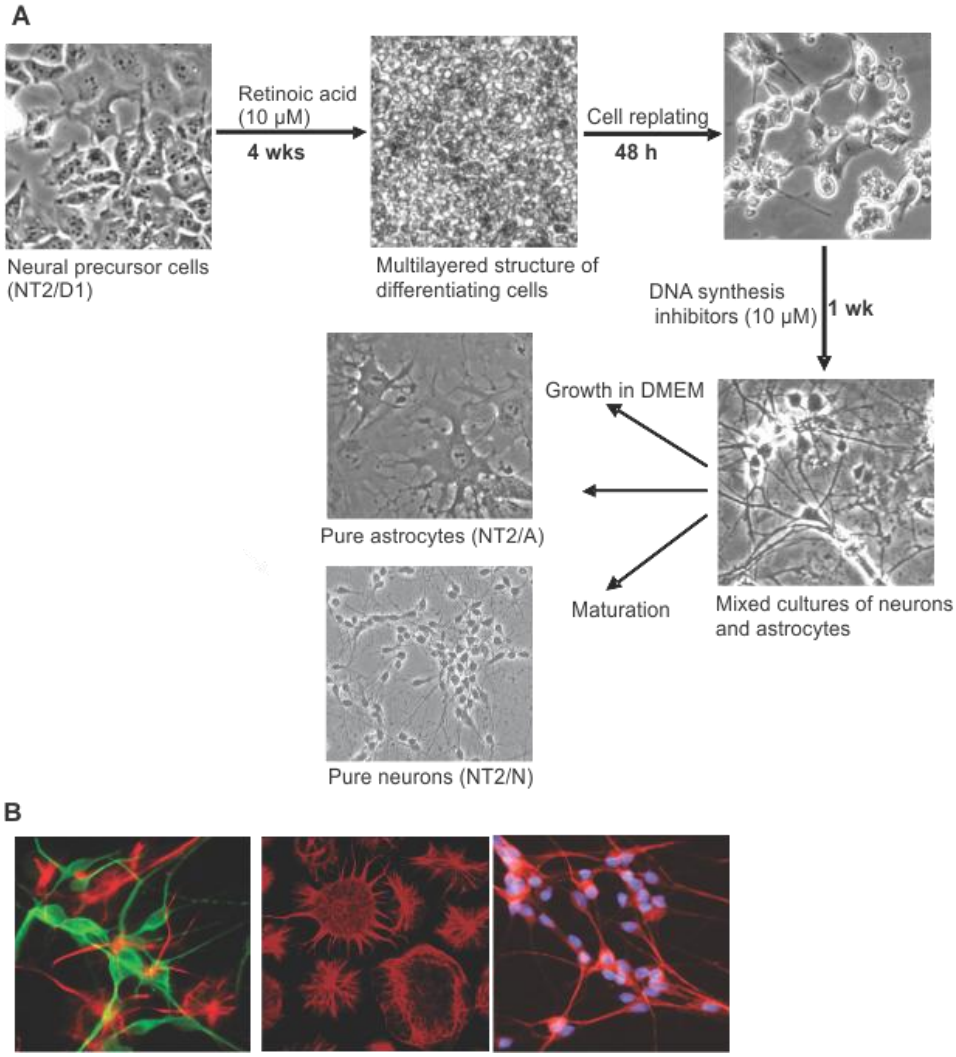

Figure 3. Human NTera-2 (NT2) experimental model system. A) NT2/D1 precursor cells were grown in high glucose Dulbecco's modified Eagle's medium (HG/DMEM) containing 10% fetal bovine serum (FBS) (complete medium). For differentiation, cells were seeded at 2×10^6 in T75-cm^2 flasks and treated 3 times a week for 4 weeks with 10 μM all-*trans*-retinoic acid (RA). After 4 weeks of RA treatment, the culture appeared as an extremely dense multi-layered structure of small cells piling on each other. Cells were then harvested using trypsin-EDTA and replated into T-175cm^2 flasks in complete medium for 48 h in the absence of RA. Cultures were fed twice weekly for 1 week with complete medium supplemented with DNA synthesis inhibitors 1-β-D-arabinofuranosylcytosine (1 μM), 5-fluoro-2'-deoxyuridine (10 μM), and uridine (10 μM) to inhibit the division of NT2/A astrocytes. During this period, cultures were inspected microscopically on regular basis for the number of astrocytes. When a monolayer of astrocytes remained under the NT2/N neurons, DNA synthesis inhibitors were removed and cultures were further allowed to mature in complete medium. From these mixed cultures, NT2/A or NT2/N could be isolated and established as highly enriched cultures. Typically, cultures can be maintained as NT2-N/A mixed cultures consisting of approximately 40% neurons and 60% astrocytes, whereas the enriched cultures contain > 99% NT2/N or NT2/A. B) NT2/A express glial fibrillary acidic protein (GFAP) and NT2/N express microtubule associated protein (MAP2) in either mixed or enriched cultures.

Accumulating evidence suggests that oxidative stress might originate in glial cells and alterations in glial functions are important contributors to the pathogenesis of neurodegenerative diseases, such as PD [50]. Accordingly, inhibition of GSH biosynthesis by BSO has been shown to induce an inflammatory phenotype in glial cells, which was accompanied by an increase in the inflammatory mediators, TNF-α, IL-6, upregulation of iNOS, and production of nitric oxide [57]. TNF-α and IL-6 produced by GSH depleted glial cells are not toxic *per se*, but might synergistically act with yet unidentified factor(s) to enhance neurotoxicity. Because astrocytes are the major suppliers of GSH in the brain, a compromised astroglial GSH system could contribute to a lower defense capacity in brain against free radical damage and subsequently to increased susceptibility of neurons to injuries.

3. Strategies to Increase GSH Levels

Based on the outcomes of numerous experimental studies pointing to a critical role of the GSH system in the brain, attempts have been made to upregulate intracellular GSH level as a neuroprotective strategy. In contrast to glutamate and glycine which are found in millimolar concentrations in the brain, cysteine is present only in micromolar amounts in the brain [58]. Because cysteine is the rate limiting substrate for GSH biosynthesis [59], most research has focused on supplying cysteine as an indirect way to upregulate GSH. *N*-acetyl-L-cysteine (NAC) acts as a cysteine donor for GSH synthesis and has direct antioxidant activity in scavenging free radicals. NAC has also been shown to directly inhibit the transcription factor NFκB and block nitric oxide production by inhibiting iNOS and inflammatory cytokines [60]. Oral administration of NAC increases brain GSH level, brain synaptic mitochondrial complex I activity [61], and also increases neuronal survival and improves behavioral read-outs in MPTP-induced parkinsonian degeneration in mice [62,63]. In SAMP8 (Senescence Accelerated Mouse) mice and transgenic mouse models of AD, oral administration of NAC also improves cognition and reduces markers of oxidative stress [64,65]. NAC is currently approved for use in the United States, Europe and Canada for the treatment of acetaminophen-induced hepatotoxicity [66]. Because of the ease of its administration and lack of toxicity in humans, NAC has been under clinical evaluation for the treatment of schizophrenia, bipolar, Gaucher's disease, AD and PD [67-70].

Another means of increasing GSH levels is the use of γ-glutamylcysteine ethyl ester (GCEE). Attachment of the ethyl ester moiety to γ-glutamylcysteine increased its cell permeability and delivery across the blood brain barrier, which has proven to be an effective way to boost GSH levels and protect against oxidative damage in many experimental models of disease [71-73]. Systemic administration of GCEE in mice and rats after traumatic brain injury restored brain GSH levels, reduced markers of autophagy, decreased levels of protein carbonyls, 3-nitrotyrosine and improved behavioral indices [74-76].

The outcomes of these treatments are highly encouraging and suggesting that NAC, GCEE or similar compounds could be used to increase the intracellular GSH level and treat disorders associated with its depletion. Here we present the effects of curcumin, a natural polyphenolic compound, on neuronal and astroglial GSH content and neuroprotection.

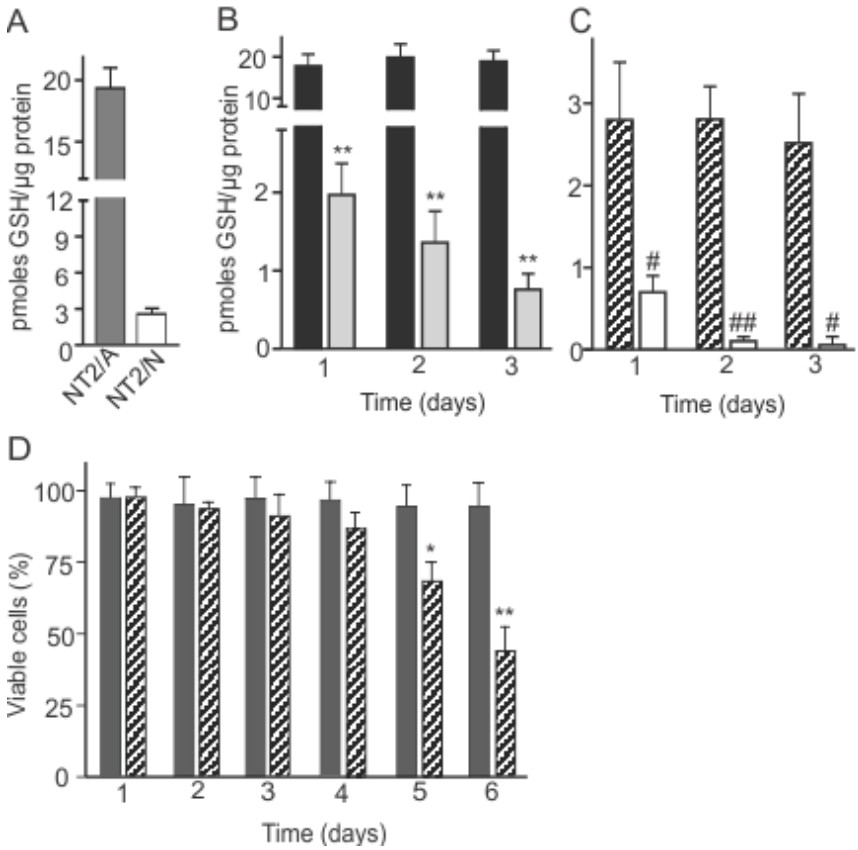

Figure 4. GSH levels and neuronal cell survival. From the mixed cultures of NT2-N/A, either untreated or treated with 100 µM BSO, NT2/N were separated from the underlying NT2/A using 0.015% trypsin-EDTA, and GSH levels were analyzed separately using HPLC as previously described [100]. A) Basal GSH levels in NT2/A (■) and NT2/N cells (□). B) GSH levels in NT2/A exposed to 100 µM BSO for up to 3 days. C) GSH levels in NT2/N exposed to 100 µM BSO for up to 3 days. Statistically significant differences between untreated (■) and BSO treated (▒) NT2/A astrocytes are shown as ** (P<0.001) and between untreated (▨) and BSO treated (□) neurons are shown as # (P<0.05), ## (P<0.001). D) Cell viability was analyzed every 24 h for up to 6 days in NT2/A and NT2/N using trypan blue dye exclusion. Statistically significant differences in neuronal survival (▨) in BSO-treated mixed cultures is shown as * (P<0.05) and ** (P<0.001). No change was seen in astrocyte viability (■). Data are expressed as mean ± SEM from 3 separate experiments analyzed in triplicate. Data analysis was performed using GraphPad Prism version 4.0 using one way ANOVA followed by *post-hoc* Bonferroni's multiple comparison test.

3.1. Effects of Polyphenolic Compound Curcumin on GSH Metabolism and Neuronal Survival

Numerous experimental, clinical and epidemiological data reveal protective effects of dietary phytochemicals, in particular polyphenolic compounds, against neurodegenerative diseases [77]. Polyphenols are a large and diverse family of compounds synthesized by plants as secondary metabolites. They have been shown to exert beneficial effects on the CNS by protecting neurons against oxidative stress-induced injury, in part, due their antioxidant activity and suppression of pro-inflammatory responses [78,79]. A wide variety of

polyphenolic compounds have been evaluated for neuroprotection. Curcumin therapy for age-related neurodegenerative diseases, such as AD, is gaining some credence mainly due to epidemiological data showing a lower incidence of AD in India that consumes large amounts curcumin as compared to the United States [80,81].

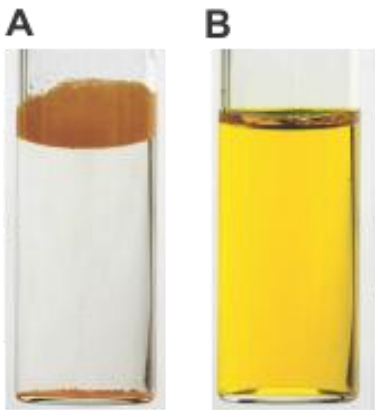

Figure 5. Water-soluble formulation of curcumin (WS-Cur). A) Suspension of curcumin in water. Most of the curcumin is insoluble in water and floats on the surface. B) Curcumin formulated using Ubisol solubilization technology (WS-Cur) at 10 mg/mL. WS-Cur is completely soluble in water and appears as a yellow solution.

Curcumin, the principal curcuminoid found in the rhizomes of curcuma longa (turmeric), is known for its beneficial anticancer, anti-inflammatory and antioxidant properties [78,82-85]. Despite its well-recognized health benefits, a major obstacle in the medicinal administration of curcumin has been its poor solubility and low bioavailability making reliable testing of its exact clinical efficacy very difficult [86]. Previously our laboratory devised a method to convert insoluble natural compounds, including curcumin, to water-soluble nano-micellar formulations. The method takes advantage of the amphiphilic properties of polyethylene glycol (PEG) and sebacic acid derivatised sterols and tocopherols (Ubisol technology developed at the National Research Council Canada and licensed to Zymes LLC, [87-89]). In the case of curcumin we used a plant sterol, beta-sitosterol and chemically converted it to PEG 600-β-sitosteryl sebacate (PSS) by conjugating it to PEG 600 *via* bi-functional sebacic acid as previously described [90]. PSS and curcumin form a non-covalent complex at a molar ratio of 3: 1 (3 molecules of PSS per 1 molecule of curcumin), which is soluble in aqueous media (e.g., water, physiological saline and phosphate buffered saline). Suspensions of curcumin in water and curcumin-PSS complex (WS-Cur) in water are depicted in Figure 5. In contrast to curcumin added directly to water, which resulted in surface floating of the insoluble material (Figure 5A), WS-Cur was fully water-soluble (Figure 5B). We treated the NT2-derived neuron-glia co-cultures (NT2-N/A) with 50 µM curcumin added directly to tissue culture media as WS-Cur formulation. A 24 h treatment resulted in significantly increased GSH levels in both astrocytes (~2.54-fold, Figure 6B) and neurons (~4.25-fold, Figure 6C) as compared to control cultures. Subsequently we challenged these WS-Cur–treated co-cultures with a toxic dose of 6-hydroxydopamine (6-OHDA) [43]. 6-OHDA is known to induce oxidative damage, which is reflected in the elevation of reactive oxygen species and reduction of cellular GSH content [91]. Thus, NT2-N/A cultures were treated with 50 µM WS-Cur for 24 h and then challenged with 100 µM 6-OHDA. As shown

in Figure 6, WS-Cur treated cells were protected from the effects of 6-OHDA (Figure 6). No evidence of cell death was seen in cultures treated with 50 µM WS-Cur for up to 48 h (Figure 6A). In cultures treated with 6-OHDA alone, neurons showed typical characteristics of cell death (i.e., retraction and loss of neuronal processes and nuclear condensation, Figure 6E). No cell death was seen in the underlying astrocytes. Quantitative assessment of these cultures showed that ~40% of the neurons lost viability following exposure to 100 µM 6-OHDA (Figure 6D). By contrast, neurons were completely protected against the same challenge with 100 µM 6-OHDA in cultures treated with WS-Cur (Figure 6D), suggesting that the curcumin-elevated GSH levels might have contributed to the observed neuroprotection. This is consistent with our previous observations showing that neuronal cells are more susceptible to 6-OHDA induced toxicity when GSH levels are depleted with BSO [43]. It should be noted that although WS-Cur is a two component formulation, i.e., PSS and curcumin, there is no available information regarding any role of plant sterols, such as sitosterol, in the GSH metabolism. This led us to suggest that the observed increase in the GSH content was due to the action of curcumin, but whether this was a direct or indirect effect is at present unknown.

The mechanism of action of curcumin is complex and multifaceted and, in part, is regulated by the activation of nuclear transcription factor Nrf2/Keap1 and other cytoprotective proteins of the phase II detoxification response [92]. Curcumin has been shown to suppress the transcription factors NF-κB and SIRT1 which control expression of pro-inflammatory mediators, chemokines, and certain cytokines, such as IL-1, IL-6, IL-8 as well as inhibiting the action of TNF-α [93,94]. Curcumin can bind and disrupt amyloid-beta plaques in animal models of AD. It can also reduce the aggregation of α-synuclein that occurs in PD as well as protect dopaminergic neurons against protein oxidation, and preserve mitochondrial complex I activity [95-99]. The data presented here has shown that curcumin could increase the intracellular GSH levels in both neurons and astrocytes; hence, some of its beneficial effects might stem from this phenomenon.

Conclusion

In this chapter, we present arguments and supporting data on the significance of the intracellular GSH system in brain defense against oxidative stress. The main producers of GSH in the brain are astrocytes.

They also provide precursors for GSH synthesis in neurons; hence, neuron-glia metabolic coupling is critical for neuronal cell survival. Neurons are supported and can cope with adverse conditions as long as astrocytes retain their normal physiological functions permitting intact neuron-glial interactions. Impairments in astrocytic GSH metabolism and/or chronic oxidative stress that lead to a critical GSH depletion renders astrocytes incapable of protecting neurons.

In this manner, a defective GSH metabolism could, indeed, facilitate the progression of neurodegeneration. The ability of WS-Cur to modulate GSH levels in neural cells and exert neuroprotection might prove to be an effective strategy for the prevention and/or treatment of oxidative stress-induced neurodegeneration.

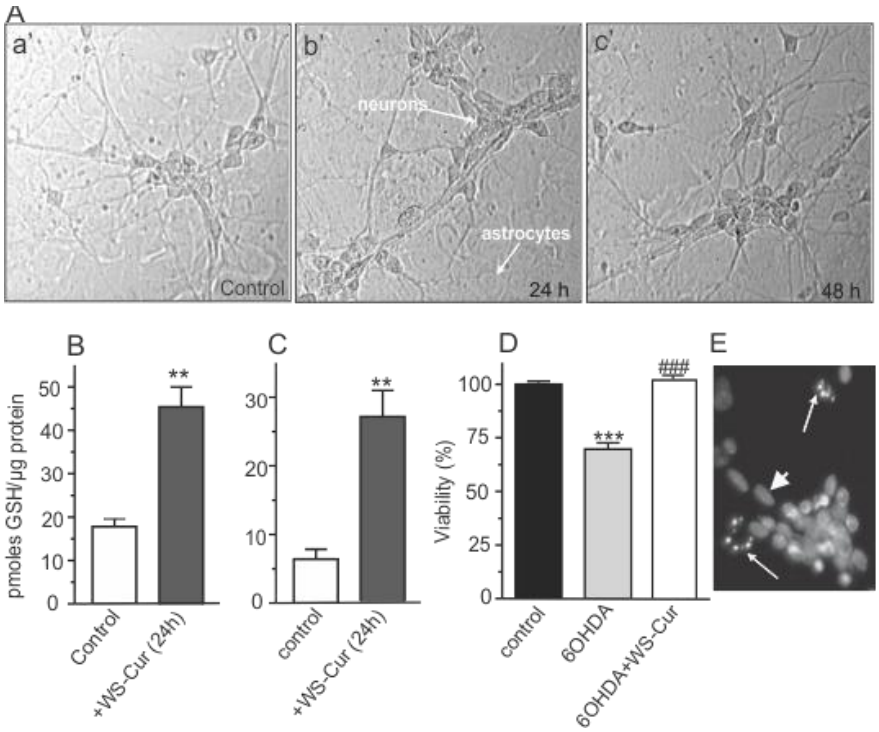

Figure 6. Effects of WS-Cur on GSH levels and neuronal viability in mixed cultures of NT2-N/A. WS-Cur was prepared using the Ubisol solubilization technology (US patent #6,045,826), developed in our laboratory. A) Mixed cultures consisting of 60% astrocytes and 40% neurons (a') were treated with 50 µM WS-Cur for 24 h (b') or 48 h (c'). No morphological changes resulting from the treatment were evident. B) Astrocytic and C) Neuronal GSH levels following 24 h of WS-Cur treatment. Data shown are mean ± SEM of two separate representative experiments, each involving 4-8 replicate dishes per experimental point. Statistically significant differences between untreated (□) and WS-Cur treated NT2/A astrocytes or NT2/N neurons (■) is shown as ** (P<0.01). D) Neuroprotection against 6OHDA-induced neurotoxicity. NT2-N/A mixed cultures were pretreated with 50 µM WS-Cur for 24 h and then challenged with 100 µM 6-OHDA for 15 min. The medium containing 6OHDA was washed out and then replaced with fresh medium. Following an incubation of 16 h, cell viability was determined by the MTT assay. Data are expressed as percent cell survival compared to untreated control cells. Data shown are mean ± SEM of two separate experiments involving 3-6 replicate wells for each treatment. Data analysis was performed using GraphPad Prism version 4.0 using one way ANOVA followed by *post-hoc* Bonferroni's multiple comparison test. Statistically significant differences between cell viability in untreated (■) and 6OHDA treated (▭) cultures are shown as *** (P<0.001). Statistically significant differences between the cell viability following 6OHDA treatment (▭) versus cultures pre-incubated with WS-Cur for 24 h and treated with 6OHDA (□) are indicated by ### (P<0.001). E) Morphology of neuronal nuclei after 6OHDA treatment visualized by Hoechst staining. Arrows point to apoptotic neuronal nuclei and arrow head indicates intact neuronal nuclei.

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In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter VI

N-Acetylcysteine Amide (NACA), a Novel GSH Prodrug: Its Metabolism and Implications in Health

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Abstract

Glutathione (γ-glutamyl-cysteinyl-glycine; GSH), the most abundant non-protein thiol, plays a pivotal role in various cellular functions. Glutathione deficiency leads to oxidative stress which is implicated in aging and in a number of pathologies, including Alzheimer's disease, Parkinson's disease, liver disease, cystic fibrosis, sickle cell anemia, HIV, AIDS, cancer, heart attack, stroke, and diabetes. Since GSH cannot be replenished directly due to its unfavorable biochemical and pharmacokinetic properties, a logical approach to enhancing antioxidant protection would be the use of GSH prodrugs. GSH may be increased by supplying GSH prodrugs like esters of GSH, γ-glutamyl cysteine, cysteine, cysteine esters, and N-acetylcysteine (NAC). However, large doses of these prodrugs are required due to their poor bioavailability and toxicity which limit their use as a therapeutic agent. A potential candidate that possesses far better characteristics for development as a GSH prodrug to address oxidative damage is the low molecular weight thiol antioxidant, N-acetylcysteine amide (NACA). NACA's characteristics as a drug were improved over NAC by neutralizing the carboxylic group of NAC, which makes the NACA molecule more lipophilic and, therefore, enhances its ability to penetrate cellular membranes. The enhanced ability to penetrate cells allows NACA to be administered at a lower dose than NAC, giving the drug a greater therapeutic index and lowering the risk of side effects that traditionally have been associated with higher doses of NAC. NACA is an excellent source of sulfhydryl (SH) groups that can be converted by the cells into

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metabolites capable of stimulating glutathione synthesis. The molecule can also promote intracellular detoxification and act directly as a free radical scavenger. NACA functions as a carrier of NAC and its antioxidant and free radical scavenging abilities are equal to or better than those of NAC. Promising results with NACA have provided additional momentum for research on GSH prodrug-based approaches to treat oxidative stress-related disorders. This chapter will focus on the role of NACA in protection against oxidative damage by increasing GSH levels in numerous "Oxidative Stress"-related diseases.

Abbreviations

3-NT	3-Nitrotyrosine
4HNE	4-Hydroxynonenal.
8-oxodG	8-oxo-7,8-dihydro-2´-deoxyguanosine
AMD	Age Related Macular Degeneration
AZT 3'	Azido-2', 3'-deoxythymidine
BBB	Blood- Brain Barrier
BSO	L-Buthionine-(S,R)-Sulfoximine
CAT	Catalase
CNS	Central Nervous System
DA	Dopamine
DEP	Diesel Exhaust Particle
DEE	Diesel Engine Exhaust
gp120	HIV-1 envelope glycoprotein 120
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GST	Glutathione-S-Transferase
GSH	Glutathione
GS	Glutamine Synthetase
HIV-1	Human Immunodeficiency Virus
HAND	HIV-1- Associated Neurocognitive Disorders
HAART	Highly Active Antiretroviral Therapy
IDV	Indinavir
MDA	Malondialdehyde
METH	Methamphetamine
MOG	Myelin Oligodendrocyte Glycoprotein
MPT	Mitochondrial Permeability Transition
MPTP	Mitochondrial Membrane Transition Pore
mtDNA	Mitochondrial DNA
NAC	N-AcetylCysteine
NACA	N-AcetylCysteine amide
PUFAs	Polyunsaturated Fatty Acids
PM	Particulate Matter
PLA2	Pphospholipase-A2
PAHs	Polycyclic Aromatic Hydrocarbons

RBE4	Rat Brain Endothelial Cells
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
Tat	Transregulatory protein
TJ	Tight Junctions

1. Introduction

Glutathione (γ-L-glutamyl-L-cysteinylglycine, GSH), a tripeptide of L-glutamate, L-cysteine, and glycine is the predominant low-molecular-weight thiol in animal cells with concentrations up to 10 mM [1, 2]. The tripeptide exists intracellularly in two states: oxidized (glutathione disulfide (GSSG)) and reduced (GSH). Intracellularly >98% of the tripeptide is kept in its reduced state by glutathione reductase (GR) enzyme with the remainder as glutathione disulfide and glutathione conjugates (GS-R) [3]. GSH is known to be involved either directly or indirectly in a number of biological phenomena and is mainly responsible for maintaining cellular redox status in endothelial cells. GSH scavenges free radicals ($R^{\bullet}$) and other reactive oxygen species (ROS), and neutralizes toxic metabolites by condensing with them both enzymatically and nonenzymatically [4]. Because a major physiological function of GSH is to provide cells with a reducing environment and to destroy the reactive oxygen species and free radicals formed in metabolism, it is crucial for normal cell function and survival.

2. Oxidative Stress

Oxidative stress is the loss of balance between the antioxidant defense and oxidant production in the cells (Figure 1). Normal metabolic processes in an aerobic organism produce ROS including free radicals, an excess of which results in oxidative stress. ROS can be produced endogenously or exogenously. Mitochondria are the most common source of ROS *in vivo* with more than 90% of ROS being produced during normal aerobic respiration. In addition to the mitochondrial sources, ROS can be derived from xanthine oxidase, NADPH oxidases, cyclooxygenases, lipoxygenases, and uncoupled nitric oxide synthase. Exogenously ROS can be formed from a variety of stressors like ionizing radiation, diesel exhaust particles, medicinal drugs, HIV infection, methamphetamine use, exposure to lead, organic solvents, anesthetic gases, hyperoxic environments, and pesticides [5, 6].

Cellular defense against ROS involves direct acting small molecular weight compounds, like ascorbic acid (vitamin C), tocopherols (vitamin E), GSH, etc. as well as antioxidant enzymes, like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and GR. One of the consequences of oxidative stress and free radical generation is decreased levels of cellular antioxidants. Since GSH is the most abundant intracellular free thiol, the loss of antioxidant capacity in an oxidatively stressed cell is mainly due to a decrease in GSH and/or an increase in GSSG. Thus, oxidative stress *in vivo* mainly translates to deficiency of GSH and/or its precursor, cysteine. Glutathione, in its reduced form, is the most powerful

intracellular antioxidant and the ratio of reduced to oxidized glutathione (GSH/GSSG) serves as a representative marker of the antioxidative capacity of the cell.

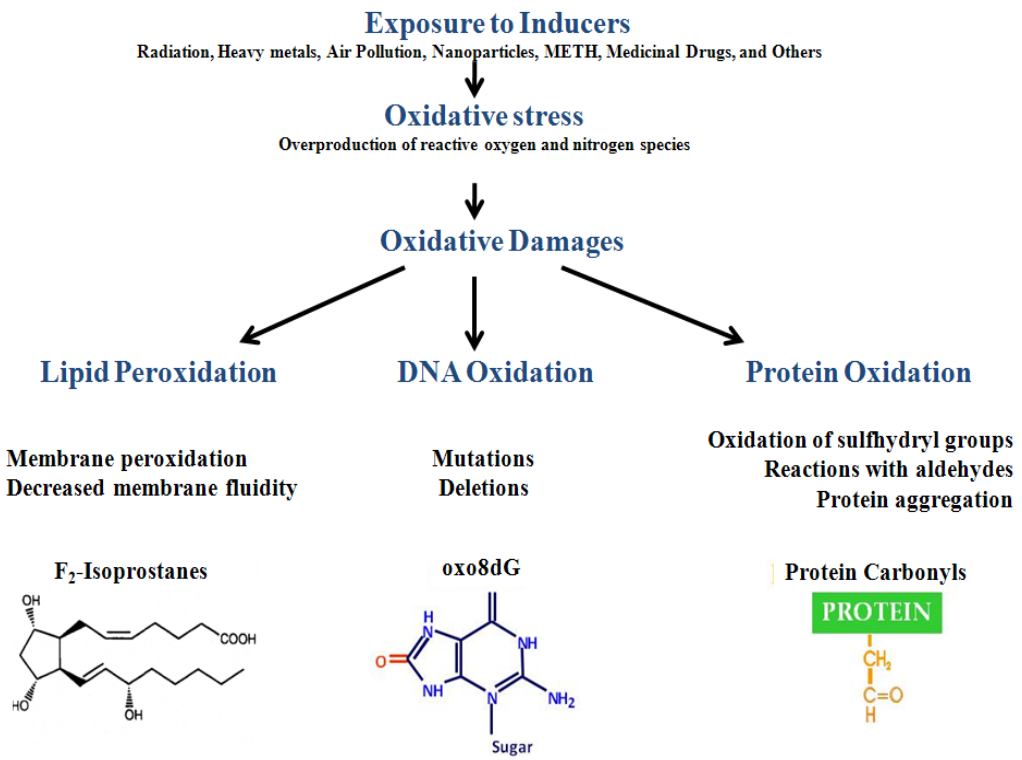

Figure 1. Consequences of oxidative stress.

The three major antioxidant enzymes are SOD, CAT, and GPx. SOD and CAT act as a first line of defense against ROS [7]. SOD converts superoxide anion to hydrogen peroxide (H_2O_2) and oxygen [8] and the H_2O_2 produced is removed by peroxidase and catalase enzymes [8]. CAT, which is an antioxidant enzyme that catalyzes the conversion of H_2O_2 to molecular oxygen and water, is an established indicator of oxidative stress. Although H_2O_2 is not considered to be a free radical, it can easily form hydroxyl radical, one of the most powerful oxidants. Removal of H_2O_2 by CAT is one of the most important antioxidant defense systems [8, 9]. GPx is another enzyme that is responsible for detoxification of H_2O_2, as well as other peroxides like steroid hydroperoxides. GPx uses glutathione as its substrate and oxidizes it to glutathione disulfide, which is then reduced to GSH by another enzyme, GR, thereby supporting the antioxidant defense system indirectly. Glutathione-S-transferase (GST) is also a glutathione-dependent enzyme, mainly involved in xenobiotic and lipid peroxide detoxification.

A decrease in GSH and an increase in ROS set off a cascade of further oxidative damage. ROS are capable of causing oxidative damage to all major macro-molecules in cells, including lipids, proteins, and nucleic acids. Oxidized derivatives of these substrates are studied as markers of oxidative stress. Polyunsaturated fatty acids (PUFAs), major components of cell membranes, can undergo free radical attack, producing lipid peroxidation products that are frequently used to quantify oxidative stress-related injury, and can be

assessed by a number of methods that include the measurement of either primary or secondary peroxidation end products like malondialdehyde (MDA) and 4-hydroxynonenal. MDA levels provide a suitable index for measurement of lipid peroxidation *in vivo* [10]. In general, once toxic lipid peroxides are formed by free radicals, GPx attempts to eliminate them at the expense of GSH. Meanwhile, GSH is either oxidized or consumed by free radicals thereby making the GSH/GSSG ratio very low. Unavailability of GSH as a substrate for GPx stalls the process of lipid peroxide decomposition, thus building up the levels of MDA. Oxidative attack on proteins results in the formation of protein carbonyls, oxidized amino acid side chains, protein fragments, and advanced glycation end products [11]. Increased protein carbonylation is a reliable parameter of oxidative stress, as it lacks interference from other non-protein substances [12], and measures carbonylation of various protein residues, including lysine and arginine [13]. ROS can also induce oxidative damage to DNA, resulting in strand breaks, base and nucleotide modifications, with the most common site being sequences with high guanosine content. Although more than 20 oxidized purine and pyrimidine bases have been identified, only a fraction of these have been studied as oxidative stress markers, most notably 8-oxo-7,8-dihydroguanine (8-oxoGua) and its 2´-deoxynucleoside equivalent, 8-oxo-7,8-dihydro-2´-deoxyguanosine (8-oxodG) [14].

ROS decrease mitochondrial membrane potential which, together with oxidation of pyridine nucleotides and glutathione, promote mitochondrial damage and the onset of a phenomenon known as mitochondrial permeability transition (MPT). MPT is a sudden increase in the permeability of the inner mitochondrial membrane, loss of $\Delta\Psi m$ (inner mitochondrial membrane potential), uncoupling of oxidative phosphorylation, mitochondrial swelling, and the release of intramitochondrial ions through the mitochondrial membrane transition pore (MPTP) [15]. Mitochondrial permeability transition is recognized as an initiating phenomenon, important for both necrosis and apoptosis [15]. Swelling of the mitochondria, due to increased permeability, ruptures the outer membrane of the mitochondria releasing the cytochrome *c* into the cytoplasm. Cytochrome *c* can, in turn, cause apoptosis of cells by activating pro-apoptotic factors [16]. Early events in apoptosis include the release of cytochrome *c* (from mitochondria) and calcium from the endoplasmic reticulum into the cytosol, which are necessary for apoptosis. A small amount of cytochrome *c*, released from mitochondria, can bind to and promote calcium conductance through inositol-1,4,5–triphosphate receptors in the endoplasmic reticulum membrane. The released calcium then triggers a massive exodus of cytochrome *c* from all mitochondria in the cell, thereby activating the caspases and nuclease enzymes that finalize the apoptotic process [17]. Several members of the caspase family play important roles as effector molecules in apoptosis. A potent “executioner” caspase, caspase-3, is a central component of the proteolytic cascade during apoptosis [18]. Activated caspase-3 is responsible for the breakdown of several cellular components related to DNA repair and regulation during apoptosis. Evidently, calcium uptake into the mitochondria is secondary to an increase in the cytoplasmic calcium concentration as a stimulus for the massive release of cytochrome *c*. It is believed that mitochondrial dysfunction is followed by an extensive release of intracellular calcium and cytochrome *c*, which results in cell death.

Various oxygen radical stressors have been shown to result in GSSG formation and short-term depletion of GSH. Changes in the redox state of the cell may affect signaling pathways resulting in altered activities of a number of biological processes, such as enzyme catalysis, protein synthesis, and receptor binding. Thus, maintaining a proper GSH/GSSG ratio is

crucial for several critical cellular functions and disruption of these processes leads to multitude of human pathologies.

GSH deficiency increases a cell's susceptibility to oxidative damage, which plays an important role in the onset and progression of many diseases, including cancer, inflammation, kwashiorkor (predominantly protein deficiency), seizure, Alzheimer's disease, Parkinson's disease, sickle cell anemia, liver disease, cystic fibrosis, HIV, AIDS, infection, heart attack, stroke, cataract, age related macular degeneration (AMD), and diabetes [3,19]. Depletion of GSH through conjugation or by excretion of GSSG necessitates replenishment. Therefore, one possibility to protect cells from damage caused by ROS is to restore the intracellular glutathione levels. Since GSH is not taken up directly by most cell phenotypes, it is predominantly replenished by *de novo* synthesis. Increased levels of GSH and other thiols have been associated with increased tolerance to oxidant stressors resulting in disease prevention or treatment in humans.

3. GSH Prodrugs

GSH deficiency has been implicated in a number of oxidative stress- related diseases and, therefore, a logical approach for treatment of these disorders would be to replenish GSH levels. Unfortunately, high doses of GSH are required due to its poor oral bioavailability and a short half life (<3 min) with intravenous administration [19-21]. Since GSH cannot be replenished directly, an alternative approach to enhancing antioxidant protection would be the use of GSH prodrugs. Researchers have focused on developing a potential therapeutic alternative for GSH and a few of these are discussed below.

3.1. GSH Esters

Esters of GSH are effective at increasing cellular GSH levels owing to their membrane permeability [22-25]. Methyl, ethyl, and isopropyl esters of GSH, in which the carboxyl group of glycine residue is esterified, undergo rapid deesterification with effective intracellular hydrolysis and transport [22-25]. Mono methyl ester of GSH has been reported to prevent acetaminophen-induced hepatotoxicity in fasting adult mice by enhancing GSH levels in the liver and kidney [26].

In addition, mono ethyl ester of GSH was able to protect against liposaccaride-induced liver damage in mice, ischemic damage, heavy metal induced toxicity, antitumoral agents, and reperfusion injury by increasing GSH concentrations [27-31]. GSH levels have been reported to be efficiently restored by diethyl ester of GSH, which is intracellularly converted to monoethyl ester. The resulting monoethyl ester does not efflux and subsequently hydrolyzes to yield GSH [32, 33]. However, esters cannot be used as GSH prodrugs since high doses are required to maintain desired GSH levels as they are rapidly cleared from the body. In addition, some of their cleavage products such as methanol, ammonia, and alcohols are toxic [22].

3.2. Cysteine

The availability of amino acids for GSH synthesis is a fundamental factor in its regulation. Under physiological conditions, cellular levels of glutamic acid and glycine (but not cysteine) are plentiful. Consequently, GSH synthesis depends on the availability of cysteine, which is a rate-limiting factor in the synthesis of GSH [34]. Since cysteine is toxic at high concentrations and is readily oxidized to cystine, it cannot be administered directly. As a consequence, compounds that can be metabolized to cysteine could be used as prodrugs to increase cysteine levels and, consequently, GSH levels.

3.3. N-Acetylcysteine (NAC)

N-acetylcysteine (NAC) is the acetylated derivative of the amino acid L-cysteine. The presence of the acetyl moiety reduces the reactivity of the thiol, as compared with that of cysteine. NAC is less toxic, less susceptible to oxidation and subsequent dimerization, and more water soluble when compared with cysteine, making it a better GSH/cysteine prodrug [1, 35-37].

Historically, NAC has been used as a mucolytic agent in chronic respiratory illnesses, as well as an antidote for hepatotoxicity due to an acetaminophen overdose [38]. Animal and human studies have shown NAC to be a powerful antioxidant and potential therapeutic agent in the treatment of cancer, heart disease, HIV infection, heavy-metal toxicity, age-related neurodegenerative diseases, cystic fibrosis, and diabetes [39-41]. NAC has also been shown to be of value in Sjogren's syndrome, influenza, hepatitis C, and myoclonus epilepsy.

NAC's effectiveness is primarily attributed to its ability to reduce extracellular cystine to cysteine, and as a source of sulfhydryl groups [37]. NAC stimulates glutathione synthesis, enhances glutathione-S-transferase activity, promotes liver detoxification by inhibiting xenobiotic biotransformation, and is a powerful nucleophile capable of scavenging free radicals.

Although NAC can directly scavenge free radicals, the rate constants for reaction with ROS are several orders of magnitude lower than those of antioxidant enzymes such as SOD, CAT, and GPx. Thus, the direct free radical scavenging activity of NAC is not likely to be of great importance for its antioxidant activity *in vivo*.

Use of NAC has been limited by several drawbacks, including low membrane penetration and systemic bioavailability [42-43]. Researchers have estimated the oral bioavailability of the intact NAC molecule to be between 4 and 10 percent. Disulfide linkage to proteins and deacetylation of NAC in the intestinal mucosa and lumen are probably the greatest factors in this apparent low oral bioavailability of NAC.

3.4. N-Acetylcysteine Amide (NACA)

In an attempt to ameliorate the problems associated with NAC, a low-molecular weight thiol antioxidant, N-acetylcysteine amide (NACA, AD4), was developed by Atlas et al. [44]. In NAC, the carboxyl group is negatively charged at physiological pH, limiting the drug's

ability to cross cell membranes. However, in NACA the carboxyl group is neutralized [Figure 2] which makes it more hydrophobic and membrane permeable [44]. In fact, NACA has been shown to increase cellular levels of GSH either by providing the limiting substrate, cysteine, for GSH biosynthesis or reducing its oxidized form, glutathione disulfide (GSSG), in a thiol exchange reaction [45, 46]. The molecule can also promote intracellular detoxification and act directly as free radical scavenger. NACA acts as a carrier of NAC and its antioxidant and free radical scavenging abilities are equal to or better than those of NAC.

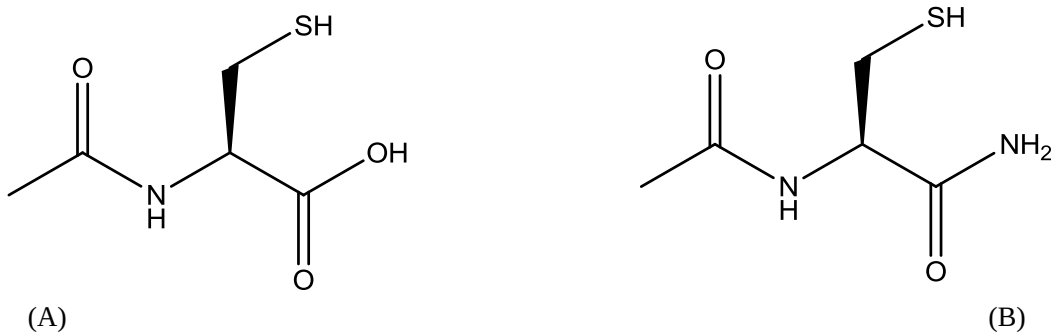

Figure 2. The structures of (A) N-acetylcysteine and (B) N-acetylcysteineamide.

Evidence of the efficient membrane permeation of NACA was demonstrated in a study which assessed NACA for its antioxidant and protective effects in a model using human red blood cells [45]. NACA crosses the blood–brain barrier (BBB), scavenges free radicals, chelates copper, and attenuated myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis in a multiple sclerosis mouse model [47].

Moreover, studies have demonstrated that treatment with NACA results in a remarkable restoration of intracellular thiols, a more effective protection against hemoglobin oxidation, and a substantial reduction of intracellular oxidation compared with NAC [45, 47]. It was also reported to act as a sulfhydryl group donor for GSH synthesis in GSH synthesis-arrested cells [47]. In addition, our own studies demonstrated that NACA supplies significantly more thiols in plasma and liver tissues than NAC. The enhanced ability to penetrate cells allows NACA to be administered at a lower dose than NAC, giving the drug a greater therapeutic index and lowering the risk of side effects that traditionally have been associated with higher doses of NAC.

4. Implications of NACA in Various Oxidative Stress-Related Conditions

Depletion of GSH renders cells particularly vulnerable to oxidative stress. The resulting damage is the key step in the onset and progression of many diseases. We have investigated the role of NACA in neurotoxicity, Diesel Exhaust Particle (DEP)-induced toxicity, Methamphetamine-induced toxicity, metal ion toxicity, radiation-induced toxicity, and cataracts.

4.1. Neurotoxicity

4.1.1. Glutamate-Induced Toxicity

Oxidative stress plays an important role in neuronal cell death associated with many different neurodegenerative conditions such as cerebral ischemia, Alzheimer's disease, and Parkinson's disease. Glutamate, an excitatory amino acid, is one of the major neurotransmitters in the central nervous system (CNS) [48-50]. Elevated levels of glutamate are thought to be responsible for CNS disorders through various mechanisms. Two mechanisms have been proposed for glutamate toxicity. The first mechanism explains the excitotoxicity of glutamate as being mediated through three types of excitatory amino acid receptors [51]. Besides receptor-mediated glutamate excitotoxicity, it has also been proposed that elevated levels of extracellular glutamate inhibit cystine uptake, which leads to a marked decrease in cellular GSH levels, resulting in the induction of oxidative stress [52-54]. Depletion of GSH results in decreased antioxidant capacity of the cell, accumulation of ROS, and ultimately apoptotic cell death. Several studies have demonstrated the induction of oxidative stress by glutamate in various cell lines, including immature cortical neurons [55, 56], oligodendroglia [57], cultured rat astrocytes [58], neuroblastoma cells [52], and PC12 cells [59].

Supplementing cells with antioxidants, such as NAC, lipoic acid (LA) [60], tocopherol [61], and probucol [62] has shown protective effects against glutamate toxicity, mostly by replenishing GSH.

However, in certain neurological diseases, such as cerebral ischemia and Parkinson's disease, enhancement of tissue GSH in brain regions may not be attained because many antioxidant agents are unable to cross the BBB efficiently [63, 64]. Therefore, we investigated the protective effects of GSH prodrug, NACA, and the mechanism behind its protection against glutamate-induced cytotoxicity in the neuronal cell line, PC12 [65]. Incubation of PC12 cells with glutamate resulted in reduction of GSH and cysteine levels when compared to the control group.

Reduced levels of cysteine indicated that the presence of excess glutamate inhibited cystine uptake, which led to decreased GSH levels. Depletion of intracellular GSH may also be due to glutamate-induced accumulation of ROS within the cell. NACA treatment was able to increase GSH and cysteine levels and effectively reverse the inhibitory action of glutamate. The possible mechanism for NACA to facilitate the supply of cysteine was attributed to its membrane permeability and deacetylation to cysteine [66]. Significant increases in ROS and MDA levels were observed in glutamate-treated cells which were reversed upon pretreatment with NACA. NACA supplied an adequate amount of GSH as a substrate for glutathione peroxidase, to effectively decompose lipid peroxides in the cells, thereby reducing MDA levels.

It was demonstrated that NACA protects PC12 cells against glutamate-induced cytotoxicity by preventing glutamate-induced loss of cellular GSH and inhibiting lipid peroxides.

Considering the protective effects of NACA against glutamate-induced cytotoxicity, in which oxidative stress seems to be involved, NACA may be helpful in neurodegenerative disorders, such as cerebral ischemia [67] and Parkinson's disease [64], where GSH levels are depleted in certain regions of the brain.

4.1.2. Human Immunodeficiency Virus 1 (HIV-1)-Associated Neurotoxicity

HIV-1 infection is associated with a high prevalence of disorders related to the CNS [68-70]. About one-third of the infected population develops HIV-1-associated neurocognitive disorders (HANDs) such as dementia, motor impairments, and behavioral dysfunctions [71, 72]. Oxidative stress plays an important role in the pathogenesis of HANDs causing damage to lipids, proteins, and DNA. The brain is especially vulnerable to oxidative damage as a result of its high oxygen consumption rate, its abundant lipid content, and its relative paucity of antioxidant enzymes compared to other tissues.

The BBB, composed primarily of the brain microvascular endothelial cells, forms a tight seal due to the presence of well-developed tight junctions (TJ) that restrict the entrance of circulating molecules and immune cells into the brain [73]. The major components of the TJ include transmembrane proteins, occludin and claudin, and the submembranous peripheral ZO proteins [74, 75]. TJ proteins are also highly sensitive, and respond to the changes in their microenvironment by alteration and dissociation of the occludin/ZO complex, leading to impairment of the BBB [76].

The BBB helps to maintain the homeostatic environment of the brain, supplies the brain's nutritive needs, and plays a role in communication between the brain and peripheral tissues [77-82]. The role of the BBB is important in the development of HIV-associated dementia because it serves as the conduit by which free virus and infected immune cells enter the brain from the circulatory system [83-85]. It also prevents the effective accumulation of many anti-retrovirals in the brain [86, 87] and releases substances in response to immunological (including viral) challenges [88-93]. A mild disruption of the BBB is more frequent in AIDS patients with dementia, as compared with AIDS patients without dementia or seronegative controls [94]. Such problems may be related to the trafficking of immune cells across the BBB [95]. In the last few years, there has been a body of evidence that HIV-1 envelope protein gp120 or Tat (trans-activator of viral replication) may cause BBB changes. There is a possibility that the binding of these proteins to brain endothelial cells may cause many of the changes seen in the BBB of HIV-dementia patients [96]. For example, one study found a significant increase in permeability (up to 47%) in cultured endothelial cells after exposure to gp120 [97]. Another study confirmed that gp120 is capable of changing and activating the vascular component of the BBB *in vivo* [98]. Yet another study of HIV-1 Tat protein demonstrated that exposure of endothelial cells to HIV-1 Tat protein resulted in a dose-dependent increase in oxidative stress and a decrease in intracellular GSH [94]. GSH is responsible for maintaining redox balance in the brain endothelial cells, thereby protecting them from oxidative stress, as well as playing a crucial role in maintaining the BBB's normal functions [99].

We studied oxidative stress induced by HIV-1 proteins gp120 and Tat *in vitro* in an immortalized cell line of rat brain endothelial cells, RBE4 cells [100]. Exposure to gp120 and Tat proteins depleted intracellular GSH, enhanced MDA levels, and reduced CAT, GPx, and GR activities in cultured rat brain endothelial cells indicating that HIV proteins induce oxidative stress in RBE4 cells. Increased cellular oxidative stress may be the major mechanism causing possible HIV-1 protein-induced disruption or alteration in transport systems of the BBB in HIV patients with dementia. In order to prevent the oxidative stress of these viral proteins, prophylactic antioxidant administration might be considered for HIV-1 proteins.

HIV-1 infected patients showed a decrease in GSH and cysteine levels; therefore, it seemed to be a logical solution to replenish GSH and cysteine. Oral NAC increased the intracellular GSH levels, decreased the mortality rate of patients, and prevented the loss of $CD4^+$ T cells. NAC analogs have been introduced as an additional regimen for the treatment of HIV infection [101], but with low therapeutic efficiency owing to their low permeability.

Therefore, we evaluated the effects of NACA on gp120- and Tat-induced oxidative stress and cytotoxicity in RBE4 cells [102]. Various oxidative stress parameters, including GSH, GSSG, CAT and GR activity, as well as MDA levels, were used as measures of oxidative stress. NACA significantly increased the levels of intracellular GSH, CAT, and GR and decreased the levels of MDA in RBE4 cells, showing that oxidatively-challenged cells were protected. GSH depletion has been indicated as being a part of the cell death effector machinery, and it goes hand-in-hand with ROS production during apoptosis in similar systems [103, 104]. It has been reported that HIV-1 Tat and gp-120 induce endothelial cell apoptosis through caspase-3 activation [105, 106]. Exposure to gp120 and Tat was seen to induce apoptosis in RBE4 cells. NACA treatment did reverse the increased caspase-3 activity following gp120 and Tat exposure. The oxidative stress induced by HIV-1 viral proteins can be effectively blocked by NACA and its effectiveness was further evaluated in an *in vivo* model [107].

Many HIV-positive patients use addictive drugs like methamphetamine (METH), which is a well-known neurotoxicant [108-110]. Since METH has been shown to induce oxidative stress, it was of significant interest to understand if METH potentiated the oxidative stress induced by HIV-1 proteins gp120 and Tat at the BBB. The role of NACA in protecting the BBB from oxidative stress-induced damage in animals exposed to gp120, Tat, and METH was investigated [107].

Animals exposed to HIV viral proteins (especially gp120+Tat) had significant decreases in GSH levels in their brains, as compared to controls. The greatest decline in the level of GSH was observed in animals treated with both HIV viral protein (gp120+Tat) and METH, indicating that METH was potentiating the oxidative stress induced by the viral proteins. However, pretreatment of the animals (in the gp120+Tat+METH group) with NACA increased the GSH levels significantly, indicating that the antioxidant NACA was able to partially abrogate oxidative stress-induced damage in these animals. A significant decrease in the activity of GPx was observed in animals treated with gp120+Tat and gp120+Tat+METH, as compared to the controls, indicating that the overwhelming oxidative stress induced by these toxins deplete the antioxidant enzyme in the brain. However, animals in the gp120+Tat+METH group, pretreated with NACA, had GPx activity similar to that of the control. This is in agreement with previous studies, where NACA has been reported to inhibit METH-induced oxidative stress in an *in vitro* model of BBB [111].

In addition, HIV patients abusing addictive drugs like METH have been reported to have exacerbated neurodegenerative changes [112-116], and one of the most critical factors in the development and progression of these changes is the loss of integrity of the BBB [117-119]. A decrease in the expression of tight junction proteins, ZO1 and occludin, in animals treated with gp120+Tat+METH, pointed to the alteration of BBB permeability. Treatment with NACA resulted in increase in expression of these tight junction proteins. In addition to this, TJ proteins like occludin and claudin 5 were also found to be modulated by 4-HNE, which is one of the major biologically active aldehydes generated from peroxidation of membrane lipids [120], and has been implicated in actin cytoskeleton remodeling and disruption of the

endothelial cell barrier in the lungs [121]. One of the initial reactions of 4-HNE in the cells is the protein modification by the formation of Michael adducts [122-124] which, in turn, are capable of invoking a wide range of biological activities by modulation of different cell signaling pathways [121]. These adducts have also been reported to increase paracellular transport of albumin across the human umbilical endothelial cell monolayer [125] and permeability of the BBB [126]. An increase in the expression of Michael's adducts was observed in the TJ proteins (occludin and claudin 5) of animals treated with gp120+Tat+METH, indicating altered permeability of the BBB. Further, studies by Usatyuk et al. [121] have shown that Michaels adducts induce oxidative stress by depleting the intracellular GSH level, modulating mitogen activated protein kinase (MAPK) activation, and altering the endothelial cell barrier function. Michael adducts formations in TJ proteins (claudin 5 and occludin) were partially blocked by pretreatment with the thiol protectant NACA. In addition to Michaels adduct, damage to the BBB have also been linked to 3-Nitrotyrosine (3-NT), a specific and stable marker for peroxynitrite formation [127, 128]. An intense and widespread deposition of 3-NT has been observed in the autopsy CNS tissues of patients with AIDS-associated dementia. However, no 3-NT was detected in patients who had died with HIV encephalitis not associated with dementia [129]. Further, the presence of 3-NT was also detected in patients suffering from multiple sclerosis [130]. TJ protein, claudin 5, was modulated by 3-NT in animals treated with gp120+Tat+METH, pointing to the role of 3-NT in the alteration of BBB permeability in HAD.

In summary, addictive drugs like METH potentiate oxidative stress induced by gp120 and Tat in concert, by decreasing the levels of the antioxidants GSH and GPx in the brain. The free radicals also modify the lipid and protein molecules in the brain, as indicated by the increase in MDA and protein carbonyl levels. Treatment of animals with gp120+Tat+METH, also alters the expression of the TJ proteins, in addition to modulating it, leading to a compromised BBB integrity. However, pretreatment of these animals with the thiol antioxidant NACA, confers protection to these animals and, thus, could be considered as a viable therapeutic option for patients suffering from HAD and other neurodegenerative diseases.

4.1.3. Highly Active Antiretroviral Therapy (HAART)-Induced Neurotoxicity

The introduction of HAART, the combination of three or more classes of antiretroviral drugs, has reduced the prevalence of opportunistic infections such as cytomegalovirus and toxoplasmosis but the clinical impact of HAART toxicity on HIV-1-associated neurocognitive disorders is still controversial [131-133]. Most of the clinical studies report that cognitive impairment is still a significant clinical problem in HAART-treated patients [102, 134]. Accumulated evidence indicates that prolonged use of HAART to treat HIV patients may contribute to an overall increase in amyloid and hyperphosphorylated Tau deposition, two of the key hallmarks of neurocognitive disorders such as Alzheimer's disease [135, 136]. Studies have also shown that neurocognition improved significantly after discontinuation of HAART in subjects with a preserved immune function [137]. Although several *in vitro* studies have shown that HIV proteins and methamphetamine can cause dysfunction in the BBB, the effects of HAART drugs on the BBB endothelial cells and their role in exacerbating HAND are not clear. We studied the effect of a combination of 3'-Azido-2', 3'-deoxythymidine (AZT) and Indinavir (IDV), one of the most commonly recommended HAART regimens on free radical generation and mitochondrial dysfunction-mediated

endothelial cell death, and also the ability of NACA to confer protection against AZT+IDV-induced oxidative stress [138].

HAART drugs were toxic to BBB endothelial cells in a dose-dependent manner. Endothelial cells, exposed to individual drugs, showed that AZT was more toxic than IDV. AZT, a nucleoside reverse transcriptase inhibitor, has been shown to decrease the levels of mitochondrial DNA (mtDNA) *in vivo* and clinically [139, 140] by inhibiting γ-DNA polymerase. The decrease in mtDNA inhibits the synthesis of adequate proteins for oxidative phosphorylation. A subsequent energy loss and increase in production of ROS have been reported in other studies involving HAART treatment [141, 142]. We observed a significant concentration- dependent increase in total ROS, indicating an increase in oxidative stress in response to the treatment.

Long-term AZT treatment has been shown to deplete GSH in rats treated acutely and in cell cultures [143]. A dose-dependent decrease in GSH levels after HAART treatment suggested that AZT+IDV induced ROS production with subsequent decreases in GSH levels. AZT+IDV may have caused an increase in levels of hydroperoxides that exceeded the metabolic capabilities of the glutathione system to maintain glutathione in the reduced form. AZT+IDV treatment increased the levels of lipid peroxidation metabolites because of oxidative damage. Mitochondria are logical targets for oxidative stress, based on their ability to generate free radicals, and may be primarily involved in oxidative stress associated with AIDS treatment. A decrease in the $\Delta\Psi m$ after treatment, along with a dose-dependent decrease in ATP levels after treatment, was observed. Decreases in $\Delta\Psi m$ and ATP levels indicate disrupted mitochondrial function. Elevated levels of cytochrome *c* and procaspase-3 were observed in cytosolic fractions of the treated groups compared to those of the control groups. It was proposed that mitochondrial dysfunction is followed by an extensive release of intracellular calcium and cytochrome *c* which, in turn, might have caused the execution of cell death after exposure to the combination of AZT+IDV in blood–brain vascular endothelial cells. Endothelial cell apoptosis has been demonstrated in an atherosclerotic lesion and has been considered a mechanism for atherogenesis in HAART-treated patients [144]. AZT treatment induced apoptosis in placental cells and parathyroid cancer cells [145, 146]. In contrast to these studies, AZT+IDV did not induce apoptosis at therapeutic concentrations in primary human umbilical cells and various other studies [147, 148]. A noticeable decrease in TEER and an increase in dextran permeativity provided evidence that HAART drugs altered the permeability across the endothelial cell monolayer. To further support the fact that HAART drugs induced oxidative stress in hCMEC/D3 cells, the thiol antioxidant NACA was used to inhibit the effects of the drugs. The GSH and ROS levels in NACA-pretreated cells were similar to those of control. NACA replenished GSH levels, which may be useful in reversing some of deleterious effects of oxidative insults. Thus, treatment with the AZT+IDV drug combination may affect the BBB, and inclusion of antioxidants in HAART therapy may protect HIV-infected patients, particularly those with neurocognitive disorders, against drug-induced oxidative stress.

4.2. METH-Induced Toxicity

Methamphetamine (METH) is an amphetamine analog that causes degeneration of the dopaminergic system in mammals and subsequent oxidative stress. Magnetic resonance

imaging of the brain has shown that chronic use of METH causes neuronal damage [149] and produces numerous adverse effects on the CNS [150]. Further, damage to dopaminergic nerve terminals has also been reported in the brains of METH-treated animals [151-153]. Numerous mechanisms for METH-induced neurotoxicity have been proposed, of which the release of dopamine (DA) in the nucleus accumbens [154, 155] and the successive selective degeneration of striatal DA terminals [156] are the most commonly known. Recently, however, the focus has been on generation of METH-induced oxidative stress. In the brain, DA oxidizes under physiological conditions to form short-lived, toxic ROS [157]. DA dependent oxidative stress has been observed after pharmacologically relevant levels of METH have been administered [158]. Additionally, extracellular concentrations of GSSG also increased after METH administration [159], suggesting either another mechanism of toxicity or an effect of oxidative stress. Impairment of the mitochondrial function has also been linked to pathways involving ROS, leading to a further oxidized environment and a decrease in ATP production [160]. These findings indicate that oxidative stress may play a pivotal role in the neurotoxic damage caused by METH abuse.

Although the initiating cause of toxicity is not known, it seems that oxidative stress acts as a propagating force in spreading the toxicity to mitochondria and other systems that are susceptible to oxidation, as observed in various neurodegenerative diseases like Parkinson's and Alzheimer's diseases [161]. Indeed, evidence of increased oxidative stress has been observed both *in vitro* (SH-SY5Y neuroblastoma cells) and *in vivo* (rat brain tissues), following METH abuse [162-164]. METH abuse has also been reported to cause degeneration in the various regions of the brain [165]. In the literature, brain degeneration has been associated with modifications of the BBB [117-119]. GSH depletion leads to a loss in protein sulfhydryls, which are believed to be essential for membrane functions such as enzyme activities and transport systems [99]. We observed that METH caused oxidative stress to BBB cells, as demonstrated by decreased intracellular GSH, increased MDA levels and intracellular ROS production, and decreased GPx activity. In addition, METH treatment also altered the integrity of the BBB by increasing the permeability of the cells. These toxic effects of METH were reversed, however, by pretreatment of the cells with NACA. This antioxidant restored the levels of GSH, and scavenged the ROS produced by treatment with METH, thereby maintaining the permeability of the BBB.

Oxidative stress is believed to play a crucial role in METH-induced toxicity in the brain and other tissues, as evidenced by findings in previous studies [166-169]. However, a comparison of the oxidative effects of METH in different organs has not been sufficiently studied. We compared the *in vivo* oxidative effect of METH on the brain, liver, and kidney tissues of CD-1 mice. The potential of NACA to prevent oxidative stress-related damage to these organs was also evaluated. Results (unpublished data) indicated that NACA prevented oxidative damage, possibly by scavenging existing ROS, while halting the production of further ROS, lipid peroxidation, and protein carbonylation. In addition, NACA acted by increasing levels of GSH, as well as the activity of the detoxification enzyme, GPx.

Depletion of GSH by METH initiates a myriad of events. There is an initial increase in free radicals that overwhelms the scavenging ability of the GSH-dependent enzymes (glutathione peroxidase), which leads to oxidation of lipids and proteins. Riviere et al. [170] studied the disposition of METH and its metabolite amphetamine and reported the rank order of (+)-METH tissue accumulation after i.v. dosing was kidney >spleen >brain > liver >heart > serum, with terminal elimination half-life values ranging from 53 to 66 minutes. In another

study, Volkow et al. [171] studied METH distribution and accumulation in the human body via Positron Emission Tomography and reported that the liver, brain, and kidney had relatively higher uptakes of the drug. It was distributed through most organs. They reported that the rank order of uptake was lungs, liver > brain > kidneys. METH's clearance was fastest in the heart and lungs; slowest in the brain, liver, and stomach; and intermediate in the kidneys, spleen, and pancreas. We observed (unpublished data) maximum oxidative stress in the kidneys followed by that in the liver and brain. Despite different results reported by the two groups, a high accumulation of METH in most body organs is likely to contribute to the medical complications associated with METH abuse and, therefore, treatment of METH abusers should focus on multi-organ damage.

Currently, no medications are available to safely reverse life-threatening METH overdoses quickly. Medications that could help METH users recover rapidly from the toxic effects of chronic use are highly desirable for compliance to the current treatment regimen. Based on encouraging results from the use of NAC and other antioxidants, we believe that NACA would be more effective in treating METH-induced toxicities because of its better permeability across membranes and low toxicity profile. Combining NACA with current treatment medications, as a neuro and tissue protective agent, might be an effective approach to treating METH abusers. Additional studies are needed to further delineate and clarify strategies that might improve treatment of METH-induced toxicity.

4.3. DEP-Induced Toxicity

Diesel engine exhaust (DEE) is a complex mixture of organic and inorganic gases (NOx, SOx, CO), and particulate matter (PM). Diesel exhaust particles (DEPs), a by-product of DEE, are one of the major components of airborne PM in the urban environment. They are composed of carbon, heavy carbohydrates, hydrated sulfuric acid, polycyclic aromatic hydrocarbons (PAHs) and their derivatives: quinones, semi-quinones, and trace amounts of heavy metals such as iron, copper, chromium, and nickel [172-174]. DEPs have mean diameters of 0.2 µm or less, which render them easily respirable and capable of being deposited in the airways and the alveoli.

DEPs can easily penetrate and settle deep inside the lungs and induce oxidative stress leading to damage of the lungs. Thiol antioxidants have been shown to be effective in treating oxidative effects in the respiratory tracts [175, 176]. We investigated the role of the novel thiol antioxidant, NACA, in protecting the lungs from DEP-induced oxidative damage [177]. We determined the oxidative stress parameters by measuring the levels of GSH, cysteine, and lipid peroxidation by-product (MDA), and the activity of the antioxidant enzyme CAT, in animals exposed to DEP/filtered air. The cytotoxic effects of DEP on lungs were assessed by measuring the lactate dehydrogenase levels and the number of macrophages in these animals. The lungs are constantly exposed to xenobiotics and PM that can damage them. Macrophages, an important target for DEPs [178-181], are the first lines of cellular defense in the lungs, and clear the air space by phagocytosis of the toxic or allergic particles that evade the respiratory tract. After phagocytosis, macrophages respond in a hierarchical fashion to increasing particle load and incremental levels of oxidative stress [182]. A significant increase in the number of macrophages in animals exposed to DEPs and a dose-dependent increase in particle load in the macrophages were observed (Figure 3). These effects suggest

that the macrophages were ingesting the DEPs in an effort to clean the xenobiotics from the lungs. Several enzymes, like cytochrome P4501A1, that are present in the macrophages, have been reported to aid in the detoxification of the xenobiotics in the lungs [183-186]. However, activation of these enzymes contributes to the generation of ROS [186] that causes damage to the lungs. DEP-exposed animals that were pretreated with NACA had a significant decrease in the number of macrophages, indicating the potent role of this antioxidant in reducing oxidative-stress related damage.

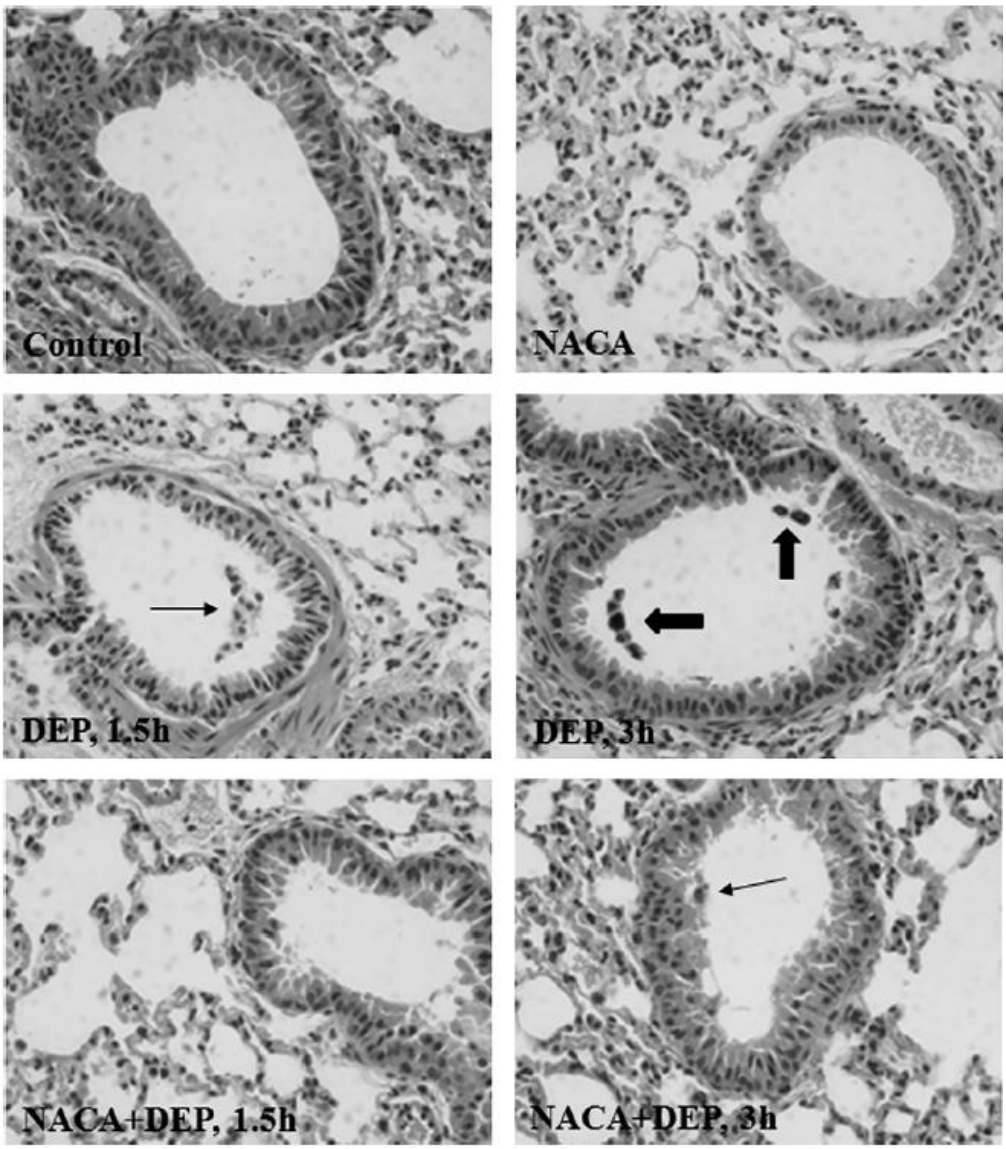

Figure 3. Representative photomicrographs of H&E stained lung section of male C57BL/6 mice exposed to DEPs or clean filtered air for 9 days and sacrificed 24 h thereafter. Large arrows indicate macrophages filled with DEPs. Small arrows indicate macrophages with little or no DEPs. Magnification 40×.

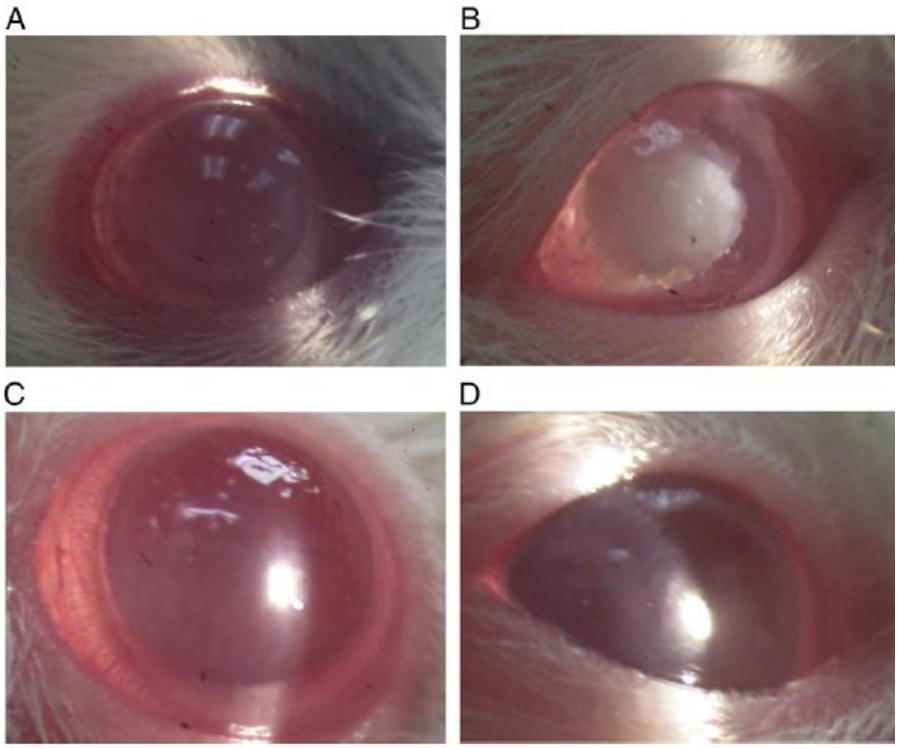

Figure 4. Images of cataract formation, utilizing a slit-lamp microscope at 10× original magnification and a digital camera in macro mode are shown. These pictures were taken when the rat pups were 15 days of age, 1 day before sacrifice. A representative picture of the lenses observed for each group is shown. (A) Control lens. (B) BSO-only lens. (C) NACA-only lens. (D) NACA+BSO lens.

Although the lungs have a well-developed antioxidant system [187], any increase in ROS or depression in the antioxidant system has been reported to result in epithelial cell damage, cell shedding, and bronchial hyperactivity [188, 189]. Studies with animal models have indicated that oxidative stress contributes to airway hyper-responsiveness by increasing damage to the oxidant sensitive-adrenergic receptors, as well as decreasing mucociliary clearance [190]. Consistent with these observations, DEP-exposed animals had a significant decrease in mucous clearance. However, pretreatment of the DEP-exposed animals with NACA significantly improved this condition, indicating that NACA attenuates the hyper-responsiveness in the lungs of DEP-exposed animals by reducing oxidative stress.

GSH, a direct scavenger of ROS [162], has been reported to be a vital component in defending the airspace epithelium from damage in response to oxidants and inflammation. In addition, GSH redox status is also critical for the transcriptional regulation of many pro-inflammatory genes [191]. Decreases in GSH levels in the lungs have been reported in various pulmonary diseases like idiopathic pulmonary fibrosis and acute respiratory distress syndrome [192, 193]. GSH has also been reported to attenuate IL-13-induced asthma in mice [194]. This indicates that low levels of GSH may render individuals susceptible to the deleterious effects of exposure to inhaled toxicants and may also perpetuate inflammatory responses in their lungs. Results showed that animals exposed to DEPs have significant decreases in their GSH levels, indicating that exposure to DEPs induces oxidative stress in these animals. In the DEP-exposed group, however pretreatment of the animals with NACA,

increased their GSH levels. Further, NACA-treated animals also had significant increases in their cysteine levels, as compared to the untreated group. Results also indicated that exposure of animals to DEPs significantly decreases the CAT levels. Pretreatment with NACA increased the CAT levels, thereby protected the lungs by removing hydrogen peroxide and superoxide radicals. Exposure to DEPs induced a significant increase in MDA levels in these animals, as compared to the NACA-treated group, thereby pointing to the role of this antioxidant in protecting animals from DEPs-induced damage. Increases in the release of LDH have been reported to occur as a result of ischemia, starvation, dehydration, injury, and chemical poisoning [231, 232]. Increases in LDH levels were observed in animals exposed to DEPs, and pretreatment of the animals with NACA resulted in a decrease in the LDH levels in the lungs. These results demonstrated a positive correlation between LDH activity and MDA levels in the NACA-treated animals and the untreated DEP-exposed animals, suggesting that the release of LDH was a result of damage to the cell membranes due to lipid peroxidation. In addition, cytotoxicity in the lungs may also be attributed to increased apoptosis of the macrophages [180] due to DEPs. Apoptosis of the macrophages involves shedding of the apoptotic bodies after death, which spreads the toxic chemicals to the neighboring cells. This further induces cytotoxicity, as these apoptotic bodies may contain active chemicals like PAHs, and their uptake by the surrounding inflammatory cells may further induce cytotoxicity and contribute to pathogenesis of respiratory diseases [180].

Data indicates that, after exposure to DEE, DEPs enter into the lungs, where they are engulfed by the macrophages. These particle-laden macrophages induce oxidative stress in the lungs, as indicated by decreases in GSH and CAT levels, and increases in MDA levels. Administration of NACA however, resulted in significant reductions in macrophages and oxidative damage in lungs, thereby suggesting a therapeutic potential for this novel antioxidant.

4.4. Radiation-induced Cytotoxicity

The search for more effective radioprotectors has intensified recently due to increased use of ionizing radiation in radiotherapy for the treatment of malignant tumors. Radiotherapy treatment modality relies on the generation and use of ROS to eradicate tumors [197] and, in the process, non-target tissues are also damaged [198]. Therefore, the application of ionizing radiation to the treatment of malignant tumors has been limited by the need to avoid extensive damage to normal tissues [199]. The highly toxic hydroxyl radicals produced by ionizing radiation, attack the DNA molecules, causing single and double strand breaks. Additionally, ROS also cause lipid peroxidation and protein oxidation. These deleterious changes impact the cellular functions negatively, resulting in cell death, thereby lowering cell viability. Radiotherapy for cancer patients can be greatly enhanced by the use of radioprotectors, capable of protecting normal tissues from radiation induced oxidative stress. Thiol antioxidants, namely, cysteine, GSH, NAC, and β-mercaptoethylamine (cysteamine) have been shown to protect mice and rats against the harmful effects of radiation [200, 201]. However, some of these thiols, such as cysteamine, have been reported to have lethal and behavioral toxicities [202]. Some studies have also demonstrated that NAC can act as a radioprotective agent against oxidative damage induced by UV, ionizing radiation, and

gamma rays [203-207]. However, low bioavailability of NAC limits its use as a radioprotectant.

We therefore investigated the possible protective effects of NACA in radiation-challenged CHO K1 cells, in comparison to NAC [208]. Assessment of the radioprotective effects of NACA and NAC was performed by measuring the parameters (such as GSH, cysteine, and MDA levels) in both the treatment and control groups, as well as the activities of antioxidant enzymes, namely, GPx, GR, and CAT. A comparison was made of all parameters measured in the different treatment groups to identify the antioxidant capable of providing better radioprotection, with minimal toxic effects. Both antioxidants had a similar capability in replenishing GSH and cysteine levels that had been depleted by radiation treatment. However, NACA had better abilities to prevent radiation-induced cytotoxicity and lipid peroxidation as compared to NAC. Both NACA and NAC protected the cells from radiation-induced apoptosis by controlling the levels of caspase-3, the enzyme that triggers programmed cell death. Radiation significantly decreased the activities of CAT, GPx, and GR, possibly due to oxidation of sulfhydryl groups of the residues present in their active sites, or due to other structural and functional changes induced in the enzyme by ionizing radiation [209]. Treatment with NAC and NACA restored enzyme activities to control levels. However, NAC was comparatively less effective in bringing the activities of these enzymes to control levels. Previous investigations have shown that exposure to ionizing radiation does decrease activities of antioxidant enzymes, while antioxidants that help restore these activities have a protective effect on the cells [210]. It can be concluded that NACA is indeed better than NAC as an antioxidant thiol radioprotector. The effectiveness of NACA in *in vitro* models shows that further investigation is warranted in *in vivo* models, to determine its effectiveness in protecting normal cells in animals subjected to irradiation. NACA thus holds promise for being used as a supplement in situations that require protection from ionizing radiation.

4.5. Metal Ion Toxicity

Lead is a ubiquitous environmental toxicant. Severe and acute lead poisoning can cause encephalopathy, convulsion, coma, and even death. Despite several efforts to reduce lead levels in the environment, lead exposure continues to be a major public health problem, particularly in urban areas in the US as well as in third world countries [211]. Lead adversely affects the CNS, and low level exposure is associated with behavioral abnormalities, decreased hearing, and cognitive impairment [212]. The CNS symptoms of infants and children who have lead poisoning include impulsive and aggressive behaviors, greater difficulty in paying attention, and problems in following directions [211]. Although the biochemical and molecular mechanisms of lead-induced toxicity are poorly understood, studies suggest that at least part of the lead-induced cell damage may include excitotoxicity, apoptosis, adverse influences on neurotransmitter storage and release processes, interference with calcium-mediated cellular processes, and activation of protein kinase *c* (PKC) [213]. Although several mechanisms may mediate these effects, lead's ability to mimic calcium is a key factor in its toxic effects [214]. Lead is able to cross the BBB and may activate PKC by mimicking calcium, which in turn, may increase ROS production [214]. Studies indicate that lead-induced neurotoxicity has a glutamatergic component [215]. The neurotoxic effects of

lead are attributed to its deposition in astrocytes and its continuous release into the synaptic spaces [216]. Glutamate, an excitatory amino acid, is one of the major neurotransmitters in the CNS. It is released into the synaptic spaces and binds to the glutamate receptors which facilitate normal synaptic transmission, synaptic plasticity, and brain development [217].

Studies indicate that accumulation of glutamate in the extracellular space overly stimulates the glutamatergic receptors, which may be excitotoxic to neurons [50]. This process is associated with an influx of calcium and translocation of PKC, which mediates cellular processes [52, 214]. Reports indicate that glutamate may interact with lead, and this interaction may result in neuronal damage [218]. PKC activity changes seem to be a key factor in glutamate-induced ROS production and in the amplification of glutamate-induced ROS production by lead. Therefore, glutamate excitotoxicity, lead-induced neurotoxicity, and oxidative stress may be related phenomena causing neuronal damage [219-220]. Furthermore, one of the studies indicates that under conditions of lead toxicity in an adult rat brain, the hippocampus is most vulnerable to excitotoxicity because of an impaired clearance of the released glutamate [221]. The results discussed above, concerning the effects of lead on glutamatergic functions, raise the reciprocal question of whether glutamate has effects on lead-induced cell death. The possible involvement of oxidative stress in both lead and glutamate toxicity suggests that antioxidants might be effective in reducing the toxicity of glutamate and lead. Antioxidants have been the choice of agents for use against several oxidative stress conditions. In our previous studies, we have shown the protective capabilities of different antioxidants, including taurine [222], captopril [223], alpha-lipoic acid [224], and N-acetylcysteine [225], against lead-induced oxidative stress *in vitro* and *in vivo*. Lead crosses the BBB and accumulates in astrocytes, where it may impair cell function and perturb glial-neuronal interactions [226-228]. Therefore, antioxidants that cannot cross the BBB may not be effective against lead-induced neurotoxicity and glutamate excitotoxicity. NACA, which is able to cross the BBB, might be a potential therapeutic in treatment of lead-induced neurotoxicity.

We have previously shown the protective effects of NACA against glutamate-induced cytotoxicity in PC12 cells [65]. We also studied the cytotoxicity in PC12 cells caused by exposure to a combination of lead and glutamate [229]. Results suggested that glutamate (1 mM) potentiates lead-induced cytotoxicity by increased generation of ROS, decreased proliferation (MTS), decreased GSH levels, and depletion of cellular adenosine-triphosphate (ATP). Consistent with its ability to decrease ATP levels and induce cell death, lead also increased caspase-3 activity, an effect potentiated by glutamate. Exposure to glutamate and lead elevated the cellular MDA levels and phospholipase-A2 (PLA2) activity and diminished the glutamine synthetase (GS) activity. NACA protected PC12 cells from the cytotoxic effects of glutamate plus lead, as evaluated by MTS assay. NACA reduced the decrease in the cellular ATP levels and restored the intracellular GSH levels. The increased levels of ROS and MDA in glutamate–lead treated cells were significantly decreased by NACA. Data showed that glutamate potentiated the effects of lead-induced PC12 cell death by a mechanism involving mitochondrial dysfunction (ATP depletion) and oxidative stress. NACA had a protective role against the combined toxic effects of glutamate and lead by inhibiting lipid peroxidation and scavenging ROS, thus preserving intracellular GSH.

Involvement of a glutamatergic component in lead-induced toxicity and role of NACA, as a potential chelator against the entire toxic phenomenon of lead in comparison with NAC was also evaluated in an *in vivo* model [230]. NACA was found to be effective in lowering

blood lead levels, when compared to NAC. This may be attributed to the greater permeability of NACA, than that of NAC. Our results indicated that decreased GSH levels are accompanied by increases in GSSG levels in RBCs, livers, and brain tissues, following exposure to lead. High levels of MDA were seen in RBCs, brain, liver, and kidney of lead-exposed animals. Also an increased Glu/Gln ratio, accompanied by decreased GS activity in lead-exposed animals was observed. This may be possible because GS, the astrocyte-specific enzyme, converts the toxic glutamate to non-toxic glutamine. Therefore, this observation suggests that, under lead exposure, brain tissue is vulnerable to the excitotoxic cell damage arising from the impaired clearance of the released glutamate. Furthermore, activation of glutamatergic receptors may alter the activity patterns of a calcium-sensitive enzyme such as phospholipase. Increased PLA2 activity was observed in lead-exposed animals, when compared to that in control animals. Administration of glutamate potentiated the detrimental effects of lead by altering the GSH and MDA levels in the RBCs and brains. The results of GSH, MDA, and PLA2 activity indicate the involvement of free radicals in potentiation of lead-induced toxicity by glutamate. NACA was more effective than NAC at counteracting the glutamate and lead induced neurotoxicity. Considering current chelation therapy and the toxic effects of chelators, multi-functional NACA may be effectively included in the treatment of lead poisoning.

4.6. Cataracts

Cataracts are the most common cause of treatable blindness worldwide and develop as a result of the progressive loss of transparency of the lens [231, 232]. It has been demonstrated that oxidative stress plays an important role in cataract etiopathogenesis, as with many age-related diseases [233-238]. A developing lens has numerous antioxidant enzymes and a high concentration of ascorbate and GSH that protect the lens against oxidative damage [9, 239]. The resistance of the lens to oxidative damage diminishes in correlation with the age-related decrease in GSH levels and the degradation of GR [240-242]. These age-related changes in antioxidant defense mechanisms cause crosslinking, aggregation, insolubility, and fragmentation of crystalline proteins (main structure of the lens), which result in the formation of cataracts [240, 242-245]. Cataracts are the most common cause of blindness, accounting for almost half of all cases worldwide [246]. GSH is a crucial intracellular thiol that protects the lens against oxidative stress and helps maintain lens transparency [239]. GSH is an indispensable and primary lenticular antioxidant [242]. There is a wide body of evidence that indicates loss of GSH occurs because of the oxidation of GSH to GSSG, because GSSG levels increase drastically once the cataracts develop. In addition, hyperbaric oxygen causes cataracts, as it is characterized by the loss of GSH and protein–SH, as well as protein insolubilization [247]. At present, the treatment for cataracts requires removal of the natural lens that has developed opacification, through surgery, and replacing it with a synthetic lens to restore the lens's transparency. Although cataract surgery is considered to be one of the safest procedures, treatment is relatively expensive and there is a significant rate of post-surgical complications, including the development of a posterior capsular opacification [248]. Therefore, an alternative method for treating or preventing the occurrence of cataracts would be through the use of a potent thiol-exchange compound.

The effects of NACA on cataract development in our lab were evaluated in Wistar rat pups (249). Cataract formation was induced in these animals with an intraperitoneal injection of a GSH synthesis inhibitor, L-buthionine-(S,R)-sulfoximine (BSO). The pups were sacrificed on postpartum day 15, after examination under a slit-lamp microscope. Their lenses were analyzed for selective oxidative stress parameters, including glutathione (reduced and oxidized), protein carbonyls, CAT, GPx, GR, and MDA. The lenses of pups in both the control and the NACA-only groups were clear, whereas all pups within the BSO-only group developed well-defined cataracts. It was found that supplemental NACA injections during BSO treatment prevented cataract formation in most of the rat pups in the NACA+BSO group. Only 20% of these pups developed cataracts, and the rest retained clear lenses (Figure 4).

Further, GSH levels were significantly decreased in the BSO-only treated group, but rats that received NACA injections during BSO treatment had these levels of GSH replenished. GSH levels were restored in rats to 90% of that of the control groups during BSO exposure. Supporting these data, it was observed that NACA caused an increase in GR activity in BSO-treated rats. Increases in GSH levels and the GSH/GSSG ratio may be attributed to the increased activity of GR by preserving the integrity of cell membranes and by stabilizing the sulfhydryl groups of proteins. Further, NACA itself may act as a sulfhydryl group donor for GSH synthesis and thereby decreasing the loss of protein sulfhydryl groups, as well as opacification of the lens. NACA was also able to reduce MDA levels by supplying an adequate amount of GSH as a substrate for glutathione peroxidase to effectively decompose lipid peroxides in the rats. Oxidative damage to proteins, as reflected by protein carbonylation, was significant in BSO-treated animals and this was reversed by NACA. NACA was also able to restore both CAT and GPx to those levels seen within the control group. The possible mechanism for the restored CAT activity in BSO-exposed rats, when treated with NACA, may be the scavenging of free radicals by NACA. However, further investigation is needed to confirm this theory. Increased GPx activity is probably due to higher levels of GSH, which GPx uses as a substrate for its action.

In summary, these results strongly suggest that antioxidants have the ability to protect against, or to delay, the onset of cataract formation by reducing oxidative damage. NACA could confer a protective effect by providing a substrate for the generation of GSH and the ability to maintain antioxidant levels within the lens and, possibly, through disulfide-exchange mechanisms. Treatment with NACA may prove to have a major therapeutic role. In future studies, we will focus on the prophylactic role of NACA on cataract formation induced by various agents and investigate the development of a topical formulation for the application of this antioxidant.

Conclusion

In conclusion, NACA, a novel GSH prodrug, is a potent therapeutic option for treatments aimed at maintenance of GSH homeostasis and cellular antioxidant levels. The current interest in GSH prodrugs will lead to development of newer more potent GSH prodrugs with higher bioavailability and enhanced membrane permeability which would reduce the toxicity associated with higher doses of current prodrugs. GSH prodrugs are favorable therapeutic

substitutes for GSH in treating oxidative stress-related disorders, the potential of which will be realized in the near future.

Acknowledgments

We appreciate the efforts of Barbara Harris in editing the manuscript.

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In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter VII

Role of Glutathione in Infection and Cardiovascular Diseases

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ABSTRACT

Integral to the effective maintenance of cellular homeostasis, and indeed survival, is the neutralization of reactive oxygen intermediates (ROIs). ROIs are generated by a vast array of metabolic, physiological, and immunological processes. The first-line defense against such "oxidative stress," as it has been coined, is a compound known as reduced glutathione (GSH). GSH is only available in finite quantities; hence, during times of large oxidative insults, GSH levels become depleted. As a result, membrane phospholipids become disorganized which may impair proper functioning of vital membrane anchored and embedded transport proteins. Findings from the recent studies signify the importance of GSH in augmenting the immune responses against human immuno-deficiency virus (HIV) and *Mycobacterium tuberculosis* (*M. tb*) infections as well as against atherosclerosis. This chapter provides a comprehensive review of the beneficial effects of GSH in augmenting the functions of macrophages, T lymphocytes and natural killer cells in controlling *M. tb* infection in both healthy individuals and individuals with HIV infection. The review also discusses the beneficial effects of GSH against atherosclerosis.

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Abbreviations

BSO: buthionine sulphonine
CAD: coronary artery disease
GCLC: catalytic GCL
GCLM: modified GCL
GGT: γ-glutamyl transpeptidase
GPx: GSH peroxidase
GSH: glutathione
GSNO: nitrosoglutathione
GSS: GSH synthetase
GSSG: GSH disulfide
HDL: high density lipoproteins
HMDM: human monocyte derived macrophages
iNOS: nitric oxide synthase
LDL: low density lipoprotein
LGSH: liposomal GSH
MDA: malondialdyde
MDR TB: multi-drug TB
MIP-1: Macrophage Inflammatory Protein-1
mmLDL : modified LDL
M. tb: Mycobacterium tuberculosis
NAC: N-acetylated L-cysteine
NK: natural killer
NO: nitric oxide
NOS: nitric oxide synthase
Ox-LDL: oxidized-LDL
PBMC: peripheral blood mononuclear cell
RNI: reactive nitrogen intermediates
ROIs: reactive oxygen intermediates
TB: tuberculosis
TLRs: toll-like receptors
XDR TB: drug resistant TB

1. Introduction

The propensity of an organism to regulate its internal environment by various feedback mechanisms, so as to stabilize its function and maintain a condition of equilibrium or stability is termed homeostasis. Within the human body generation of metabolic by-products such as ROI, are continuously assuming a destructive role as they interfere with that homeostasis. The continued effects of ROI create stress and strain on the integral molecular structures which uphold the basic architecture and function of the cell. Antioxidants, such as GSH provide first-line of defense against such “oxidative stress”, and are vital to the continued maintenance and survival of the cells [1-4].

2. Synthesis of GSH

Reduced GSH (GSH) is the principal molecule used in the intracellular compartment of cells to neutralize potentially damaging oxidative species. This potent antioxidant is synthesized in a two-step series of adenosine triphosphate (ATP) dependent reactions, but synthesis is dependent upon facilitated transport of cystine into the cytoplasm from the extracellular environment by the Xc-transport, a cysteine/glutamate antiporter system which can be selectively inhibited by high plasma glutamate. Inside the cytoplasm, cystine is converted to cysteine which is then utilized for the synthesis of GSH [1-4]. N-acetylated L-cysteine (NAC) is the most commonly used pharmacologic agent to replenish the intracellular levels of GSH. NAC serves as a reservoir for sulfhydryl groups (thiol groups) which account for GSH's biological activity [1-4].

Once NAC is successfully taken up by the cell, the acetyl group is removed and the rate-limiting enzyme of GSH synthesis, gamma-glutamylcysteine synthetase (glutamate cysteine ligase, γGCL) combines cysteine with gamma-L-glutamate to form gamma-glutamylcysteine (GSH).

In turn this rate-limiting step is selectively inhibited by buthionine sulphonine (BSO). The final step of GSH synthesis involves the addition of a carboxy-terminal glycine to gamma-glutamylcysteine by the enzyme GSH synthetase (GSS).

3. Biochemistry and Mechanism of Action

Once produced, GSH can combine with oxidative molecules through thiol groups on cysteine to reduce intracellular oxidative stress [1-4]. GSH is converted to an oxidized form (GSSG) once cysteine is oxidized to cystine; a system exists for the constant recycling of GSSG back to GSH via an enzyme, GSH reductase. It is important to note that the body's principal mechanism of combating infection involves the generation of free radicals, notably by the NADPH oxidase and nitric oxide synthase (NOS) systems [1-4]. These systems in turn may stress the antioxidant capacity of the intracellular GSH pool, leading to oxidative damage before GSH reductase is able to effectively function, hence a bottleneck occurs at this enzymatic step and large quantities of GSSG accumulate, leading to a decreased GSH: GSSG ratio resulting in oxidative stress. The principal mechanism of cellular damage by oxidative stress is thought, atleast initially, to involve damage to the inner leaflet of the plasmalemma by oxidation of membrane lipids.

A laboratory marker for membrane lipid oxidation is malondialdyde (MDA), a free radical is generated as a byproduct in the reaction; it functions to further amplify the oxidative damage to the plasma membrane.

As expected, MDA levels have been shown to correlate with decreased intracellular GSH levels [5]. Once membrane lipids begin to be denatured from their native configuration, the integrity of protein channels begin to be compromised, notably the channel used for intracellular uptake of cystine (Xc-transporter).

This will result in a situation where no *de novo* GSH can be made to neutralize the exponentially increasing pool of ROI [6].

4. GSH and Bacterial Cells

While GSH production seems to be ubiquitous in eukaryotes, only a subset of prokaryotes produces this antioxidant. However, some of the bacteria that are not capable of producing GSH can import it into the bacterial cell from an external source. *Escherichia coli* is one bacterium that can synthesize GSH. Though GSH is dispensable for *E. coli* during the logarithmic phase of growth probably due to the existence of redundant systems, its synthesis does contribute to the resistance to oxidative damage. In stationary phase growth, mutant *E. coli* bacteria that cannot synthesize GSH and have two-fold decreased growth rate possibly due to increased oxidative stress conditions during this phase of growth [7]. *E. coli* uses two enzymes that participate in synthesis of GSH, γGCL and GSS. In *E. coli* these enzymes are induced by hydrogen peroxide through the tetrameric transcription factor OxyR which responds to oxidative stress [7]. The oxidized form of the transcription factor activates stress responsive promoters some of which pertain to GSH synthesis and homeostasis and thus serves as a mechanism to sense environmental oxidative stress. In another Gram negative organism, Salmonella sp., GSH synthesis is induced by the stringent response [8]. This occurs under conditions of starvation when the alarmone ppGpp is produced. This in turn activates DnaK suppressor protein (DskA) which is a response regulator that controls expression of genes whose products are responsible for production of GSH. This response serves to increase resistance of the bacterium to oxidative damage during stationary phase growth.

In bacteria as in eukayotes, GSH serves to resist oxidative stress and is itself oxidized so that two molecules are linked by a disulfide bond. In *E. coli* under oxidative conditions, as reduced GSH is consumed and oxidized GSH predominates, the enzyme GSH reductase is induced [9]. GSH reductase is also induced in an OxyR dependent manner in *E. coli* in response to H_2O_2 [10]. Thus GSH reductase can recycle oxidized GSH back to a reduced form to regenerate oxidative damage fighting molecules that aid the bacterium in resistance to oxidative damage. In *E. coli* much of the GSH that is synthesized is located within the cytoplasm of the cell where reducing conditions predominate and most SH groups are in the reduced form [10]. In contrast, the periplasm of the bacterial cell is more oxidized, with many proteins possessing disulfide bonds and GSH concentrations in this subcellular compartment very low. Surprisingly, GSH can be transported extracellularly in *E. coli* to accumulate in the growth media [10]. This perhaps functions to neutralize toxic oxidative stress that may occur extracellularly. The bacteria may seek to prevent damage before it reaches the bacterium. Cyanobacteria also synthesize GSH. As these bacteria are involved in aerobic photosynthesis, ROI may be produced that can damage the host cell. GSH in these species are limited to the cytoplasm and cell wall [11].

Gram positive bacteria can synthesize GSH using two enzymes, γGCL and GSS as occurs in *E. coli*, or using one enzyme that contains both functions as occurs in *Streptococcus agalactiae* [12]. In fact a number of Gram positive organisms synthesize GSH which include streptococci, enterococci, lactobacilli, listeria, and clostridium which aid these species in resisting oxidative damage. In addition, Gram positive organisms possess GSH transferases that serve to neutralize toxic compounds that could inhibit growth of the bacteria [13, 14]. These GSH transferases can attack electophilic groups or hydrophobic groups on hydrophilic toxic compounds.

Many bacteria do not synthesize GSH but import it from an external source such as the *in vivo* milieu of a human host. The imported GSH may be used to increase resistance to oxidative stress or it may be used as a source of cysteine to support growth. *Francisella tularensis (F. tularensis)* a fastidious organism is unable to grow in media lacking cysteine. GSH can substitute for cysteine in minimal media lacking this required nutrient to support *F. tularensis* growth [15]. Francisella requires γ-glutamyl transpeptidase (GGT) to cleave GSH and transport it into the bacterium. The transporter GGT is required for growth in media lacking cysteine and intracellular multiplication within animal cells. Thus in this system GSH is cleaved before being imported into *Francisella*. Other bacteria such as *Streptococcus mutans* can transport GSH directly across the cell wall [16]. *Haemophilus influenzae* cannot synthesize GSH but can import it from the growth media [17-19]. Imported GSH protects against oxidative stress and also provides a source of sulfur [17]. Much as for *Francisella*, GSH transport into the bacterium is essential for *H. influenzae* growth in media lacking cysteine. Another bacterium *Helicobacter pylori* also uses GGT to transport GSH [15]. Interestingly mycobacteria contain GGT and can import GSH into the mycobacterial cell.

Bacteria such as *Lactococcus lactis (L. lactis)* can import GSH which can help to protect the bacteria against oxidative damage. The presence of GSH can result in increased stability of the strain. This stability translates into increased resistance in the face of aerobic growth which may be important for the use *of L. lactis* as a starter culture in cheese production. *L. lactis* also produces GSH reductase, thus reducing oxidized GSH from imported pools. GSH reductase increases under conditions of aerated agitated cultures. This enzyme is also variable among strains indicating that certain strains are more resistant than others. It may be an important characteristic of a successful starter culture to possess initially greater quantities of GSH reductase. Other bacteria such as *Streptococcus thermophilus* and *Enterococcus faecalis* at increased oxygen concentrations also augment levels of GSH reductase in the bacterium.

Furthermore, some bacteria may use GSH to aid in their virulence. The dental pathogen *Treponema denticola* is an oral pathogen that is associated with periodontitis. This bacterium is located within deep periodontal pockets within the gingiva. Cultivation of this bacterium in media which contains GSH resulted in H_2S production which is thought to be damaging to host cells [20].

Thus utilization of GSH present in a host can lead to a product which may increase the size of the periodontal pockets and encourage further growth of these treponemes. In addition GSH can be metabolized to pyruvate which promotes bacterial growth and further encourages colonization of dental pockets by the bacteria.

In addition to oxidative damage, GSH can protect against other stresses as well. In the Gram positive organism *Lactobacillus sanfranciscensis*, addition of GSH to growth media protects this microorganism against freeze-thawing and cold treatments [21]. It is hypothesized that it does this by preventing peroxidation of membrane fatty acids. This presumably helps to stabilize membranes and aid as a barrier to environmental stresses. In. *E. coli* which can synthesize GSH in response to oxidative stress, this system can also lead to resistance to heavy metals [22].

In particular in a background of mutant bacteria that lack a mechanism to remove heavy metals from the microorganism as they are deficient in metal efflux systems, GSH is important to sequester heavy metals.

5. GSH and Antibacterial Effects

In addition to its role as an antioxidant, GSH has also been demonstrated to exhibit antimicrobial activity. In contrast to the previous examples where GSH protects bacteria and promotes growth, GSH is toxic to *M. tb*. In fact when the mycobacterial GGT is deleted from the genome, the deletion mutant is resistant to the antimycobacterial effects of the GSH, indicating that it must be imported to have an effect [23]. As mycobacterial contain other antioxidant molecules, mycothiols, it may be possible that GSH disturbs the redox balance within these cells and results in toxicity [24]. In fact macrophages which produce elevated levels of GSH have enhanced antimycobacterial activity [25]. It has been proposed that GSH is an evolutionary remnant of eukaryotic-derived antibiotics. This hypothesis is based on the observation that GSH resembles penicillin, a first-generation cephalosporin antibiotics; it has been proposed to convert into a beta-lactam form known as glutacillin which may interfere with bacterial cell wall propagation [26]. It has been shown that GSH must be cleaved from the naïve tripeptide form into an active dipeptide by the removal of glutamate by the GGT, a ubiquitous bacterial enzyme. The dipeptide form of GSH is able to enter the bacterial cell membrane through active transport via the enzyme dipeptide permease [27, 28].

6. Antimycobacterial Activity of GSH

Further studies on the antibacterial effects of GSH by Venketaraman *et al*; demonstrated that mutant strains of mycobacteria that lack GGT are resistant to the deleterious effects of GSH. The effects of GSH are multifaceted; 10mM of GSH was demonstrated to be bacteriostatic; while, 10mM of nitrosoglutathione (GSNO) was bacteriocidal to *in vitro* intracellular mycobacteria. Hence, activation of the nitric oxide (NO) system within the macrophage is a requirement for bacteriocidal action. When this system is activated, ROS are produced as an inevitable byproduct; to buffer this increased oxidative load, GSH production is also inducted and serves not only as a carrier for NO in the dipeptide form, but also directly reduces ROS load within the macrophage [29, 30].

M. tb is the causative organism responsible for tuberculosis (TB). Prior to the discovery of streptomycin in 1943, TB was considered one of the most deadly infections known to man. TB is engrained in human history, skeletal remains dating back to before biblical times have demonstrated abnormalities likely caused by Pott's disease; Antediluvian Greeks called it *phthisis*, Romans called it *tabes*, the Hindus called it *rajayakshma*, and the Victorian English called it *consumption* [31]. All these names reflect the severe cachexia associated with the infection. The best estimates suggest that TB was globally endemic by 1000 AD. After digesting these facts, then contemplating that at present, treatment of TB requires a multidrug regimen taken daily for 8-12 months, you may begin to develop respect for *M. tb* [32]. TB remains the oldest infectious disease in human history, and disturbingly, seems refractive to eradication; and even worse, is rapidly evolving into multi-drug (MDR TB) and extremely drug resistant (XDR TB) strains. TB disproportionally affects individuals living in the developing world, and the need for cost effective treatment is at the pinnacle of public health concern [33].

6.1. GSH and Macrophage Control of *M. tb* Infections

To understand the mechanism of defense against *M tb* a closer look at the immune cells involved and their function is necessary, especially the role of monocytes. Monocytes are derived from bone marrow precursor cells, when released into the blood they typically survive no more than 48 hours in the circulation. If monocytes are signaled by chemokines to leave the circulation and enter the peripheral tissues they are able to differentiate into mature macrophages capable of long-term residence [34]. These macrophages are the principle effector cells of the innate immune responses, but also serve as a reservoir for intracellular pathogens in various chronic infectious diseases. While monocytes within the circulation maintain the nitric oxide system, macrophages lose this system during the process of differentiation; albeit, unless they are activated by IFN-γ. NO is synthesized within monocytes and activated macrophages by inducible nitric oxide synthase (iNOS); once NO is generated in sufficient concentration it can be used to induce oxidative damage to both extracellular and internalized pathogens [30].

Macrophage activation not only requires direct contact with the pathogen through interaction with membrane bound toll-like receptors (TLRs) but also requires IFN-γ for complete activation. Once murine macrophages are activated they generate NO [36]. Our studies indicate that in murine macrophages, GSH acts as a carrier molecule for NO; when these two molecules combine the result is the formation of GSNO (nitrosothiol) and GSNO degrades internalized *M. tb*. However, non-activated macrophages remain as a potential reservoir for pathogen growth [36, 37].

Findings from several laboratory studies using human monocyte derived macrophages (HMDM) clearly indicate that addition of exogenous IFN-γ to HMDM neither result in NO production nor activation of HMDM. If pathogens are able to prevent activation of their resident HMDM; they are able to hide from the immuno-surveillance of neutrophils, gamma-delta T-cells, and the complement system, from within the HMDM itself.

It has also been demonstrated that GSH has direct antimycobacterial effects on the growth of *M. tb* as evident from the studies using macrophages isolated from iNOS knockout mice which were able to maintain mycobacterial growth stasis following NAC treatment, suggesting that malfunctioning macrophages *in vivo* may possibly lack sufficient bacteriostatic glutacillin.

The complete picture of GSH involvement in human innate defenses is beginning to be understood by two different *in vitro* models; HMDM and Whole Blood Model. In the HMDM model, differentiated macrophages were infected *in vitro* with *M. tb*. Once internalized, *M. tb*-infected HMDM were treated with NAC and the growth of *M. tb* was monitored during 7 days post-infection. We observed inhibition in the growth of *M. tb* in GSH-enhanced HMDM compared to a control group which did not receive NAC following direct infection with *M. tb*; a third group of HMDMs which were treated with BSO showed an increased intracellular survival of *M. tb*. This model suggests that GSH plays a direct antimycobacterial role. Data demonstrates that maximal decrease in the growth of *M. tb* occurs when 10mM NAC is added to HMDM culture; while one would think there is never too much of a good thing, results have shown that when the NAC concentration is doubled to 20mM, mycobacterial growth velocity actually accelerates, suggesting a delicate range of intracellular redox states exist which must be maintained for effective antibacterial action. Once deficient of intracellular

GSH, HMDMs begin to function illogically which research suggests is initially due to ROI accumulation leading to mitochondrial damage. Brown *et al*., has demonstrated that GSH deficient HMDMs have a significantly reduced rate of phagocytosis compared to control, this seems to be the earliest sign of intracellular oxidative stress [38]. As ROIs accumulate, HMDMs begin to upregulate expression of CD36, a known scavenger receptor with high affinity for oxidized-LDL (ox-LDL); eventually, excessive uptake of ox-LDL transforms HMDMs into foam cells; furthermore, ox-LDL accumulation has been demonstrated to result in HMDM upregulation of Macrophage Inflammatory Protein-1 (MIP-1) which pathologically activates neighboring stroma to release pro-inflammatory cytokines [39]. When these aberrant foam cells are present in the circulation, particularly in the abdominal aorta and aortic arch, they form a nidus for atherosclerosis development. It may be surprising to hear that while ox-LDL is responsible for foam cell development, ox-LDL is generated due to deficiency of GSH in the plasma [40]. The circulating LDL lipoprotein contains GSH peroxidase, this enzyme is reliant on circulating GSH to keep LDL in the reduced form, during GSH deficiency, pathological ox-LDL becomes available to macrophages which are similarly GSH deficient [40]. Shortly thereafter, excessive ox-LDL accumulation within defective macrophages results in activation of programmed cell death. Literature suggests that ox-LDL accumulation results in direct destruction of mitochondrial membranes with concomitant intracellular calcium release and caspase activation. The above paradigm suggests complex multi-system homeostatic mechanisms involving various organ systems which could be as interconnected in normal physiology as the glucose-alanine cycle.

While the HMDM model does explain some aspects of antimycobacterial action by GSH, it does not explain concurrent activation of all cellular components of the immune environment. To shed greater light upon the *in vivo* picture, the Whole Blood Model was used. The Whole Blood Model attempts to explain how human monocytes are activated *in vivo*. It is known that monocytes/macrophages must be activated by IFN-γ which comes from both CD4+ T cells and in some cases natural killer cells (NK) cells. This model co-cultures mycobacterial-infected monocytes and other immune cells that have been either treated or not with NAC. The utility of GSH has been under-appreciated in the past; albeit, in hepatic metabolism, however, recent discoveries have the scientific community poised to place GSH as an integral component of physiologic peripheral blood mononuclear cell (PBMC) cell signaling. Low intracellular GSH levels in CD4+ T cells have demonstrated a pathologic predilection for pro-inflammatory and humoral specific (TH2) cytokine cascade activation when challenged with an intracellular mycobacterial infection, thwarting effective clearance of the pathogen [5]. Most excitingly, the addition of NAC to CD4+ T-cells, *in vitro*, demonstrated the ability to reverse the pathologic cascade resulting in complete stasis of intracellular mycobacterial growth in previously healthy individuals [5]. Additionally, NK cells have been shown to be dependent on significant intracellular concentrations of GSH for proper signal transduction through the FASL/CD40L pathway; likewise, supplementation of NAC to GSH-depleted NK cells was able to restore antimycobacterial innate function in previously healthy individuals [5]. As will be discussed below, this combination of substrate and cytokines leads to both NK cell and CD4+ T cell activation, both of which have been shown to activate effector monocytes. This novel discovery demonstrates that NAC supplementation results in complete activation of monocytes through indirect mechanisms, most notably, via increased IFN-γ release through selective activation of TH1 CD4+ T-cells.

As discussed in more detail below, *M. tb* infection is characteristic of a pro-inflammatory cytokine cascade; NAC was demonstrated to suppress IL-1, IL-6, and TNF-α, most likely through activation of TH1 CD4+ T-cells. While the indirect mechanism is likely dominant *in vivo*, the ability of GSH to sequester NO within HMDMs is not to be overlooked as a significant component to NAC mechanism of action; however mechanisms involving T-cell activation of macrophages, are also abrogated by GSH depletion [41-43].

6.2. Natural Killer Cells (CD56+NK Cells)

NK cells function as the first-line defense against tumorgenesis and viral infections. These cells are constantly surveying the surface of our native cells, looking for altered expression of surface markers. If such altered expression is detected, NK cells induce programmed cell death of the altered cell. NK cells express two surface proteins known as FASL (CD95) and CD40L (CD154), when these ligands engage their respective receptors on the inappropriately altered target cell, a series of intracellular events is triggered which culminates in activation of intracellular caspases, a rise in intracellular calcium, mitochondrial breakdown, and ultimately apoptosis of the cell [44]. This mechanism prevents irreversibly damaged and malfunctioning cells from impairing physiologic homeostasis. NK cells also function to release IFN-γ upon stimulation, IFN-γ is a potent cytokine involved in activation of the TH1 arm of the adaptive immune system; notably in the activation of macrophage iNOS which facilitates intracellular killing of phagocytosed microbes such as *M. tb*.

It has recently been discovered by Venketaraman *et al*., the pivotal role intracellular GSH plays in NK cells. Using an eloquently designed model, this group has demonstrated that decreased levels of intracellular GSH within NK cells impairs their ability to function maximally in both healthy and HIV+ individuals when co-cultured with autologous monocytes previously challenged with *M. tb*. Particularly fascinating results showed that the same amount of viable intracellular *M. tb* remained when NK cells where treated with BSO, a GSH depleting substance, as when NK cells were treated with either anti-FASL/anti-CD40L; demonstrating that a significant gradient of intracellular GSH is absolutely necessary for functionality of NK cell mediated apoptosis of infected monocytes (Figure 1).

While the exact mechanistic role of GSH within NK cell signal transduction remains unclear at the present time, Venketaraman *et al*., have demonstrated that increased intracellular GSH levels, especially when combined with an activated TH1 cytokine cascade, clearly increases expression of NK cell surface markers, specifically, NK Activating Receptor (NKG2D/DAP10), FASL/CD40L, NKp44 (natural cytotoxicity triggering receptor), and NKp30 (natural cytotoxicity triggering receptor). This is a novel discovery within immunology, demonstrating for the first-time that the intracellular redox state of NK cells plays a direct role in their functionality. It is likely that disease states which pathologically deplete intracellular GSH impair the innate immune system, as demonstrated here with NK cells, by impairing expression of CD40L and inhibiting release of IL-12 and IFN-γ; a situation favoring a pathologic humoral response unable to eliminate opportunistic infections, such as *M. tb*, CMV, *T. gondii*, HIV, *C. albicans*.

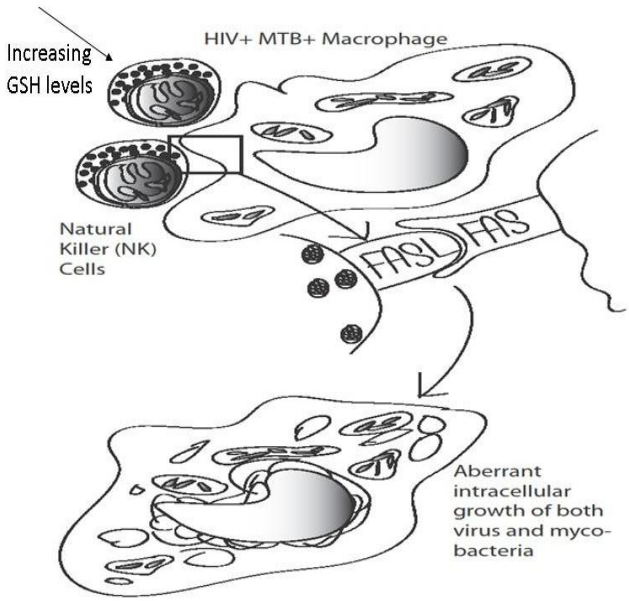

Figure 1. Cotrol of M. tb in macrophages by GSH-enhanced NK cells.

6.3. CD4+ T Lymphocytes

While the classifications of T lymphocytes are becoming more specific, it is generally believed that CD4+ lymphocytes, belonging to the cell-mediated immune response, operate through a TH1 cytokine cascade. These lymphocytes play a critical role in activating the innate immune system in response to intracellular infection [45]. Once activated, these cells release a characteristic set of autocrine and paracrine cytokines which ultimately result in activation of NK cells and macrophages. Of central importance to this TH1 cascade are the cytokines IL-2, IL-12, and IFN-γ. IL-2 is a signaling molecule which functions to amplify the TH1 response; whereas, IL-12 is needed to transform undifferentiated TH0 lymphocytes into TH1 lymphocytes, however, both are also required for full activation of NK cells. IFN-γ is the principal cytokine responsible for activation of macrophages [46].

It has been demonstrated for the first time by Venketaraman *et al*., that low levels of intracellular GSH within T lymphocytes impairs production of specific cytokine cascades, hinting that intracellular signaling involving GSH is much more complex than previously imagined. This group has demonstrated that low levels of GSH within T cells result in a TH2 specific cytokine profile. Of utmost importance is the observation that GSH depleted T cells express greatly increased concentration of IL-10 [100]. This may help elucidate the mechanism behind the high correlation of HIV viremic load and TNF-α plasma levels. The TH2 cytokine shift in HIV infection may be integral to viral pathogenesis; it has been documented that TNF-α directly increases NFκb transcription factor expression within CD4+ T cells, a phenomenon necessary for HIV viral replication and eventual depletion of the CD4+ T cell pool. Of paramount importance is the observation by Venketaraman *et al*. that replenishing GSH levels within these HIV+ T lymphocytes restores the physiologic TH1 cytokine profile which consequently decreases intracellular macrophage bacterial growth

velocity; albeit, not to the same extent of complete stasis observed in healthy individuals (Figure 2).

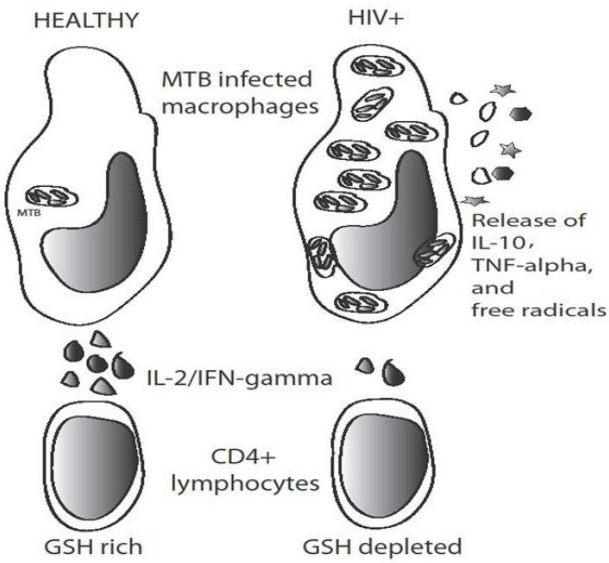

Figure 2. Control of *M. tb* in macrophages by GSH-enhanced T cells.

7. HIV

HIV pandemic has significantly impacted human existence and that research is actively investigating potential ways to cure or prevent infection may quite possibly be a monumental understatement. There are two major pathologic strains of HIV, HIV-1 and HIV-2; while HIV-2 is found almost exclusively in sub-Saharan Africa, HIV-1 is considered to be globally endemic [47]. Fascinatingly, HIV has emerged as the premier model of GSH deficiency, and documentation has been compiled demonstrating extensive derangement of the immunological cell-mediated axis secondary to this deficiency [36]. Underlying this discovery is a potential shift in the way HIV pathogenesis is viewed, and ultimately is treated. Once viewed as a virus selectively deregulating and destroying CD4+ T-cells, it is now likely that an intracellular deficiency of GSH among all PBMCs impairs cell-mediated immune function by impairing all effector cells of the cell-mediated response, leading to a situation primed for opportunistic infections [36].

While all PBMCs and even red blood cells (RBCs) have been evidenced to harbor decreased levels of intracellular GSH in HIV infected individuals, it seems that this deficiency is the most concerning within the CD4+ T-lymphocytes, macrophages, and NK cells. Venketaraman *et al*., have observed that decreased intracellular GSH in this cell population most likely leads to chronic oxidative damage to membrane lipids, but more strikingly, leads to increased release of humoral cytokines (TH2 cytokines) such as IL-10. Increased IL-10 paracrine signaling actively inhibits the cell-mediated cytokine (TH1 cytokine) cascade. This creates a situation similar to leprosy, where an inappropriate immune response is triggered to clear an intracellular pathogen; while the complement system may be

enhanced by humoral activation, intracellular infections are left unchecked. The macrophage, once able to strike fear into the heart of opportunistic or intracellular pathogens, is miraculously transformed into the Swiss bank of the microbial world, a safe haven where surveillance is not allowed. HIV infection creates a unique situation where GSH deplete effectors of the cell-mediated immune response are instructed to self-destruct or frankly become nonfunctional, but there is selective survival of macrophages which serve as pathological breeding grounds for opportunistic organisms. It is surely no coincidence that end-stage HIV, a time when such opportunistic infections are prevalent, is highly correlated with elevated plasma levels of IL-10; perhaps not surprising either is a decrease in TH1 cytokines such as IL-12, IL-2, and IFN-γ; however, what may be surprising is the observation of greatly elevated TNF-α and the ramifications of TNF-α excess.

Venketaraman *et al*. have observed an association between decreased GSH within PBMCs and increased TNF-α production by a malfunctioning TH1 axis involving CD4+ T-lymphocytes, macrophages, and NK cells. The effect of this aberrant TNF-α release is perhaps a central player in HIV pathogenesis [100]. Elevation of TNF-α during GSH deficiency directly induces ROS which damage immune cell membranes when most vulnerable to oxidative stress [48-50]. TNF-α excess in the face of GSH deficiency leads to activation of NFκb in PBMCs, directly resulting in increased HIV provirus replication [51]. Elevated TNF-α has systemic effects as well, triggering activation of a pro-inflammatory cytokine cascade involving IL-1, IL-6, and IL-8 functioning to induce viral replication, and directly lead to excessive inflammatory damage and toxicity characteristic of fever, anorexia, and cachexia.

It is possible that oxidative stress is a central mechanism of HIV pathogenesis via immunocellular PBMC derangement, but may be systemic in nature. Current evidence has shown that GSH is decreased in plasma, RBCs, and lung epithelial lining fluid (normally a significant barrier to airborne infection) as well. Of plausible significance is the observation of excessive plasma glutamate in HIV infection, remember, glutamate is a known inhibitor of the Xc-transport system required for uptake of cystine for *de novo* GSH synthesis [6]. While the root cause for the GSH deficiency in PBMCs during HIV is not known, evidence has clearly demonstrated malfunction and decreased activation of macrophages, NK cells, and TH1 CD4+ T cells. GSH-deficient macrophages have reduced antimicrobial activity due to decreased capacity of NO; additionally, macrophages clearly have reduced IL-12 expression, resulting in impaired differentiation of TH0 CD4+ T cells into TH1 CD4+ T cells, and impaired activation of NK cells. HIV-infected and GSH-depleted macrophages release excessive pro-inflammatory cytokines that further impair the TH1 axis and lead to systemic symptoms of chronic illness. Similarly affected NK cells are unable induce apoptosis through FASL or CD40L, perhaps explaining how macrophages act as reservoirs during opportunistic infection. Lastly, CD4+ T-lymphocytes that are able to differentiate into TH1 cells are not able to activate macrophages due to blockade of IFN-γ expression; furthermore, excessive TH2 CD4+ T-lymphocyte differentiation strongly amplifies cell-mediated antagonistic cytokines. Venketaraman *et al*. offers observations of HIV+ individual's immune response compared to those of healthy controls; and offers a novel unifying theory of HIV etiology and pathogenesis, that aberrations at all levels of the TH1 axis occur secondary to GSH deficiency [36].

Recent observations have demonstrated a synergistic, even symbiotic relationship between HIV and TB; a true match-made-in-heaven, and at the heart of the love affair, appears to be immunosuppression secondary to GSH depletion. It has been known for quite some time that individuals with HIV are more likely to develop TB. Treatment of these co-infected individuals represents a major hurdle because of various drug-drug interactions that limit the efficacy of treating either condition. Needless to say, new therapies are being actively sort after to control TB in HIV+ individuals. GSH has emerged as a candidate, and Venketaraman *et al*. offer convincing evidence suggesting a novel mechanism of action. This group have documented novel observations that GSH in clearly depleted in pulmonary TB independent of HIV infection, and that this decreased GSH is directly correlated with increased *M. tb* growth velocity and pro-inflammatory cytokine release.

Research demonstrates that there are two major cellular mechanism involved in controlling growth of MTB within macrophages. The first mechanism involves direct activation of macrophages by TH1 CD4+ T-lymphocytes via IFN-γ; the second mechanism involves receptor-mediated cell death of infected macrophages via NK cell FASL/CD40L engagement. Both mechanisms are been shown to be upregulated in HIV+ individuals upon administration of NAC. Moreover, evidence demonstrates that NAC supplementation to HIV+ macrophages results in stasis of mycobacterial growth velocity; hence GSH itself has direct antimicrobial action against tuberculosis, albeit a cidal effect requires TH1 CD4+ T-cell mediated activation of macrophages to induce iNOS and eventually GSNO production.

In recent years, research has shed light on the on the utility of NK cells. NK cell function was known to be deranged by various infectious organisms, notably, *C. albicans*, CMV, *T. gondii*, and of course, HIV [52]. It appears that *M. tb* also impairs functionality of these cell-mediated effectors through multiple mechanisms—all of which however, are at least restored to moderate functionality by NAC supplementation, highlighting GSH as an important player in the underlying mechanism. NAC was demonstrated to increase expression of NKG2D (the NK cell activating receptor), FASL/NKp30/44 (receptors capable of activating programmed cell death on target cells), and CD40L (an activating ligand that is capable of activating macrophages to release IL-12, activating the TH1 adaptive immune system).

HIV and TB infections are considered to be synergetic on many levels, but notably, macrophages are induced to express uncharacteristically high levels of IL-6, a pro-inflammatory cytokine, which directly inhibits activation of TH1 CD4+ T-lymphocytes and makes macrophages refractive to IFN-γ. This pro-inflammatory cytokine excess is responsible for the cachexia in both HIV and TB; interestingly, IL-6 is also known as osteoclast activating factor and has been associated with induction of osteoporosis, highlighting an example of the catabolic shift characteristic of pro-inflammatory states.

Venketaraman *et al* have demonstrated that GSH deficiency is the central player regulating *M. tb* growth within the macrophage. In HIV+ individuals it was shown that NAC supplementation inhibited *M. tb* growth but this stasis was nullified by addition of BSO (an inhibitor of *de novo* GSH production). This group also demonstrated that in healthy individuals infected with *M. tb* there is no specific imbalance of cytokine cascades; however, in HIV+ patients challenged with *M. tb*, there is a TH2/proinflammatory-dominant cascade. Excitingly, *in vitro* NAC supplementation resulted in reduction in pro-inflammatory cytokines and elevation of IFN-γ, essentially upregulating cell-mediated function of all effector cells.

Several studies have demonstrated a link between increased TGF-β production and decreases in GCLC gene expression, as well as synthesis of GSH. In our recent studies [101], we observed a significant increase in the levels of TGF-β in both plasma and macrophage supernatants from individuals with HIV infection and this increase correlated with reduction in the expression GCL gene in macrophages. The results of our studies indicate that increased levels of free radicals (induced by pro-inflammatory cytokines) and decreased expression GCL (induced by TGF-β) may cause decrease in the levels of GSH in macrophages derived from individuals with HIV infection [101]. The decreased levels of GSH in macrophages from individuals with HIV infection is accompanied by enhanced intracellular survival of *M. tb*. Importantly, treatment of macrophages from individuals with HIV infection with liposomal GSH (Readisorb) resulted in more efficient inhibition in the growth of *M. tb* compared to NAC. Since there is down-regulation in the expressions of enzymes that are responsible for GSH synthesis in cells derived from individuals with HIV infection, we strongly believe that liposomal GSH (L-GSH) will be more preferred that NAC for restoring the levels of GSH and for augmenting the macrophage responses against *M. tb* infection.

GSH-replenishment may potentially be a silver bullet capable of preventing progression of, and improving prognosis of both HIV and TB. GSH will have the greatest impact in developing countries such as sub-Saharan Africa where HIV and TB confection are extremely common. Evidence suggests that GSH-supplementation would also have a significant impact on either of these individual disease states as well. Clinical trials have shown that enhancing GSH levels slows progression of HIV in those affected; based on this observation alone, recommendations should be made to include NAC/L-GSH as standard of care in combination with highly active anti-retroviral therapy (HAART), especially when TB co-infection is present. No clinical trials have been conducted to study the use of L-GSH for treatment of HIV negative patients with active or latent TB; however, as demonstrated by Venketaraman *et al*., NAC is clearly efficacious when used *in vitro* for this purpose.

8. GSH and Cardiovascular Disease

When looking at the association of GSH in cardiovascular disease it is important to look at the role of peroxidases in the pathogenesis of atherosclerosis [52]. The implications of this association are of importance due to the fact that cardiovascular diseases continues to be a major health problem [53]. The primary cause of cardiovascular diseases such as coronary artery disease, stroke and peripheral vascular disease is atherosclerosis and inflammation [54-56]. Atherosclerosis accounts for almost 25% of all deaths in the United States and each year more than 1.2 million will suffer a coronary attack while 800,000 people will suffer a stroke [57]. Atherosclerosis is a process in which deposits of fatty substances, cholesterol, cellular waste products, calcium and other substances build up in the inner lining of an artery. This build up is called plaque [58] and usually affects large –and medium-sized arteries. Plaque can cause damage in a number of ways. The plaque can grow large enough to impede blood flow through an artery, but most of the damage occurs when plaque becomes fragile and ruptures. Plaque that rupture can cause blood clots to form that can block blood flow or break off and travel to another part of the body. If the block occurs in a blood vessel that feeds the heart, it can cause a heart attack. If blood supply to the arms of legs is reduced, it can cause

decreased supply of oxygen and nutrient to the muscles and skin, resulting in difficulty walking and eventually lead to gangrene. Atherosclerosis can also lead to myocardial infarction and if bits of plaque break off, they can lodge in arteries in the brain causing stroke [57]. It has been suggested that oxidative stress and decrease in GSH availability can lead to atherosclerosis, even in notherwise healthy appearing adults [59-62].

Increases in function of GSH as well as intracellular levels of GSH have been observed with the use of LGSH in cell culture [62] and animal studies [63, 64].

The deposition of lipids in the sub endothelial layer of the artery wall with subsequent alteration of low density lipoprotein (LDL) will lead to the formation of atherosclerosis [65, 66]. The lipoprotein B in LDL cholesterol can be altered in a range of changes by aldehydes and oxidation which are termed modified LDL (mmLDL) to Ox-LDL atherosclerosis [65, 66]. The alterations of the LDL cholesterol to modified and oxidized forms converts cholesterol to forms that can be recognized by scavenger receptors on macrophages and contribute to foam cell formation [67].

Deposition of ox-LDL in atherosclerotic plaque has been demonstrated in both atherosclerosis and unstable plaque [68]. It appears that that increased plasma levels of oxidized LDL in association with coronary artery disease (CAD) may be due to their back-diffusion from the vessel wall [69]. Ox-LDL has been shown in several studies to be an independent marker of the progression of atherosclerosis [70-73]. The pathophysiology relates to the macrophage ingestion of excess ox-LDL and formation of foam cells, the acknowledged trigger of atherosclerosis [74]. The mechanism of ox-LDL and the requirement for GSH to prevent oxidation of LDL cholesterol has been described in Rosenblat *et al* [63] using liposomal GSH (LGSH) as the source of GSH in both human blood, *in vitro* studies and in the mouse model of atherosclerosis. The presence of reduced GSH is critical as it has been shown that both LDL and high density lipoproteins (HDL) contain the antioxidant enzyme GSH peroxidase (GPx)[63]. GSH is the single substrate for GPx, so a continuous supply of GSH in the reduced state is needed to prevent the oxidation of LDL and HDL cholesterol. The metabolism of cholesterol also appears to be related to the availability of GSH as studies have shown GSH supplied by LGSH is able to maintain HDL-mediated macrophage cholesterol efflux [63, 75]. It appears that since ox-LDL is formed in arteries [69] that the presence of excess amounts of ox-LDL may be an indicator of general macrophage dysfunction as ox-LDL has been shown to stimulate the release of macrophage pro-inflammatory cytokines [74] in the intima of arteries as well as other immune responses.

Depletion of GSH has been shown to play a pivotal role in cardiovascular disease. It has been shown that the level of plasma-reduced GSH was independently associated with cardiac events after undergoing post myocardial infarction revascularization procedures [76]. Polymorphisms on the 5' flanking region of the GCL enzymes known as catalytic GCL (GCLC) and modified GCL (GCLM) genes are associated with an increased risk of myocardial infarction and endothelial dysfunction [78, 78]. Manipulation of the GSH levels by a cysteine source [75], by the direct addition of GSH in liposomes [63] and by genetic enhancement of GSH production [79] has been shown to alter atherosclerotic lesion development in hyperlipidemic ($ApoE^{-/-}$) mice.

Oxidative stress in the form of ROI and reactive nitrogen intermediates (RNI) have been associated with atherosclerosis. For ease of discussion, it is easier to describe the reactive species as ROS, which includes hydrogen peroxide (H_2O_2), pero-xyntitrite ($ON\text{-}OO^-$) and

hypochlorous acid (HOCl) as well as free radicals such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$) and nitric oxide ($NO^{\bullet}$)[80]. Increased ROI production has been associated with hypertension, diabetes mellitus, hypercholesterolemia, restensosis, heart failure and atherosclerosis [81-86]. Superoxide dismutase generates H_2O_2 from $O_2^{\bullet-}$, as well as direct production of H_2O_2 from xanthine oxidase and glucose oxidase donating two electrons to oxygen [87]. In the presence of transition metal such as Fe^{2+}, H_2O_2 spontaneously converts to $HO^{\bullet}$ by the Fenton reaction [88] and can be detoxified by catalase, GPx and peroxiredoxin to H_2O and O_2[86] GPxs catalyze H_2O_2 and lipid peroxidases to water or their respective alchohols using reduced GSH [89]. In $ApoE^{-/-}$ mice decreased availability of GPx leads to progression of atherosclerosis [90, 91]. GPx1 activity is inversely proportional to coronary artery disease and acute myocardial infarction [92-94].

Normal macrophage function requires GSH. When macrophage GSH is limited, cellular functions such as phagocytosis and microbial clearance are compromised [95-96]. In the natural state GSH levels are maintained by both the synthesis of GSH and by GSH reductase, an enzyme that catalyzes the reduction of GSH disulfide (GSSG) to GSH with NADPH as the reducing cofactor. Increasing the expression of GSH reductase, the enzyme that returns GSH to the biochemically reduced (functional) state in macrophages, decreases atherosclerotic lesions [97]. The importance of the level of GSH in macrophage function is demonstrated by the maintenance of macrophage function will decrease atherosclerotic lesion size. Decreased atherosclerotic lesion size in hypercholesterolemic mice was shown to maintain macrophage metabolism of cholesterol in $ApoE^{-/-}$ mice fed LGSH orally [63]. A study which increased the GSH production selectively in macrophage cells while leaving a systemic decrease in GSH by knocking out GCL function shows a similar reduction in atherosclerotic plaque formation [79].

While antioxidant therapies have shown benefit in animal studies, clinical trials of antioxidants including vitamin C and E, carotenoids, NAC as preventative therapy for cardiovascular diseases have been primarily negative [98].

One strategy for attenuating atherosclerosis and overcoming LDL and HDL oxidation may be to administer GSH entrapped in liposomes, as suggested by Rosenblat *et al*. [99]. LGSH administered to a mouse model of atherosclerosis diminished blood plasma and macrophage oxidative stress levels and macrophage cholesterol mass [99].

Conclusion

An organism is driven by its tendency to maintain homeostasis and stability within its internal environment. Metabolic by products from the consistently active physiological machinery of an organism creates destructive ROI's. This review outlined one of the most important defenses against these destructive ROI's, GSH and its function as it relates to neutralization of these destructive by products. A detailed review of generation, synthesis, regulation and mechanism of action of GSH has been presented. The antimicrobial and cardiovascular benefits of GSH by enhancement of macrophages and other immune cell functions, has been demonstrated through experimental animal models. The direct antimicrobial abilities of GSH against *M. tb* is especially important since TB still remains one of the most infectious diseases worldwide, and it effects millions of populations across the

globe. In addition, GSH has shown to increase immune function to fight *M. tb* in HIV patients by acting on macrophages, NK and T cells. Furthermore, the link between formation of atherosclerosis and depletion of GSH has been outlined on a molecular level in association with levels of ox-LDL and direct effects of ROI, and since cardiovascular disease remains the leading cause of death in the US, then the beneficial effects of restoration of GSH levels becomes extremely important. In conclusion, it is important to note the many beneficial effects of GSH, and its potential role as a front line defense against many diseases and ailments.

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In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter VIII

Glutathione and Glutathione S-Transferases: Risk Factors in Multifactorial Diseases

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Abstract

Thiol-containing compounds like glutathione (GSH) have a central role in many biochemical and pharmacological reactions due to the ease with which they are oxidized, and the rapidity with which they can be regenerated. It has long been established that the thiol moiety of GSH is important in antioxidant defense, xenobiotic and eicosanoid metabolism, and regulation of cell cycle and gene expression, and that conjugation with GSH is an essential aspect of both xenobiotic and normal physiological metabolism. GSH forms conjugates with a great variety of electrophilic compounds nonenzymatically, when the electrophile is very reactive, or more often through the action of glutathione S-transferases (GST). The role of GSH and GSTs as cell housekeepers engaged in the detoxification of xenobiotics, but also their involvement in stress response, cell proliferation, apoptosis, oncogenesis, tumor progression and drug resistance, along with the interindividual GST variability, has led to associations with several pathologies. This chapter reviews the most recent knowledge about the central role of GSH and GSTs in the pathophysiology of human diseases and focuses on their involvement in cancer cell growth and differentiation, and other multifactorial diseases like diabetes or heart diseases.

Abbreviations

Ala – alanine
Arg – arginine
Asn – asparagine
Asp – aspartic acid
BRCA1 – breast cancer gene 1
BRCA2 – breast cancer gene 2
Cys – cysteine
FAA – fumarylacetoacetate
Glu – glutamic acid
Gly – glycine
GSH – glutathione
GST – glutathione S-transferase
GSTT2P – GSTT2 pseudogene
HDL – high density lipoprotein
Ile – isoleucine
Lys – lysine
MAA - malaylacetoacetate
MAAI – maleylacetoacetate isomerase
MAPEG - membrane-associated proteins in eicosanoid and glutathione metabolism
Met – methionine
NAT – N-acetyltransferase
PAHs – polycyclic aromatic hydrocarbons
Pro – proline
RNS – reactive nitrogen species
ROS – reactive oxygen species
Ser – serine
SLE – systemic lupus erythematosus
Thr – threonine
Tyr – tyrosine
Val – valine
YY1 – Yin Yang 1

1. Introduction

DNA and cells of the human body are constantly exposed to attacks of an oxidative nature. These attacks can be exogenous, like exposure to ionizing radiation, oxidizing chemicals or UVA solar light, and endogenous through cellular signaling, metabolic processes or inflammation [1]. These endogenously induced DNA lesions can often reach a much higher level than the ones induced by environmental factors like ionizing radiation, even if in low doses, contributing significantly in the accumulation of mutations in cells and tissues. The primary damage inductors are reactive oxygen species (ROS) and reactive nitrogen species (RNS) [2]. Glutathione *S*-transferases (GSTs) are a family of Phase II detoxification enzymes that have co-evolved with glutathione (GSH) and are abundant

throughout most life forms. GSTs detoxify environmental chemicals and are involved in oxidative stress pathways. They catalyze the conjugation of GSH to a wide variety of endogenous and exogenous electrophilic compounds. Several allelic variants of polymorphic GSTs, mainly deletion polymorphisms, show impaired enzyme activity and are suspected to increase the susceptibility to various diseases [3].

2. Glutathione S-Transferases

Glutathione S-transferases (GSTs; EC 2.5.1.18) constitute a superfamily of ubiquitous, multifunctional enzymes that play a key role in cellular detoxification, protecting macromolecules from attack by reactive electrophiles [3]. They catalyze nucleophilic attack by reduced glutathione (GSH; g-Glu-Cys-Gly) on nonpolar compounds that contain an electrophilic carbon, nitrogen, or sulphur atom. Their substrates include halogenonitrobenzenes, arene oxides, quinones, and α,β-unsaturated carbonyls like, for example, products of oxidative stress, environmental pollutants and carcinogens [4]. The addition of GSH to the exogenous and endogenous chemicals neutralizes their electrophilic sites, transforming them in products with higher hydrophilicity, what facilitates their elimination from phase III enzymes. GSTs can also function as peroxidases, isomerases and thiol transferases and have non-catalytic functions such as non-substrate ligand binding and modulation of signaling processes [3]. Human GSTs are divided into three distinct super family members: mitochondrial, cytosolic and microsomal, currently named membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) GSTs [5]. Cytosolic and mitochondrial GSTs share some similarities in their three-dimensional fold [6] but have no structural resemblance to MAPEG enzymes [7]. Human cytosolic GSTs represent the largest and most complex family and they are considered the most relevant to disease investigation. They can catalyze thiolysis of 4- nitrophenyl acetate, display thiol transferase activity, reduce trinitroglycerin, dehydroascorbic acid, and monomethylarsonic acid, and catalyze the isomerization of maleylacetoacetate and Δ^5-3-ketosteroids [8]. Seven classes of cytosolic GSTs were created based on amino-acid sequence similarities, physical structure of the genes and immunological crossreactivity, and are termed Alpha (α), Mu (μ), Pi (π), Theta (θ), Omega (Ω), Sigma (ς) and Zeta (ζ). Kappa (κ) class constitutes the mitochondrial family member [9, 10]. GSTs that share more than 60% on the highly conserved N-terminal domain were merged into a class [10]. The active site of a GST protein is formed by the glutathione-binding site, which is preserved in the different classes, and the hydrophobic substrate-binding site, that has variations in the forming residues, leading to wide substrate specificity [11]. Almost all soluble GSTs are active as homodimers or heterodimers of subunits, and each dimmer is encoded by independent genes [5], resumed in Table 1.

GSTs expression can be induced by structurally unrelated compounds known to result in chemical stress and carcinogenesis including phenobarbital, planar aromatic compounds, ethoxyquin, butylated hydroxyanisole, and trans-stilbene oxide. Some of the compounds known to induce GSTs are themselves substrates of the enzyme [12]. Human cytosolic GSTs display polymorphisms that may contribute to interindividual differences in responses to xenobiotics. Those genotypes, alone or in combination, may identify subjects as “low metabolizers”, and consequently more likely to suffer formation of DNA adducts and/or

mutations that confer major susceptibility to complex multifactorial diseases, with genetic and environmental influences, such as cancer [5]. Despite individuals carrying a variant, or combinations of variants, in these low penetrating genes are estimated to have low risk to develop cancer when compared to carriers of mutations in high penetrating genes like BRCA1 and BRCA2, the high frequency in the population of some of the variants makes the population to be interpreted as being of high risk [13]. The first studies focused individuals carrying the homozygous deletion of GSTM1 and/or GSTT1 genes (null genotype). This genotypes result in total absence of a functional gene product, thus a total absence of the respective enzyme activity [14, 15]. After the discovery of allelic variants of GSTP1 that encode enzymes with altered specific activity and affinity depending on the substrate [16, 17], the hypothesis that combinations of polymorphisms in class Mu, Theta and Pi contribute to diseases, along with an environmental component, was analyzed in many researches. Concerning cancer, GSTs genotypes were investigated in relation to bladder [18], prostate [19], breast [20], colorectal [21], head/neck [22], oral [23] and lung cancer [24]. The best characterized cytosolic classes, Alpha, Mu, Pi, Theta, and the emerging class Zeta will be considered in this chapter due to their relevance in diseases in the general population (Table 2). Furthermore, the influence of GSTs polymorphism will be analyzed in relation to several types of cancer.

Table 1. Most relevant GSTs codifying genes and chromosome localization

Class	Chromosome localization	Gene
Alpha (α)	6p12	GSTA1, GSTA2
Mu (μ)	1q13.3	GSTM1, GSTM2, GSTM3, GSTM4, GSTM5
Pi (π)	11q13.3	GSTP1
Theta (θ),	22q11.23	GSTT1, GSTT2
Omega (Ω)	10q24.3	GSTO1
Zeta (ζ)	14q24.3	GSTZ1

3. Genetic Variants of Cytosolic GST Family

3.1. GST Alpha (α) Class

The human GST α class is encoded by genes clustered within chromosome 6p12. The cluster consists of five genes: GSTA1, GSTA2, GSTA3, GSTA4 and GSTA5 [8]. GSTA1, GSTA2 and GSTA4 are widely expressed in all human tissues but mainly expressed in liver, while expression of GSTA3 is rare and expression of GSTA5 has not been detected in human tissues until now [25]. It has been shown that both GSTA1 and GSTA2 genes, the major hepatic GSTs, are polymorphic and variability is thought to affect the efficiency of detoxification of xenobiotics [26-28]. A genetic polymorphism of GSTA1 is characterized by two alleles, GSTA1*A and GSTA1*B. They contain three linked basis substituted in the proximal promoter region, at positions -567, -69 and -52. GSTA*1 have T, C and G at these positions and individuals with GSTA*B have G, T and A. Specifically, the –52 (G/A) substitution has been shown to increase promoter activity in GSTA1*A, thus making it more

highly expressed [29]. Also, liver from individuals who carried the variant GSTA1*B showed reduced levels of GSTA1 enzyme [28]. GSTA2 has not been extensively studied, but it is known to have five variants (GSTA2*A–E), and they are thought not to affect GSTA2 activity. However, the variant GSTA2E shows reduced catalysis rates when compared to variants A–D [30]. Besides GSTA1 and GSTA2, also GSTA3, a GST selectively expressed in steroidogenic tissues involved in steroid hormone biosynthesis, has been proved to be polymorphic [31]. The polymorphism was found exclusively in African populations and was hypothesized that it could affect steroid biosynthesis through altered protein levels or function [27].

Table 2. Polymorphic human cytosolic GSTs

Gene	Allele	Gene alteration	Effect on protein
GSTA1	GSTA1*A	Wild-type	
	GSTA1*B	Promotor point mutation	Low protein levels
GSTA2	GSTA2*A	Pro110; Ser112; Lys196; Glu210	
	GSTA2*B	Pro110; Ser112; Lys196; ALa210	
	GSTA2*C	Pro110; Thr112; Lys196; Glu210	
	GSTA2*D	Pro110; Ser112; Asn196; Glu210	
	GSTA2*E	Ser110; Ser112; Lys196; Glu210	Reduced catalysis rate
GSTA3	GSTA3*A	Ile71	
	GSTA3*B	Leu71	Reduced activity
GSTM1	GSTM1*A	Lys173	
	GSTM1*B	Asn173	
	GSTM1*0	Gene deletion	No protein
	GSTM1*1x2	Duplication	Protein overexpression
GSTM3	GSTM3*A	Wild type	
	GSTM3*B 3	3 base deletion, intron 6	Unchanged protein
GSTM4	GSTM4*A	Tyr2517	
	GSTM4*B	Cys2517	Unknown
GSTP1	GSTP1*A	Ile105; Ala114	105Val – lower or higher activity and affinity depending on the substrate
	GSTP1*B	Val105; Ala114	
	GSTP1*C	Val105; Val114	
	GSTP1*D	Ile105; Val114	
GSTT1	GSTT1*A	Thr104	
	GSTT1*B	Pro104	Decreased activity
	GSTT1*0	Gene deletion	No protein
GSTT2	GSTT2*A	Met139	
	GSTT2*B	Ile139	Unknown
GSTO1	GSTO1*A	Ala140; Glu155	
	GSTO1*B	Ala140; Glu155 deletion	
	GSTO1*C	Asp140; Glu155	
	GSTO1*D	Asp140; Glu155 deletion	25% reduced activity
GSTO2	GSTO2*A	Asn142	
	GSTO2*B	Asp142	20% reduced expression
GSTZ1	GSTZ1*A	Lys32; Arg42; Thr82	
	GSTZ1*B	Lys32; Gly42; Thr82	
	GSTZ1*C	Glu32; Gly42; Thr82	
	GSTZ1*D	Glu32; Gly42; Met82	Reduced catalytic activity

3.2. GST Mu (μ) Class

The GSTM class has five described isoforms, GSTM1-5, encoded by a gene cluster located on chromosome 1p13.3 and arranged as 5'- GSTM4-GSTM2-GSTM1-GSTM5-GSTM3-3'[14]. Concerning GSTM1, three polymorphisms have been identified until now. Deletion of the entire GSTM1 gene, GSTM1*0, frequently affects both alleles, resulting in a lack of functional gene product, the so-called null allele [32]. The other two, a missense single nucleotide polymorphism also occurs in the GSTM1 gene, i.e. nucleotide 534 G/C, resulting in asparagine (Asn) to lysine (Lys) substitution at amino acid 173, corresponding to GSTM1*A and GSTM1*B respectively. No evidence of functional difference between GSTM1*A and GSTM1*B variants was found; thus, these alleles are typically categorized together as a single functional phenotype [33]. Detailed mapping of the GSTM gene cluster revealed that the GSTM1 gene is flanked by two almost identical 4.2-kb regions. The GSTM1*0 deletion is caused by a homologous recombination involving the left and right 4.2-kb repeats and the GSTM1 gene is excised relatively precisely leaving the adjacent GSTM2 and GSTM5 genes intact [32]. This homozygous deletion (GSTM1 null) has been examined extensively in epidemiologic studies. The frequency of the null genotype is around 50% in Caucasians and Asians, but only 27% in Africans [11]. Subjects with a homozygous deletion of the GSTM1 locus have no enzymatic functional activity and several studies suggest that the GSTM1 null genotype can interfere in the drug and carcinogen detoxification [13, 25]. The GSTM3 locus contains two alleles, A and B. The GSTM3*B allele has a three base pair deletion in intron 6 that introduces a recognition motif for the YY1 (Yin Yang 1) transcription factor, which is known to have a fundamental role in normal biologic processes such as embryogenesis, differentiation, replication and cellular proliferation. A linkage disequilibrium has been noted between the GSTM1*A and GSTM3*B alleles [34]. Also, two new polymorphisms have been detected in the GSTM3 gene, a rare p.G147W substitution and a more common p.V224I substitution. These two polymorphisms can combine to form four different isoforms. The p.W147 variant seems to exhibit decreased catalytic and specific activity, whereas the p.I224 variant has shown increased catalytic and specific activity [35].

3.3. GST Pi (π) Class

The GST Pi class is encoded by a single gene, GSTP1, the most studied of GSTs genes, that spans approximately 3 kb and located on chromosome 11q13 [36]. GSTP1 is expressed in many tissues including breast and lung, where it is the predominant GST [37]. GSTP1 is polymorphic with two common functional variants based on substitutions in amino acids 105, Isoleucine (Ile) to Valine (Val), and 114, Alanine (Ala) to Val, demonstrating different catalytic efficiencies due to changes in the active site. Thus, four haplotypes have been identified: the wild-type GSTP1*A (Ile105 + Ala114) and three variant haplotypes, GSTP1*B (Val105 + Ala114), GSTP1*C (Val105 + Val114) and GSTP1*D (Ile105 + Val114). Both amino acids changes lie in close proximity to the hydrophobic-binding site and affect substrate specificity to the point of distinguishing between planar and nonplanar substrates [16]. The Val105 variant has been demonstrated to have either lower or higher specific activity and affinity depending on the substrate. For example, Ile105 seems to have higher catalytic efficiency for 1-chloro-2,4-dinitrobenzene than the Val105 variant, and the

latter seems to confer higher catalytic efficiency to polycyclic aromatic hydrocarbon (PAH) diol epoxide detoxification [5, 38]. The Ala114Val polymorphism seems not to influence the enzyme activity. Only about 5–10% of Caucasians have been shown to be homozygous for the Val allele [39, 40].

3.4. GST Theta (θ) Class

The Theta class of GSTs consists of two different subfamilies: GSTT1 and GSTT2. Genes encoding both proteins are co-localized on chromosome 22q11.2 and are separated by 50 kb [41]. This class is considered the most ancient of the GSTs, and θ-like GSTs are found in almost all the investigated organisms [5]. Both GSTM1 and GSTM2 genes have five exons with identical intron/exon limits but share only 55% amino acid identity. Among the GSTT substrates, there are several environmental carcinogens found in food, air or medications, such as polycyclic aromatic hydrocarbons (PAHs), found in combustion products, diet and tobacco smoke [42]. GSTT1 is expressed in human erythrocytes, and various tissues including liver, but no expression in breast tissue has been reported [41]. Polymorphisms exist within both genes. Similar to GSTM1, homozygous deletion exists in GSTT1 gene and results in the lack of the active codified enzyme [43]. Large inter-ethnic differences have been reported in the frequencies of the GSTT1 null genotype; the prevalence of GSTT1 null genotypes is significantly lower among Caucasians (10–20%) compared to Asians (50–60%) [44, 45]. Another less common polymorphism results in a threonine (GSTT1*A) to proline (GSTT1*B) substitution at amino acid 104. The GSTT1*B allele shows a decreased catalytic activity when compared to the GSTT1*A allele, which could be attributed to the conformational change induced by the proline substitution [46]. Regarding GSTT2 gene, the transition of guanine to adenine in intron 2 gives rise to a pseudogene (GSTT2P). Evidence points to transcription of this pseudogene, although the protein product is thought to be inactive [25].

3.5. GST Omega (Ω) Class

In humans, the Omega class of GSTs contains two members (GSTO1 and GSTO2) and a pseudogene (GSTO3p). This class has different characteristics in structure and function from the other members of GST superfamily; for example, these enzymes have a cysteine residue in their active site in contrast to serine or tyrosine that is in the active sites of other subfamilies. Also, X-ray crystallography shows a unique 19-residue N-terminus extension that forms a structural unit unlike any other found in other classes. Furthermore, these GSTs exhibit poor activity with common GST substrates (such as 1-chloro-2,4-dinitrobenzene) but exhibit novel GSH dependent thioltransferase, dehydroascorbate reductase and monomethylarsonate reductase activities, and are able to modulate Ca^{2+} release by ryanodine receptors [47]. Expression of GSTO1 is abundant in a wide range of normal tissues, including liver, colon, heart, ovary, pancreas, prostate, spleen, macrophages, endocrine and glial cells. The widespread distribution of GSTO1 suggests that it has important biological functions, although they remain undefined [48]. To date, four polymorphisms have been identified, GSTO1*A–D. Among the Australian, African and Chinese populations, GSTO1*A was the

most prevalent haplotype and demonstrated a GSH-dependent reduction of dehydroascorbate, a characteristic function of glutaredoxins rather than GSTs [47]. This allele was first described as the human monomethylarsenic acid reductase, and is the rate-limiting enzyme of inorganic arsenic metabolism [49]. GSTO1*C results in an alanine (Ala) to aspartic acid (Asp) substitution at amino acid 140, which creates a non-conservative amino-acid change from hydrophobic to hydrophilic residue [47]. Thioltransferase activity differs among the GSTO1*A–C polymorphisms.

Thioltransferase activity of GSTO1*C was verified to be 75% of the wild type, reflecting that it may result in defective protection against cellular oxidation stresses and may contribute to individual capacity to metabolize arsenic [50]. GSTO2 is separated from GSTO1 by 7.5 kb on chromosome 10 and shares 64% amino-acid identity [48]. Like GSTO1, GSTO2 is ubiquitously expressed and shares GSH-dependent dehydroascorbate reductase activity. However, GSTO2 has a high catalytic activity toward chlorodinitrobenzene, and its overexpression induced apoptosis, suggesting a possible role in cell signaling. In GSTO2 gene, a transition of adenine to guanine at nucleotide position 424 in exon 4 (GSTO2*C) was reported, which results in an amino-acid difference from asparagine (Asn) to aspartic acid (Asp) in codon 142. It was reported that the GSTO2 Asp142 variant allozyme showed 20% reduction in level of expression when compared with the level of the GSTO2 wild-type (Asn142) allozyme [51].

3.6. GST Zeta (ζ) Class

GSTs Zeta class (GSTZ1) is constituted by a single gene located on chromosome 14, spanning 10.9 kb, and encoding for a 29 kDa protein [52]. The cytosolic GST Zeta class has been identified as a maleylacetoacetate isomerase, and therefore catalyzes the penultimate step in the catabolism of phenylalanine and tyrosine [8]. GSTZ1 is preferentially expressed in hepatocytes and renal proximal tubule cells where phenylalanine and tyrosine are catabolized [53].

Although literature is restrictive for this class, GSTZ1 polymorphisms have been identified (GSTZ1*A–D). The isozyme GSTZ1*A has the highest catalytic activity toward dichloroacetic acid, an investigational drug for certain metabolic disorders, and a nephrotoxic metabolite of industrial solvents [52]. In contrast, GSTZ1*D (p.T82M) has a reduced catalytic activity and has been associated with innate errors in tyrosine metabolism, although the disorders have also been attributed to mutations in other enzymes. Rodent models deficient for GSTZ1 provide insight into its role in metabolic deficiencies. GSTZ1/maleylacetoacetate isomerase (MAAI) converts maleylacetoacetate (MAA) to fumarylacetoacetate (FAA).

GSTZ1- deficient mice have an elevated urinary excretion of FAA and were subject to renal injury following phenylalanine and tyrosine overload [53]. In fact, four families have been identified that have GSTZ1-deficient members that have died within the first year of life. Although clinical data for GSTZ1 are insufficient to deduce a role for GSTZ1 in inherited genetic disease, it is plausible that a perturbation in GSTZ1-mediated tyrosine metabolism is contributory to the described pathology [25].

4. GSTs Polymorphisms and Predisposition to Multifactorial Diseases

4.1. Cancer

In the last twenty years an increasingly higher number of cancer susceptibility genes were known. At this point, the role of low penetrance genes has not been completely understood. Cancer is a multifactorial complex disease resulting from environmental/lifestyle and genetic influences. Germline mutations in the so-called high penetrance genes to cancer susceptibility appear to account for the majority of hereditary breast cancer, but they represent only a small part of all cancer cases. Thus, low penetrance genes, acting together with endogenous or life-style risk factors, can be associated with a significant percentage of cancer cases. Low penetrance genes can be found in several pathways like detoxification of environmental carcinogens, steroid hormone metabolism and DNA damage repair pathways. GSTs became interesting in the investigation of a potential association with cancer risk due to the wide range of compounds, including several environmental and endogenous carcinogens, they can detoxify. As discussed earlier, inherited genetic traits co-determine the susceptibility of an individual to metabolize toxic endogenous or exogenous chemicals. Studies regarding this issue, and relation with several types of cancers will be further discussed.

4.2. The Significance of GST Null Genotypes in Cancer

Cytosolic GSTs display polymorphisms in humans and this is likely to contribute to interindividual differences in responses to xenobiotics. GSTM1 and GSTT1 null genotypes have been the focus of several studies because of their special condition: the majority of polymorphisms affecting genes involved in carcinogen metabolism are single nucleotide polymorphisms, deletions are less common, and the complete absence of a gene in form of a null allele is rare. Deletion of the GSTM1 and GSTT1 genes results in a ‘null’ genotype characterized by a general deficit in enzymatic activity. GSTs are phase II detoxifying enzymes and the polymorphisms in the codifying genes may be associated with the increased susceptibility to cancer, as normal or increased GSTs activity may facilitate detoxification of electrophilic carcinogens, protecting susceptible tissues from somatic DNA mutations [13]. Thus, population with reduced GSTs enzyme activity, as result of homozygous deletions of GSTM1 or GSTT1, may be at greater risk for malignancies due to their impaired ability to metabolically eliminate carcinogenic compounds and reduced detoxification efficiency [8]. Thus, the earliest studies in this area addressed the question of whether individuals lacking GSTM1 and/or GSTT1, i.e., are homozygous for GSTM1*0 and/or GSTT1*0 alleles, have a higher incidence of bladder, breast, colorectal, head/neck, and lung cancer [25]. This phenotype has been studied as a predictive factor for cancer prognosis or response to therapy and the results achieved have been dependent upon tumor type and population. It has been found that GSTs genes do not make a major contribution in susceptibility to cancer, at least by themselves. GSTM1*0 seems to have a modest effect on lung cancer [55], GSTM1*0 and GSTT1*0 seem to have a modest effect on the incidence of head and neck cancer [56]. Also, GSTM1*0 and GSTT1*0, alone or in combination, seem to be associated with higher

susceptibility to breast cancer development [13]. It is important to note that a possible limitation of many studies into the biological effects of GSTM1*0 and GSTT1*0 is that only individuals who are homozygous nulled for these genes (−/−) have been identified, due to the PCR method used in the analysis. Generally, the absence of a PCR product indicates the GSTM1 or GSTT1 null/null genotype and individuals are categorized as either "present" (wild-type) or "null" genotypes. This analytical approach does not positively identify the null allele and, therefore, cannot distinguish homozygous wild-type from heterozygous present/null individuals but it conclusively identifies the null/null genotypes [13]. Consistently, individuals who are heterozygous (−/+) or homozygous (+/+) for the functional allele are not distinguished and are not analyzed separately. As a consequence, the significance of being homozygous wild type for GSTM1 and GSTT1 is rarely addressed and the benefit of such a genotype is probably underestimated in the literature because it is grouped together with the heterozygote genotype [8]. New assays that differentiate between −/−, −/+, and +/+ genotypes at the GSTM1 and GSTT1 locus have been developed, and revealed significant protection against breast cancer in homozygous GSTM1 +/+ individuals [57, 58]. Besides the effects in tumorigenesis, several studies indicate that loss of these genes increase susceptibility to inflammatory diseases, such as asthma and allergies, atherosclerosis, rheumatoid arthritis, and systemic sclerosis [59, 60, 61].

4.3. Breast Cancer

Breast cancer is the prevailing cancer among women in industrialized countries [11]. Although many risk factors for the development of breast cancer have been identified, the molecular mechanisms related to breast carcinogenesis remain unclear. Estrogens have been clearly identified has carcinogens, by inducing aneuploidy and structural chromosomal changes and stimulation of breast-cell proliferation has been proposed as the main effect of estrogens in breast carcinogenesis: as more rapidly cells proliferate, greater the chance of acquiring a potentially cancer-causing mutation. Also, metabolic by-products of estrogens are responsible for free-radical-mediated DNA damage, single-strand breaks, estrogen–DNA adducts formation, protein oxidation and lipid peroxidation, what triggers genetic instability and cellular damage [62, 63]. Inter-individual variability has been observed in estrogens biosynthesis and metabolic pathways, namely in genes encoding for proteins involved in estrogen biosynthesis, and in genes encoding for xenobiotic metabolizing enzymes, like GSTs. The results regarding the genotypes distribution of GSTM1, GSTT1, and GSTP1 in cases and controls in the Portuguese population were published by our group, as well as the results of the association in two and three-way combinations to evaluate the impact of gene-gene interaction [13]. We found that GSTM1 null genotype was significantly more common among breast cancer cases compared to controls (OR 2.592; 95% CI 1.432–4.690;P = 0.002), so as GSTT1 (OR 3.597; 95% CI 1.849–6.999; P = 0.0001). We did not find any significant increase of breast cancer risk associated with GSTP1 genotypes (OR 1.103; 95% CI 0.611–1.989; P = 0.765) when compared Val allele carriers in homozygosity or heterozygosity with Ile/Ile carriers. We also found an 8-fold increased breast cancer risk in women who carry null genotype both in GSTM1 and GSTT1 (OR 8.287; 95% CI 3.124–21.980; P = 0.0001). After analyzing the three-way combination of GSTM1, GSTT1, and GSTP1 polymorphisms we found that Ile/Ile genotype, so as presence of Val allele, seemed to be associated with risk of

breast cancer when combined with both GSTM1 and GSTT1 null genotypes (OR 12.600; 95% CI 2.358–67.315; P = 0.001 and OR 5.040; 95% CI 1.392–18.248; P = 0.016), so it appears that the increase of breast cancer risk was mainly given by GSTM1*0/GSTT1*0 genotype, rather than by any genotype of GSTP1. An investigation conducted by Mitrunen and collaborators [64] analysed the association of GSTM1, GSTM3, GSTP1 and GSTT1 polymorphisms with the risk of breast cancer development in 483 patients. They observed a positive association with GSTM1 null genotype (OR 1.49; 95% CI 1.03 – 2.15) and GSTM3*B allele. Also, the risk was significantly higher in women with GSTT1 null (OR 9.93; 95% CI 1.10 – 90). For GSTP1 Ile/Ile genotype they observed an OR of 2.07 (95% CI 1.02 – 4.18). These authors found that in the combination GSTM1*0-GSTT1*0-GSTP1 Ile105/Ile105, the OR was of 3.96 (95% CI 0.99 – 15.8). Another group analyzed seven studies of GSTM1, GSTT1 and GSTP1*B polymorphisms in 2048 breast cancer patients and 1969 controls [65]. The authors obtained no significant association of these polymorphisms and breast cancer development. As was described, and as is observed in other types of cancer, the majority of the studies concentrated on GSTM1, GSTT1 and GSTP1 genes. Although, other genotypes were analyzed, reaching the same *conflictuous* conclusions. Recently, the group of Andonova [66] did not observe any breast cancer risk associations with GSTA2, GSTM2, GSTO1, GSTO2, and GSTZ1polymorphisms. Regarding GSTA and GSTO, most of the evidence for GSTA1*B polymorphism has a negative association with breast cancer, and the same occurs with GSTO2*B [67].

4.4. Prostate Cancer

Prostate cancer is the most common cancer among men in industrialized countries. Prostate cancer is uncommon in men younger than 45, becomes more common with increasing age and the main risk factor is being the age of over 50 [68]. Increased exposure to carcinogens is implicated in multistage carcinogenesis, thus the differences in the effectiveness of detoxification of carcinogens could possible help to understand why one man may be at higher risk than another. As in the other types of cancer, it was much speculated that GSTM1, GSTT1 and GSTP1 polymorphisms could be associated with the carcinogenic development in the prostate. The results regarding this issue were, once again, inconclusive. A meta-analysis of 29 studies, comprising 4564 cases and 5464 controls concluded that GSTM1 null genotype increased prostate cancer risk (OR 1.3; 95% CI 1.15 – 1.55). In the same report, 22 studies including 3837 cases and 4552 controls were analyzed, and association of prostate cancer with GSTT1 null genotypes was not verified [69]. Another meta-analysis conducted to analyze several studies of GSTM1, GSTT1 and GSTP1 polymorphisms and prostate cancer did not obtain correlation between GSTM1 and GSTT1 null genotypes, and GSTP1*B genotype, and risk for breast cancer [70]. Concerning GSTA, it was found an association with prostate cancer risk in Japanese that carried GSTA1*B polymorphism and also an increase of risk in carriers of the combined GSTA1*AB or BB and GSTT1 “present” genotypes [71]. However, another study evaluating various GSTA1 and GSTA2 genetic polymorphisms did not find any type of association with prostate cancer risk [30]. A study performed in a southern European population found no association of prostate cancer risk with GSTM1 or GSTT1 null genotypes (OR 1.20; 95% CI 0.75 – 1.90; p = 0.420).

However, they found a 5.5-fold increased risk of breast cancer associated with GSTM3*BB genotype (OR 5.50; 95% CI 1.2 – 25.8; p = 0.016) [72].

4.5. Lung Cancer

Coal, wood, biomass smoke, and cooking oil fumes have been associated with a variety of health outcomes, namely lung cancer [72]. The associations observed between air pollution and lung cancer risk are not surprising since fuel combustion products are known to contain carcinogens. Smoky coal combustion increases levels of sulfur dioxide, carbon monoxide, fluorine, and known carcinogens such as PAHs, benzene, arsenic, and formaldehyde. Genetic variation in enzymes responsible for activating and detoxifying PAHs or other carcinogens may alter susceptibility of individuals exposed to these environmental pollutants. GSTs are involved in the metabolic detoxification of reactive electrophilic compounds, such as PAHs, that are formed during incomplete combustion of carbon-based fuels such as coal and wood [72].

As denoted previously, GSTP1 is the GST isoform most expressed in lung, having among its main metabolic agents the PAHs [37]. A meta-analysis of six studies comprising 560 cases and 635 controls evaluated GSTM1, GSTT1 and GSTP1 genotypes and their association with risk of lung cancer in Asians populations [73]. They found that carriers of GSTM1 null genotype had a borderline significant increased lung cancer risk (OR 1.31; 95% CI 0.95-1.79; p=0.10). The GSTT1 null genotype was also associated with an increased lung cancer risk (OR 1.49; 95% CI 1.17-1.89; p = 0.001) but association was observed for the GSTP1 105 Val allele. A larger meta-analysis of 27 studies (8322 cases and 8844 controls) analyzed the association of GSTP1*B (105 Val) and lung cancer risk and, once more, no significant association was found [74]. The same work included a pooled analysis of 15 studies (4282 cases and 5032 controls), in which was found a slightly increased risk of lung cancer associated with Val 105 allele, compared with Ile 105 (OR 1.11; 95% CI 1.03-1.21). Several studies focus on associations between tobacco habits, development of lung cancer, and GSTM1 and GSTT1 genotypes. A meta-analysis that included 34 studies, with 7629 cases of lung cancer and 10087 controls suggested a positive association for GSTT1 null in Asians (OR 1.28; 95% CI 1.10 – 1.49) but not in Caucasian individuals (OR 0.99; 95% CI 0.87 – 1.12). No significant interaction was observed between GSTT1 and smoking on lung cancer susceptibility [75]. In another study that enrolled 1921 lung cancer cases and 1343 healthy Caucasians the association of GSTP1*B genotypes and risk of lung cancer was not verified (OR 1.02; 95% CI 0.78 – 1.34) [76].

4.6. Gastric Cancer

Gastric cancer is the fourth most common cancer and the second most frequent cause of cancer death all over the world. It is widely accepted that gastric carcinogenesis is a multilevel multifactorial process. Despite several studies showing that infection by *Helicobacter pylori* is the cause of most gastric cancer, genetic factors are believed to play an important role in the development of gastric cancer.

Interindividual variations in the genetic and cellular mechanisms of activation and detoxification of cancer causing chemicals could confer different degrees of susceptibility to gastric cancer. Studies investigating the association between genetic polymorphisms of GSTs and gastric cancer risk have reported conflicting results [77]. A meta-analysis comprising eighteen case-control studies (2508 cases and 4634 controls) detailing a possible association between the GSTT1 null genotype and gastric cancer, observed a non-statistically significant OR for gastric cancer risk associated with GSTT1 deficiency (OR 1.09; 95% CI 0.97-1.21). By pooling data from seven studies (319 cases and 656 controls) that considered combinations of GSTT1 and GSTM1 genotypes, a statistically significant increased risk for gastric cancer (OR 1.95, 95% CI 1.42-2.67) was detected for individuals with deletion mutations in both genes compared with wild-types, suggesting that the GSTT1 null genotype may slightly increase the risk of gastric cancer [78]. Another meta-analysis enrolled 4357 gastric cancer cases and 9796 controls, and the combined results based on all studies showed there was a significant link between GSTT1 null genotype and gastric cancer risk (OR 1.14, 95% CI 1.01–1.28). In the subgroup analysis stratified by ethnic group, it was observed a positive association between GSTT1 polymorphism and gastric cancer risk among Caucasians, but not among East Asians [77]. In a European case-control study, including 304 gastric cancer cases and 427 control subjects, a 1.48-fold increased risk was found in carriers of GSTT1 null genotype, but not with GSTM1, GSTM3 or GSTP1 genotypes [79].

4.7. Hepatocellular Carcinoma

Hepatocellular carcinoma accounts for 85 to 90 percent of all primary liver cancers. Genetic variation has been postulated to influence risk for hepatocellular carcinoma and currently the most extensively studied inherited genetic risk factors for hepatocellular carcinoma are variants of GSTs [80]. Interestingly, liver and kidney are two organs that express the highest level of GSTT in the human body [5]. Recently, a meta-analysis of 24 individual case-control studies, involving a total of 3349 hepatocellular carcinoma and 5609 controls, investigated the associations between GSTs genetic polymorphisms and this disease. The results revealed an increased risk for hepatocellular carcinoma significantly associated with null genotypes of GSTM1 (OR 1.26; 95% CI 1.03-1.54, p=0.027) and GSTT1 (OR 1.28; 95% CI 1.09-1.51, p=0.002). Also, the GSTM1-GSTT1 interaction analysis showed that the dual null genotype of GSTM1/GSTT1 was significantly associated with increased hepatocellular carcinoma risk (OR 1.89, 95% CI 1.38-2.60, p<0.001) [81]. Another meta-analysis in 15 studies associated hepatocellular carcinoma risk with GSTA1, GSTA4, GSTM1, GSTM2, GSTM3, GSTT1, GSTP1, GSTO1 and GSTO2 polymorphisms in Asian, African and European populations. The authors observed that only GSTT1 (OR 1.19; 95% CI 0.99-1.44) and GSTM1 (OR 1.16; 95% CI 0.89-1.53) null carriers showed a positive association [82].

A study demonstrated that GSTT1*A carriers have an elevated risk for hepatocellular carcinoma development when exposed to halogenated solvents [83]. However, another study found that GSTT1 null individuals have an increased risk for hepatocellular carcinoma, and GSTM1 null genotype presented a protective effect [84].

4.8. Colorectal Cancer

Colorectal cancer is the third most common cancer and is globally the fourth most common cause of cancer. Large variations in rates have been found among different regions, with the lowest rates in Africa and Asia, and the highest in Europe, North America, and Australia [85]. Controversy exists about whether GST polymorphisms (GSTM1 null/present genotype, GSTT1 null/present genotype, GSTP1 Ile105Val and GSTA1*A/*B) represent risk factors for colorectal cancer. A meta-analysis involving forty-four studies, with 11998 colorectal cancer cases and 17552 controls, examined the associations between the above-mentioned polymorphisms and colorectal cancer. GSTM1 null allele carriers exhibited increased colorectal cancer risk in Caucasian populations (OR 1.150; 95% CI 1.060–1.248), and no significant association was detected for Chinese subjects (OR 1.025; 95% CI 0.903–1.163). In the same study, the authors found that GSTT1 null allele carriers exhibited increased colorectal cancer risk in Caucasian populations (OR 1.312; 95% CI 1.119–1.538). These associations were not found in the Chinese populations. Concerning GSTP1 Ile105Val and GSTA1*A/*B, no significant associations were demonstrated in either ethnic group [86]. Another meta-analysis of 36 case-control studies (10009 cases and 15070 controls) analyzed the association of GSTM1 polymorphisms and colorectal cancer. The combined data showed that GSTM1 deficiency is associated with a marginal effect on colorectal cancer risk (OR 1.13; 95% CI 1.03–1.23; $p<0.001$). When stratified by ethnic group and tumor site, significant results were only observed in Caucasians (OR 1.14, 95% CI 1.01–1.27; $p<0.001$), whereas no increased risk was detected in other subgroups, suggesting that GSTM1 polymorphism is associated with an increased risk of colorectal cancer, especially in the Caucasian population [85].

4.9. GSTs Polymorphisms and Tumor Drug Resistance

One of the primary causes of cancer treatment failure and patient relapse is an acquired or intrinsic resistance to anticancer therapies. Acquisition of drug resistance can be attributed to various factors that include avoidance of apoptotic cell death, altered expression of multidrug resistance-associated proteins and altered drug metabolism or uptake [25]. Recent studies have shown that the simultaneous polymorphisms of multiple classes of GSTs may correlate with therapeutic. Furthermore, it has been demonstrated that simultaneous polymorphisms in different classes may have more influence than a single gene by its own, maybe because various classes of cytosolic GSTs share overlapping substrate specificities and, therefore, absence of one GST isoform can be compensated by increased expression of other GSTs [88]. In cancer chemotherapy, pharmacogenetic studies have traditionally focused on candidate genes like GSTs, which function to detoxify reactive metabolites, thus interfering on pharmacokinetic characteristics of a specific drug. This polygenetic approach is also due to the combination of different anticancer agents with which patients are frequently treated, that are metabolized by different GSTs [88]. The most focused genotypes in relation to tumor drug resistance are the homozygous deletions of GSTM1 and GSTT1, and also the substitution of Isoleucine to Valine in amino acids 105 of GSTP1 gene. Recently, head and neck squamous cell carcinoma cases carrying the null genotypes of GSTM1 and GSTT1 or variant genotypes of GSTP1 showed a significant and superior treatment response [89].

Regarding colorectal cancer, it was found that mortality is significantly reduced in patients with one GSTM1 copy (HR 0.45; 95% CI 0.23-0.90; p = 0.02) and nonsignificantly reduced in those with the null genotype (HR 0.67; 95% CI 0.35-1.27; p = 0.22), compared with carriers of two copies. In this study, both GSTP1 and GSTT1 genotypes were not associated with survival [90]. In relation to ovarian cancer, was observed a significant survival advantage among carriers of GSTP1 Ile105Val GG/GA genotype (HR 0.77; 95% CI 0.61-0.99; p=0.04) and a non-significant survival advantage among homozygous women for the GSTM1 and GSTT1 deletion variants. There was also evidence of an additive effect, with a stronger survival benefit in women carrying three low function GST genotypes GSTM1 null, GSTT1 null and GSTP1 GA/GG (HR 0.47, 95% CI 0.22-1.02) [91].

4.10. Influence of GSTs Null Genotypes in other Multifactorial Diseases

Inflammation and oxidative stress are implicated in the pathogenesis of several multifactorial diseases such as heart diseases and diabetes mellitus. Oxidative stress is associated with increased generation of reactive oxygen species that leads to enhanced lipid peroxidation, the generation of hydroperoxides and other toxic compounds which may contribute to the development of atherosclerosis. Oxidative stress and inflammation biomarkers differ by GST genotype: the dual deletion, *GSTM1-0/GSTT1-0 (null genotype)*, seems to be associated with higher serum iron, total and LDL-cholesterol concentrations, and lower malondialdehyde concentrations [92-96]. The null GSTM1 genotype has been associated with elevated levels of DNA adducts and besides the fact that the DNA adducts are promutagenic and procarcinogenic, they are detectable in smooth muscle cells of human abdominal aorta affected by atherosclerotic lesions [97]. The same authors evaluated the polymorphic status of GSTM1 and GSTT1 in the population of atherosclerotic patients who had previously been investigated for presence of adducts in smooth muscle cells and they provide evidence that the levels of adducts are consistently increased in patients with GSTM1 null genotype, but no association was established with the GSTT1 polymorphism [98]. With respect to GSTs variant role in relation with heart diseases, literature remains unclear. Research concerning the influence of smoking on cancer risk demonstrated that these enzymes may result in either harmful or protective actions depending on whether GSTs activate or deactivate relevant chemicals [33, 95, 99]. Also, polymorphisms of GSTM1 and GSTT1 may modify the effects of cigarette smoking on risk of coronary heart disease and diabetes [100]. On a recent study, Tang et al [93] showed that the common variants in the GSTM1 and GSTT1 genes are related to markers of oxidative stress and inflammation in patients with coronary artery disease. Surprisingly, authors found a negative correlation between GSH levels and the GSTT1 gene variant. It seems that the reduction of GSTs level may result in the induction of a compensatory process in which other GSTs are overexpressed. GSTM1-0/GSTT1-0 subjects had higher C-reactive protein, fibrinogen and lower plasma total antioxidant status, compared to patients with wild-type GSTM1/GSTT1 genes. Stepwise elevations in age, the incidences of hypertension and diabetes mellitus, and levels of fibrinogen, as well as reductions of total plasma antioxidant status and GSH were associated with increased number of stenosed vessels [33]. Stroke, an interruption or severe reduction of blood flow arteries, is a leading cause of death in the world. Carotid stenosis resulting from atherosclerosis, hypertension, and diabetes are among the several risk factors

for ischemic stroke. Association analysis of GSTT1 and GSTM1 polymorphisms and GST total serum activity with ischemic stroke risk performed by Adah et al showed that GSTT1 and GSTM1 null genotypes, together with hypertension, may play a role in the pathogenesis of ischemic stroke [101]. An association between the GSTT1 variant, markers of inflammation and lipid peroxidation was described on Caucasian subjects with diabetes [94]. Krieger et al established an association between GST polymorphisms, triglycerides and HDL-cholesterol, and by reviewing Morrow et al and Evans et al issues, they hypothesized that GSTs are involved in the reverse cholesterol transport [102-104]. Chronic oxidative stress may also contribute to the increase of intraocular pressure by increasing resistance of aqueous humour outflow through trabecular meshwork [96]. The increase of intra ocular pressure is known to be one of the major risk factors for glaucoma, a progressive neuropathy which has a characteristic pattern of optic nerve and visual field damages. Glaucoma exhibits a heritable susceptibility consistent with a complex trait inheritance. Several studies concerning association of GST genetic polymorphisms with the risk of developing primary open angle glaucoma in different populations have been carried. GSTM1 polymorphism seems to be associated with increased risk of development of primary open-angle glaucoma among Estonians and Brazilians. A possible association of the GSTM1 positive genotype and GSTT1 null genotype, or the combination of both, with the increased risk of development of this disease was found in the Turkish population, however no evident association was found between GSTM1 and glaucoma in the Swedish population [105-108]. Concerning systemic lupus erythematosus (SLE), a chronic autoimmune disease featured by inflammation induced by autoantibodies, there are several studies postulating that GSTT1, GSTM1, and CYP1A1 polymorphisms may be involved in the development of SLE. The results are still not conclusive [109-111]. Glutathione S-transferases have an important role from social point of view as the intake of alcohol and caffeine could have different individual effects associated with the allelic variants of the enzymes. Glutathione S-transferase allelic variants have been associated with alcoholic liver disease. Carriers of GSTM1 null genetic variant or Val/Val genotype of Ile/Val GSTP1 polymorphism may have an increased risk to suffer from alcoholic liver disease and GSTs seem to be a potential therapeutic target in alcoholic liver disease [112]. Obstetric and gynecological diseases also seem to be associated with GSTs polymorphisms. A study performed by Tanaka et al in Japan found that the frequency of individuals with the GSTM1 deletion was significantly higher in cases of recurrent miscarriage compared with the control population among coffee drinkers, and concluded that GSTM1 enzyme activity may represent a risk factor for recurrent miscarriage, especially in the population of coffee consumers, as a result of impaired placental detoxification [113]. Women with the GSTM1 null genotype are reported to have a shorter gestation by almost one week compared to women with the normal genotype. This difference was not observed in the present study for either smokers or non-smokers [114]. However, risk of foetal growth restriction in mothers who smoked during pregnancy was modulated by maternal metabolic gene polymorphisms, as Guiguet et al observed on a case-control study in the Canadian population, where they reported a fourfold increased prevalence for a small-for-gestational-age outcome in homozygous mothers for CYP1A1 with a significant interaction between maternal smoking in the third trimester and CYP1A1, or absence of newborn GSTT1 [115]. These genes are linked to cytochrome P450 enzyme activity which is increased in CYP1A1 and decreased in homozygous deficient GSTT1 genotypes, indicating a genetic influence on cell metabolism which is altered by cigarette smoke exposure during pregnancy [115, 116].

The effect of polymorphic variants of GSTs have also been a study target for drug response; GSTM1 null genotype contributed to the hepatic disorder induced by hydrazine, a metabolic compound originated from isoniazid metabolism [92]. No difference was observed by GSTT1 genotype with respect to pharmacological treatment with ACE inhibitors, statins, aspirin or insulin [94]. Although this chapter focus GSTs genotypes it is not unlikely that one single low penetrance polymorphism is pivotal in carcinogenesis, atheromatosis and even longevity. Most findings regarding phase II genes reported an association between polymorphisms in NAT, GSTM and GSTT and various types of cancer or coronary heart disease. Lacczmanska et al and van Larebeke et al found that polymorphisms in genes encoding for xenobiotic metabolizing enzymes had a greater influence on reaching age 50-65 without severe morbidity than polymorphisms in genes encoding for DNA repair [117,118].

Conclusion

The importance of gluthatione is evident by the widespread expression in plants, mammals, fungi and some prokaryotic organisms. GSH is an important component of antioxidant defenses, and an antioxidant by itself, as the thiol group is a potent reducing agent. As an antioxidant, GSH plays a role in the detoxification of a variety of electrophilic compounds via catalysis by glutathione S-transferases. In addition to detoxification, GSH plays a role in several other cellular reactions, including the glyoxalase system, reduction of ribonucleotides to deoxyribonucleotides, and regulation of protein and gene expression via thiol-disulfide exchange reactions. GSTs are involved in Phase II detoxification, protecting cells from attack by reactive electrophiles or reducing the ability of the cells to metabolize toxins. Due to their relevant functions and since they display interindividual variations, special focus has been put on GSTs, associating them with several multifactorial diseases. In fact, allelic variants of relevant xenobiotic metabolizing enzymes are often considered as a differential risk of developing a disease such as cancer. Although polymorphisms have been described in several GSTs, most studies emphases polymorphisms in class Mu and Theta because GSTM1 and GSTT1 may suffer homozygous deletions. The majority of polymorphisms affecting genes involved in carcinogen metabolism are single nucleotide polymorphisms, deletions are less common and the complete absence of the entire gene in form of a null allele is rare. Thus, population with reduced GST enzyme activity, as result of homozygous deletions of GSTM1 or GSTT1, may be at greater risk for developing diseases due to their impaired ability to metabolically eliminate carcinogenic compounds and reduced detoxification efficiency. As it was extensively discussed in this chapter, the studies performed about this issue report conflicting results. The inconsistent results between studies reflect the complexity in the role of GSTs and might be due, for example, because some GSTs isoforms like GSTP1 may exhibit different activity, affinity, and thermostability according to genotype and substrates. Furthermore, GSTs are known to have overlapping substrate specificities and the absence of GST isoenzymes may be compensated by other isoforms. Also, different populations with different risk agent exposures may explain the differences in the outcomes of the reported studies conducted on this topic. Consequently, further studies with larger samples sizes, and considering gene–environmental and gene–gene interactions analysis should be performed.

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In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter IX

Structure and Catalytic Properties of Human Glutathione Transferase P1-1

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Abstract

Glutathione transferases (EC 2.5.1.18, GSTs) catalyze the nucleophilic attack of glutathione (GSH) on the electrophilic centre of a number of electrophilic compounds helping to detoxify a diverse array of toxic xenobiotics including carcinogenic, and pharmacologically active compounds. In this review, detailed descriptions are given on the structure and catalytic properties of human glutathione transferase P1-1 (hGSTP1-1) an enzyme that ubiquitously expressed in human tissues and exhibits many biological functions and multiple roles. The detoxification properties of hGSTP1-1 have been a primary research focus for the last years. However, now it has become apparent that the

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noncatalytic functions of GSTP1-1 have expanded the biological roles of this enzyme in cell survival, cell death and stress signalling mechanism.

Abbreviations

ASK1	signal-regulating kinase 1
GSH,	glutathione;
GST	glutathione transferase;
G-site	glutathione binding site;
H-site	hydrophobic binding site;
hGSTP1-1	human glutathione transferase P1-1;
JNK	c-jun N-terminal kinase;
TRAF2	tumor necrosis factor receptor-associated factor 2

1. Introduction

Glutathione transferases (EC 2.5.1.18, also known as glutathione S-transferases, GSTs) are multifunctional Phase II detoxification enzymes which protect cell from reactive endogenous or xenobiotic electrophile compounds [1-5].

GSTs catalyze the conjugation of GSH to the electrophilic center of these compounds thereby decreasing toxicity and increasing their solubility that help their excretion from the cell [6]. GSTs also exhibit more functions. For example, they are involved in several biosynthetic reactions, in GSH-dependent isomerization reactions, in reduction of toxic organic hydroperoxides, and in protection against oxidative stress [7-9]. In addition, GSTs are implicated in the intracellular transport and storage of hydrophobic molecules, such as haem, bilirubin, hormones, flavonoids, fatty acids and xenobiotics [8, 9].

GSTs are present in most aerobic organisms such as animals, plants, insects, parasites, yeast, fungi and bacteria [10-12] and can be divided into four families that include the soluble cytoplasmic GSTs, the microsomal bound GSTs, the mitochondrial and bacterial GSTs [13-15].

The soluble cytoplasmic family is widespread across all organisms and consists of a large number of enzymes that can be further divided into different classes. [16]. Human cytosolic GSTs can be grouped into seven classes on the basis of their amino acid sequence, which are: alpha, mu, pi, kappa, theta, omega and zeta [3]. Each class constitutes from different isoenzymes [17] that exhibit usually overlapping substrate specificities toward electrophilic compounds [1-4, 16, 18].

Human GSTP1-1 (hGSTP1-1), a member of the pi class, is ubiquitously expressed in human tissues and has attracted particular interest because of its multiple roles and functions. In the next sections of this chapter will be discussed the structural and catalytic properties of hGSTP1-1 and its roles in cell detoxification and stress signaling mechanism.

2. Structure and Function of Human GSTP1-1 Isoenzyme

2.1. Catalytic and Structural Properties of Human GSTP1-1 Isoenzyme

hGSTP1-1 exhibits wide substrate specificity towards a range of electrophile substrates. The structure of these substrates are diverse and include important endogenous compounds, as well as drugs, xenobiotic chemicals and the products of the transformation of xenobiotics by other enzymes. Nucleophilic displacement of an alkyl or aryl halogen or a nitro-group is catalysed effectively by hGSTP1-1 (Figure 1). Halogens or nitrogroups of these molecules are soft electrophiles and react readily with the GSH. Organic isothiocyanates, which are abundant in edible plants, also undergo conjugation with GSH by hGSTP1-1. Isothiocyanates, with benzyl-NCS and phenethyl-NCS appears to be the best substrates for hGSTP1-1 [19].

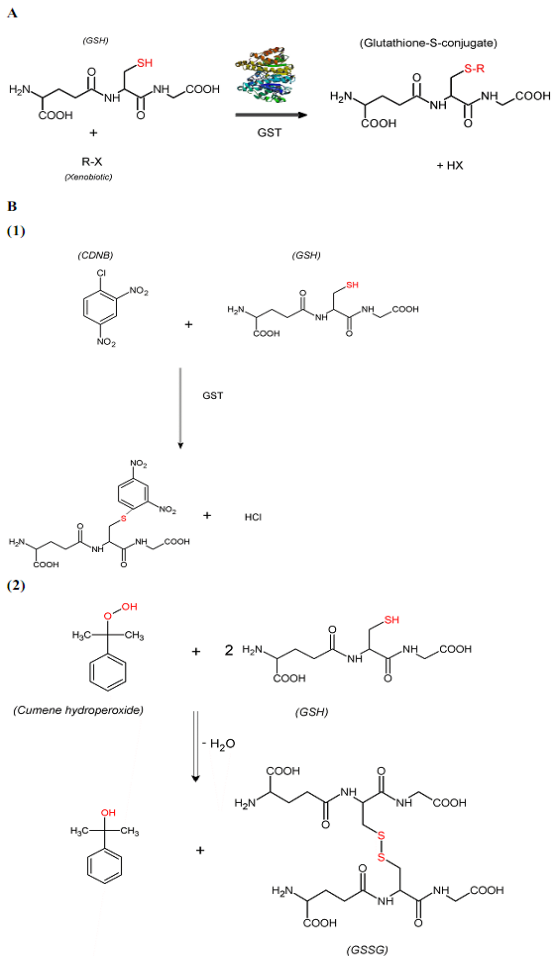

Figure 1. Glutathione conjugation to xenobiotic compounds catalyzed by hGSTP1-1. A: Glutathione conjugation to a generic xenobiotic (X) results in the formation of a glutathione-S-conjugate. B: Typical GST-catalyzed reactions. (1): nucleophilic aromatic substitution with 1-chloro-2,4-dinitrobenzene (CDNB), (2): hydroperoxide reduction with cumene hydroperoxide.

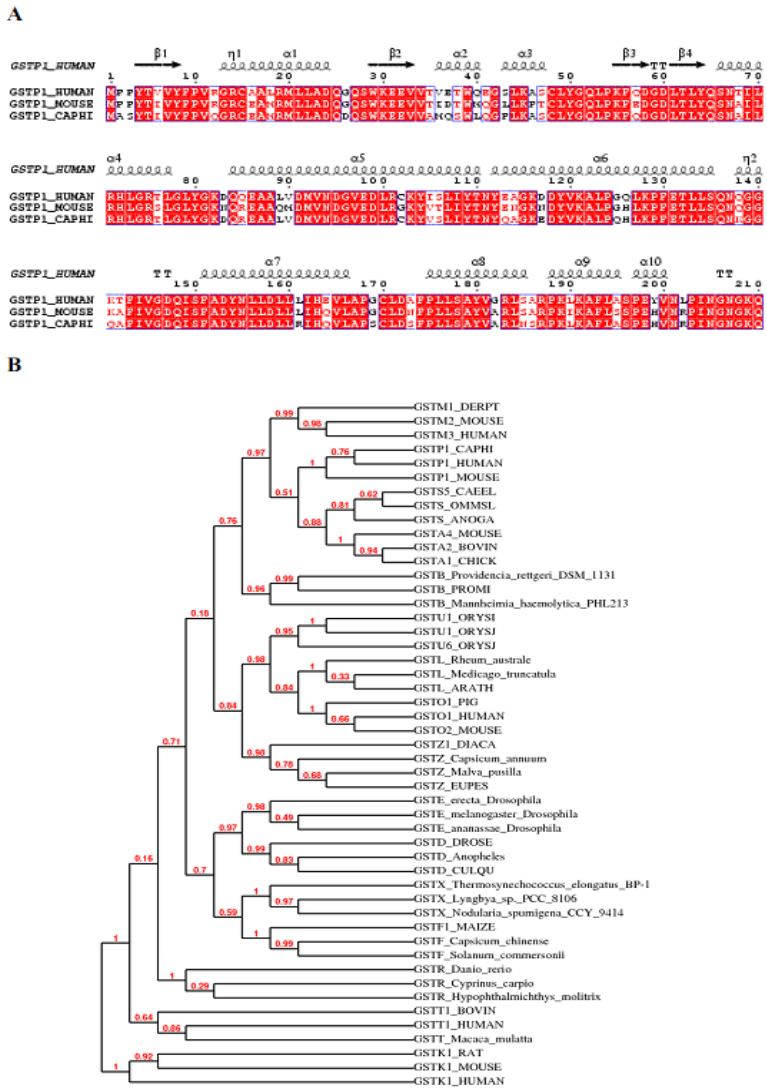

Figure 2. A: Sequence alignment of hGSTP1-1 with homologues from *Mus musculus* and *Capra hircus* produced using ESPript (http://espript.ibcp.fr/ESPript/ESPript/) [53]. Human GSTP1-1 numbering is shown above the alignment. Secondary structure elements of hGSTP1-1 are shown above the alignments. Alpha helices and beta strands are represented as helices and arrows, respectively, and beta turns are marked with TT. Conserved areas are shown shaded. A column is framed, if more than 70% of its residues are similar according to physico-chemical properties. The accession numbers of GSTP1-1 sequences that were used were: P09211, Q9TTY8, P19157. B: Phylogenetic analysis. The phylogenetic tree was created using representative members from all known GST classes (α, β, δ, ε, ζ, θ, κ, λ, μ, π, ρ, σ, τ, φ, χ, ω) and hGSTP1-1. The cladogram shows the groups which were formed after alignment of the protein sequences using ClustalW (Thompson et al. 1994) [54]. The accession numbers of GST sequences that were used were: alpha, (Q08392, O18879, P24472); beta, (P15214, D4C334, A7JQU5); delta, (B0W6B0, Q9GNE9, B4HHD9); epsilon, (B3NMR7, Q7KK90, B3MBB5); phi, (A5YWI8, Q5DUH0, P12653), kappa, (Q9DCM2, P24473, Q9Y2Q3); lambda, (B7FHT3, Q9LZ06, B5M1W3); mu, (P21266, P15626, P46419); omega, (Q9N1F5, P78417, Q8K2Q2); pi, (P09211, Q9TTY8, P19157); ro, (A7XZW2, Q0GZP3, Q1L907); sigma, (Q09596, P46088, P46428); theta, (E2RD21, P30711, Q2NL00); tau, (Q10CE7, A2XMN2, Q06398), chi, (A0ZF61, A0YYY7, Q8DMB4); and zeta, (P57108, Q84VH0, Q8H1P7, P28342).

Human GSTP1-1 also exhibits sulphonamidase activity, catalyzing the GSH-mediated hydrolysis of sulphonamide bonds. Such reactions are of interest as potential tumour-directed prodrug activation strategies (e.g. chimaeric sulphonamide derivatives of bombesin) [20].

In the cell, the formation of organic hydroperoxides is due to the attack of reactive oxygen species (ROS) on organic compounds. GSTs participate in oxidative stress defence mechanisms by catalyzing GSH-dependent reactions that inactivate such products by conjugation or reduction to the corresponding non-toxic alcohols (Figure 1B). For example, hGSTP1-1 is involved in the deactivation process of lipid peroxidation products such as oxidised DNA-bases, lipid hydroperoxides and their derivatives such as hydroxyalkenals, malondialdehydes and propenals [21, 22]. In addition, hGSTP1-1 can react directly with ROS via the modification of a reactive SH-group. This reaction leads to enzyme inactivation and disulphide bond formation that can be reversed by GSH.

The phylogeny of GST enzymes is complex. It is widely accepted that GST activity has emerged independently at least four different times throughout evolution, producing four different GST families - cytoplasmic, microsomal, mitochondrial, and bacterial [23]. The phylogenetic relationship of hGSTP1-1 with other GSTs from all known classes (α, β, δ, ε, ζ, θ, κ, λ, μ, π, ρ, σ, τ, φ, χ, ω) and a dendrogram generated by multiple amino acid sequence alignment is shown in Figure 2. Members of the classes mu, sigma and alpha form a large group, in which hGSTP1-1 is included. Their phylogeny probably reflects parallel evolutionary paths for these classes.

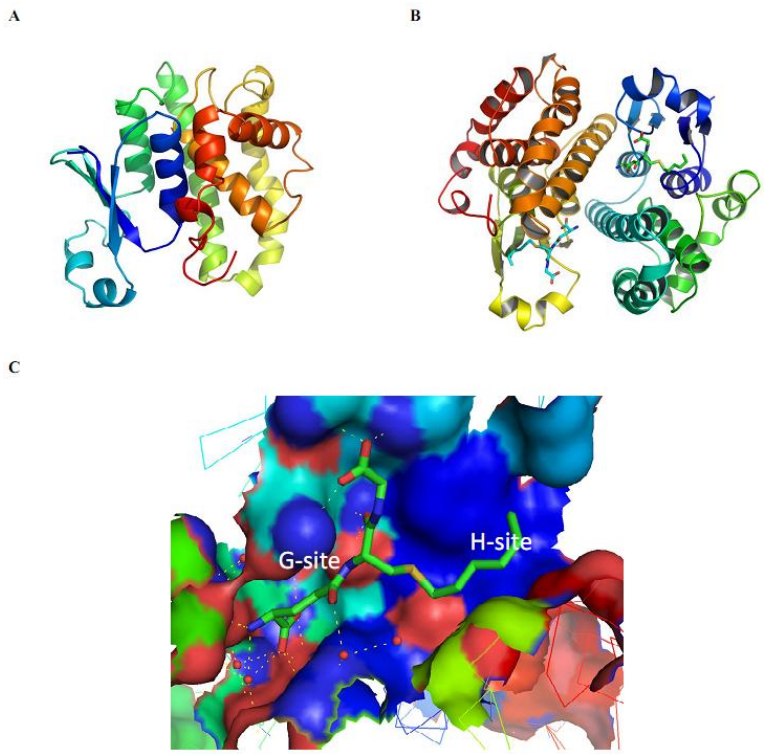

Figure 3. A cartoon representation of the hGSTP1-1 monomer (A), dimer (B) and the substrate binding site (C). The bound inhibitor S-hexyl-GSH is shown in B and C in a stick representation. The figures were produced using PyMol [55].

GSTP1-1 occurs as homodimer (Figure 3) with subunit molecular mass of 23,355.8 Da and a theoretical pI of 5.43. Each subunit of GSTP1-1 is composed of 210 amino acid residues and has one active site. The active site is suggested to consist of a GSH binding region (the G-site) and a nonspecific hydrophobic region (the H-site) that accommodates the electrophilic substrates. The G-site exhibits high specificity for GSH and includes the active site residue (Tyr8) that interacts with and activates the sulfhydryl group of GSH to generate the catalytically active thiolate anion. The H-site is not a conserved pocket and many electrophilic compounds are able to bind to it in a non-specific binding mode.

Each monomer of hGSTP1-1 constitutes two distinct domains; a smaller thioredoxin-like N-terminal domain and a larger helical C-terminal domain. It is known that all soluble GSTs have very similar polypeptide folds; however, each class exhibits unique features, particularly at the C-terminus [24]. The N-terminal small domain is an α/β structure with the folding topology βαβαββα. At the end of helix α3 begins a short linker that connects the N- and C-terminal domains. The C-terminal domain is made exclusively of helices. The active site of hGSTP1-1 appears as a large and open cavity. The G-site is located in a polar region of the N-terminal domain, formed by the beginning of helices H1, H2 and H3.

Coulombic surface analysis (Figure 4) indicates that the G-site in hGSTP1-1 exhibits positive electrostatic potential. This positive electrostatic potential of the G-site may contribute to high affinity GSH binding and –SH ionisation. The involvement of positively charged residues in the electrostatic field regulation has also been observed in other GSTs [25]. The H-site of hGSTP1-1 is formed by residues from the C-terminal domain and is hydrophobic in nature. Positive charged residues make the approach to the H-site basic. These residues form a positively charged region at the H-site, which presumably enable the enzyme to bind negatively charged substrates.

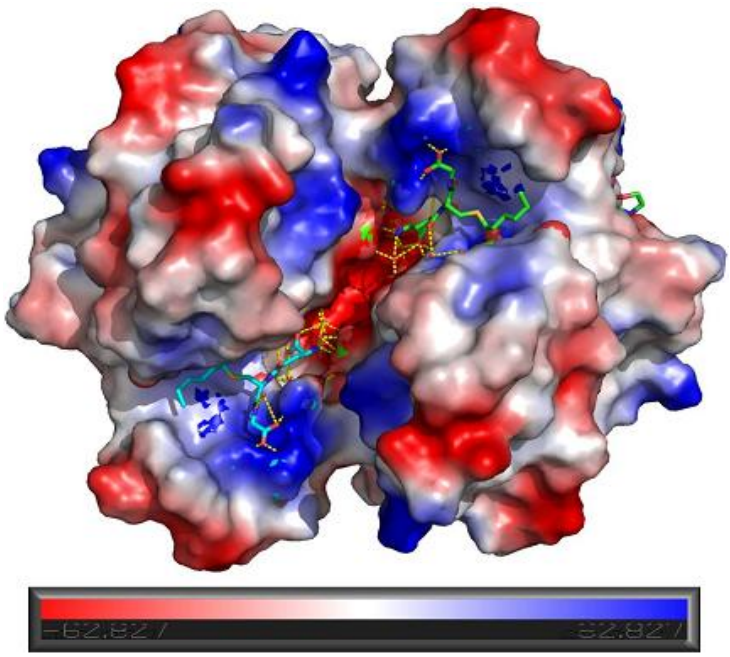

Figure 4. Coulombic surface analysis of hGSTP1-1. The bound inhibitor S-hexyl-glutathione is shown in a stick representation. The analysis was carried out using PyMol [56].

Helix H2 (Figure 2A), at the N-terminal domain, exhibits high flexibility in hGSTP1-1 and influences the catalytic activity of the enzyme. It is not clear how the mobility of this helix influences the rate of catalysis, although it is well established that the flexibility of helix H2 modulates GSH binding and product release. A plot of the crystallographic B-factors along the polypeptide chain can give an indication of the relative flexibility of the protein portions [26-28]. As shown in Figure 5, hGSTP1-1 displays a well defined flexibility pattern. Several important regions which including the helix H2 and helix H6 (Figure 2A and Figure 5) undergo large conformational changes. Comparison of the plots obtainded in the free, GSH-hGSTP1-1 and S-hexyl-GSH- hGSTP1-1 complexes it is evident that the flexibility of helix H6 is restricted in the GSH-hGSTP1-1 and S-hexyl-GSH-hGSTP1-1 complexes, indicating an induced fit mode of substrate binding and catalysis. An enzyme self-preservation mechanism of hGSTP1-1 was associated with an intersubunit communication [29].

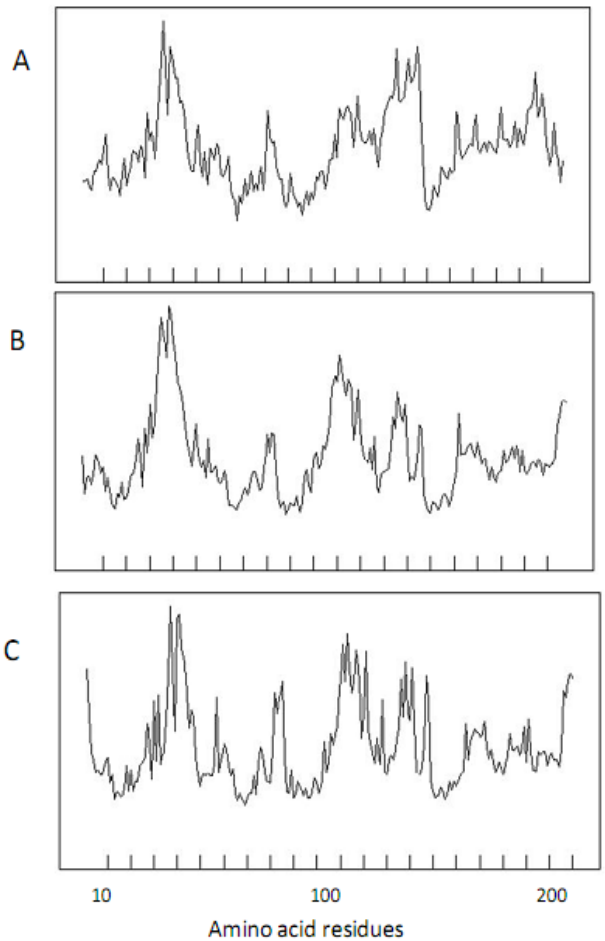

Figure 5. The flexibility of hGSTP1-1. Plot of the crystallographic B-factors along the polypeptide chain obtained from the crystal structure of free hGSTP1-1 (A); hGSTP1-1 in complex with GSH (B); hGSTP1-1 in complex with S-hexyl-GSH (C). The plots were produced using the WHAT IF software package (Vriend 1990) [56]. The height at each residue position indicates the average B-factor of all atoms in the residue.

Recent investigation has indicated that when a subunit is inactivated by a chemical or a physical factor, the second subunit triggers a different conformation to protect itself. Molecular Dynamics simulations suggested that the helix H2 has an important role in this phenomenon [29].

Analyses of crystal structures of hGSTP1-1 [30-32] have provided insights into the major interactions between subunits in the homodimers. Their subunit interfaces have three types of interactions: polar contacts, hydrogen bonds as well as hydrophobic interactions including a lock-and-key motif. In the lock-and-key motif an aromatic residue from N-terminal domain I in one subunit is wedged into a hydrophobic pocket formed by helices H4 and H5 in C-terminal domain of the other subunit. The lock-and-key motif is a common feature of pi, mu, and alpha class GSTs and the key residue is either phenylalanine or tyrosine (Tyr50 in hGSTP1-1). To investigate the importance of the key residue for dimerization and stability in hGSTP1-1, mutagenesis has been used [33]. Tyr50 in hGSTP1-1 plays both a structural role as the key residue and a functional role as part of the flexible helix H2. Hegazy et al., (2004) [34] reported that the mutant Tyr50Ala exhibits decreased k_{cat} value about 1,300-fold in comparison with the wild-type enzyme.

2.2. Functional Roles of Human GSTP1-1 Isoenzyme

hGSTP1-1 exhibits many cellular functions, therefore has multiple roles in cell survival [35]. hGSTP1-1 is the GST isoenzyme which has the broadest distribution among tissues [6, 35]. Although hGSTP1 is classified as a cytosolic GST, it has been demonstrated that it can be associated with the plasma membrane of the small cell lung cancer cell lines such as H69 and H69AR and human embryonic kidney (HEK) 293 and MCF-7 cell lines. Ali-Osman and his co-workers in 1997 isolated three human Pi gene variants from normal cells and malignant gliomas and designated them as hGSTP1*A, hGSTP1*B and hGSTP1*C. These allelic variants differ at either a single or at two codon positions [Ile104 (GSTP1*A), Val104 (GSTP1*B), Val104/Val113 (GSTP1*C)] [36]. It has been found that polymorphisms of GSTs genes are associated with patient response to chemotherapy [30, 37]. For example the alloenzyme GSTP1*B has been found to correlate with longer survival of patients with colorectal cancer who had a mixed chemotherapy [38].

An essential role of hGSTP1-1 is the regulation of stress signaling pathways [39] and the glutathionylation of cellular proteins [40]. Through protein-protein interactions, hGSTP1-1 can sequester c-jun N-terminal kinase (JNK), a key enzyme in the apoptotic cascade [41], and act as a negative regulator of this stress kinase. Furthermore, hGSTP1-1 is involved in the forward S-glutathionylation reaction, a post-translational modification that regulates the function/activity of a number of proteins. In addition it is able to form complexes with other proteins that participate in redox regulation [24].

GSTP1-1 has also been reported to associate with tumor necrosis factor receptor-associated factor 2 (TRAF2) and inhibit TRAF2-induced activation of both JNK and p38-MAP kinase [42]. GSTP1-1 inhibited TRAF2-enhanced apoptosis signal-regulating kinase 1 (ASK1) autophosphorylation and TRAF2-ASK1-induced cell apoptosis.

hGSTP1-1 has also protection role mainly against the cytotoxic effects of some alkylating agents, and their metabolites [30]. In contrast with its protective role, overexpression of hGSTP1-1 in a variety of malignancies is associated with a poor prognosis due to failure of

chemotherapy. Human GSTP1-1 is frequently overexpressed in various tumors and is the predominant GST isozyme in a wide range of sensitive and resistant cancer cells [43]. In particular, the enzyme is overexpressed in several different human cancer such as lung [32], colon [44], stomach [33, 45], kidney [46] ovary [29], mouth [47], testis [48]. In addition has been observed overexpression of hGSTP1-1 in patients with glioma [49], while negative regulation of hGSTP1-1 expression has been found in breast lesions and prostate cancer [50]. Human GSTP1-1 overexpression has been linked to multidrug resistance to chemotherapeutic agents including cisplatin, adriamycin, etoposide, thiotepa, chlorambucil, and ethacrynic acid. This can be contributed to hGSTP1-1 conjugation ability but also to the "nonenzymatic" antiapoptotic activity of hGSTP1-1 through its interaction with JNK [41]. Oxidative stress and treatment with GSTP1-1 inhibitors induce GSTP1-1 oligomerization and release of stress-activated kinases, defying apoptosis [39, 51]. Another inhibitor of GSTP1-1, the 15-deoxy-$^{\Delta 12,14}$-prostaglandin J_2 cause an impairment of GSTP1-1 protective effect on cell survival [52] through irreversible oligomerization specifically involving Cys101 [35]. Other agents that cause reversible oligomerization in different patterns are ethacrynic acid, 4-hydroxy-2-nonenal, H_2O_2 and diamide [39].

Conclusion

The human glutathione transferase pi gene is a polymorphic gene encoding active, functionally different GSTP1-1 variant proteins that are thought to function in xenobiotic metabolism and play a role in susceptibility to cancer, and other diseases. Human GSTP1-1 is capable of catalyzing several different reactions and substrates and exhibit wide substrate specificity. Structural analysis showed that hGSTP1-1 shares the same overall fold and domain organization of other cytosolic GSTs. Human GSTP1-1 can modulate cell-signalling pathways that control cancer cell survival, cell death by direct protein-protein interactions and stress signalling mechanism.

Acknowledgments

The authors thank the Ministry of Education, Lifelong Learning and Religious Affairs for the financial assistance provided. This work was partly supported by the grant programs HERACLITUS and THALES, co-funded by the European Union – European Social Fund and National Resources. The grants HERACLITUS and THALIS.-"Gluathione transferases: multifunctional molecular tools in red and green biotechnology" fall under the Operational Programme "Education and Lifelong Learning".

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In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter X

Risk of Cancer Associated with Measures of Placental Glutathione S-Transferase (GST-P 7-7): A Comprehensive Review

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Abstract

Glutathione *S*-transferases (GSTs) are a family of crucial enzymes involved in the cell detoxification of xenobiotics. GSTs exist as homo- or hetero-dimers and have been grouped into at least seven distinct classes. The main function of GSTs is to catalyze the conjugation of reduced glutathione (GSH) to an electrophilic site of a broad range of

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potentially toxic and carcinogenic compounds, thereby making such compounds less dangerous and enabling their readily excretion. Placental GST, known as GST-P 7-7, is the main isoform in normal placental tissue and comprises 67% of the total GST concentration in this phase. During development, GST-P 7-7 decreases in concentration and is absent in adult tissues. Interestingly, GST-P-7-7-positive expression have been detected in adult tissues after exposure to carcinogenic agents in several experimental test-systems being, considered a reliable biomarker of exposure and susceptibility in early phases of carcinogenesis process. In this chapter, we review a series of studies involving GST-P 7-7 positive expression and cancer risk.

1. Introduction

Glutathione S-transferases (GSTs) are a family of crucial enzymes involved in the cell detoxification of xenobiotics. GSTs exist as homo- or heterodimers and have been grouped into at least seven distinct classes [1]. The main function of GSTs is to catalyze the conjugation of reduced glutathione (GSH) to an electrophilic site of a broad range of potentially toxic and carcinogenic compounds, thereby making such compounds less dangerous and enabling their readily excretion [2].

2. Placental Glutathione S-Transferase and Cancer

Placental GST, called GST-P 7-7, is the main isoform in normal placental tissue and comprises 67% of the total GST concentration in this phase [3]. During development, GST-P decreases in concentration and is absent in adult tissues [4]. Interestingly, GST-P-positive expression have been detected in rat adult tissues during medium-term carcinogenesis assay being regarded a suitable biomaker for early detection of some neoplasms such as liver and tongue [5-7].

The Ito´s models of liver carcinogenesis and multi-organ carcinogenesis are used in conjunction and constitute an efficient and rapid bioassay for the identification of both genotoxic and nongenotoxic carcinogenic chemicals. The Ito´s model is an 8-week bioassay system that uses the number and size of foci of altered hepatocytes positive for glutathione S-transferase placental form (GST-P 7-7-positive foci) as the end-point marker. Forty-four compounds were tested using the Ito´s multi-organ model: 17 out of 17 liver carcinogens were positive, and 19 out of 22 (86%) nonliver carcinogens were positive. None of the five noncarcinogens tested were positive [8]. In particular, protein lysates from microdissected GST-P 7-7-positive foci and hepatocellular carcinomas (HCC) from livers of rats treated with N-diethylnitrosamine followed by phenobarbital at doses of 0 and 500 ppm were analyzed. Coordinated overexpression of mitochondrial chaperons prohibitin (PHB) and prohibitin 2 (PHB2), septin 9 (SEPT9), neurabin 1, and other cytoskeletal and functional proteins in GST-P 7-7-positive foci, during initiation and/or promotion stages of rat hepatocarcinogenesis, was associated with induction of cell proliferation and progression these preneoplastic lesions. Newly discovered elevation of PHB, PHB2, and SEPT9 in GST-P 7-7-positive foci and HCC,

imply that they might play important role in the onset of liver carcinogenesis and be of potential values in the studies of hepatocarcinogenesis [9].

To further elucidate the role of metal-related molecules in hepatocarcinogenesis, it was examined immunolocalization of transferrin receptor (Tfrc), ceruloplasmin (Cp) and metallothionein (MT)-1/2 in GST-P 7-7-positive foci during early stage of tumor promotion by fenbendazole (FB), phenobarbital, piperonyl butoxide or thioacetamide in a rat two-stage hepatocarcinogenesis model. To estimate the involvement of oxidative stress responses to the promotion stage, immunolocalization of 4-hydroxy-2-nonenal, malondialdehyde and acrolein was similarly examined. The results suggested that facilitation of lipid peroxidation is involved in the induction of GST-P 7-7-positive lesions by tumor promoters, and up-regulation of Tfrc and down-regulation of Cp may be a signature of enhanced oxidative cellular stress in these lesions [10]. On the other hand, several chemopreventive agents have been studied in this context. For example, licorice flavonoid oil (LFO) is a new functional food ingredient consisting of hydrophobic licorice polyphenols in medium-chain triglycerides. Recently, it was reported that licorice and its derivatives have anticarcinogenic activity against some types of tumor cell lines. However, the anticarcinogenic activity has not been identified in the liver, which is a major target organ for carcinogenesis in humans. No increase in the number of GST-P 7-7-positive liver foci was observed in all LFO groups compared with the negative control (solvent) group, and the number of foci in the 600 mg/kg LFO group was significantly lower than that in the negative control group. These results indicate that LFO concentrate solution has a significant inhibitory effect on liver carcinogenesis at 600 mg/kg [11].

Accumulating evidence suggests that GST-π is expressed in the cytoplasm, mitochondria, and nucleus in some tumour cells, and that the nuclear expression of GST-π appears to correlate with resistance to anti-cancer drugs. Although the mitochondrial targeting signal of GST-π was previously identified in the amino-terminal region, the mechanism of nuclear translocation remains completely unknown. Herein, some authors have investigated the region of GST-π195-208 that is critical for nuclear translocation, mediated by a novel and non-classical nuclear localization signal. In addition, using an in vitro transport assay, it was demonstrated that the nuclear translocation of GST-π depends on the cytosolic extract and ATP. Although further experiments are needed to understand in depth the precise mechanism of nuclear translocation of GST-π, these findings may help to establish more efficient anti-cancer therapy, especially with respect to resistance to anti-cancer drugs [12].

When DNA sequencing method was used to investigate the GSTP gene, no significant associations between exon 5 and exon 6 GSTP-1 gene polymorphisms and response to therapy or overall survival was detected. Patients carrying both variant exon 5 (Ile/Val or Val/Val) and variant exon 6 (Ala/Val) genotypes had significantly shorter TTP (5 vs. 8 months, $p = 0.04$). Moreover, patients with heterozygote exon 6 variant had presented with extensive-stage disease. No individual effect of variant alleles was found in relation to chemotherapy response, median TTP and overall survival. Therefore, the carriage of both types of variant alleles may predict worse outcome [13].

Increased expression of the glutathione S-transferase placental isoform (GST-pi) and of P-glycoprotein (P-gp) in tissues from patients with nonsmall cell lung cancer (NSCLC) has been associated with poor antineoplastic drug sensitivity, response to treatment, and survival. However, the diagnosis of advanced NSCLC often is mainly based on cytology. GST-pi and P-gp expression levels were associated inversely with response to chemotherapy and survival.

Cytologic evaluation of GST-pi and P-gp expression may predictor the response to treatment and the survival of patients with advanced NSCLC [14].

It has well been established that all colonic polyp examined were adenoma of low, mild and high- grade dysplasia as shown in the histopathological reports. Nevertheless, the examination of the above specimens by electron microscopy revealed that 3 of 9 adenoma of mild dysplasia had ultrastuctural features similar to high-grade dysplasia adenoma. GST-pi was variably expressed in adenoma, with the lowest relative levels occurring in low-grade adenoma and the highest levels found in high-grade adenoma. GST-pi was located mainly in undifferentiated colonic epithelial cells. GST-pi positive particles were found in the cytoplasm and especially in the nucleus adjacent to the nuclear membrane of these cells. The overexpression of GST-pi in mild-grade adenomas with significant subcellular changes and in the majority of high-grade dysplasia adenoma suggests that this might be related to the carcinogenetic proceeding. Immunohistochemical localization of GST-pi in combination with ultrastructural changes indicates that GST-pi might be a sensitive agent for the detection of premalignant changes in adenoma [15].

Taking into consideration that placental glutatione S-transferase (GST 7-7) and cellular proliferation play a crucial role during carcinogenesis, our research group has struglled to investigate the expression of GST-P, and proliferating cellular nuclear antigen (PCNA) by means of immunohistochemistry during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide (4NQO). GST-P 7-7-positive foci were detected in non-neoplastic oral epithelial cells at 4 weeks of 4NQO administration. In the same way, GST-P 7-7-positive cells were detected in pre-neoplastic lesions and squamous cell carcinomas induced after 12 and 20 weeks-treatment, respectively. None of the control animals expressed GST-P 7-7-positive cells. Regarding cellular proliferation, PCNA positive nuclei were higher at 12 and 20 weeks following 4NQO exposure ($p<0.05$) when compared to negative control [6]. These results suggest that the expression of GST-P 7-7 is correlated with cellular proliferation, in which GST-P is associated with risk and progression of oral cancer, whereas PCNA is closely involved during neoplastic conversion. GSTP 7-7 may facilitate cell proliferation and inhibit apoptosis, hence allowing for the expansion of a population of initiated tumor cells. The enhanced expression of GST-P at the protein level has been reported previously in chemically induced oral carcinomas in hamster buccal-pouch mucosa but the expression of GST-P 7-7 at the mRNA level has not yet been demonstrated. Therefore, some authors have investigatyed the GST-P mRNA expression in 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal-pouch carcinomas using a reverse transcription-polymerase chain reaction (RT-PCR). Placental glutathione S-transferase mRNA was demonstrated to be present amongst all the 12-week DMBA-treated hamster buccal-pouch mucosa animals, but not for the untreated animals or the animals for which the buccal pouch was treated with mineral oil. Multiple potential regulatory pathways including gene amplification, enhanced mRNA stability, chromosomal translocation/gene rearrangement, and hypomethylation of the promoter region can contribute to the overexpression of GST-P mRNA in DMBA-induced hamster buccal-pouch carcinomas. Further study is necessary to completely understand which candidate mechanism(s) will contribute principally to the increased GST-P mRNA expression in hemically-induced oral carcinogenesis [16].

The regulation of the GST-P 7-7 gene was known and the results demmonstrated that the strong enhancer element GPE1 (GST-P enhancer-1) specifically regulates the GST-P gene by interacting with specific transcription factors in normal liver and during

hepatocarcinogenesis. In particular, C/EBPalpha was required for the suppression of GST-P gene in normal liver, whereas the Nrf2/MafK heterodimer was required for the activation of this gene during hepatocarcinogenesis [17].

The rat placental glutathione S-transferase (GST-P 7-7), an isozyme of glutathione S-transferase, is not expressed in normal liver but is highly induced at an early stage of chemical hepatocarcinogenesis and in hepatocellular carcinomas (HCC). Recently, it has been postulated that the NF-E2 p45-related factor 2 (Nrf2)/MafK heterodimer binds to GST-P enhancer 1 (GPE1), a strong enhancer of the GST-P gene, and activates this gene in pre-neoplastic lesions and HCC. In addition to the positive regulation during hepatocarcinogenesis, negative regulatory mechanisms might work to repress GST-P 7-7 in normal liver, but this remains to be clarified. A chromatin immunoprecipitation analysis showed that C/EBPalpha bound to GPE1 in the normal liver in vivo but did not bind in pre-neoplastic hepatocytes. Introduction of the C/EBPalpha gene fused with the estrogen receptor ligand-binding domain into HCC cells, and subsequent activation by beta-estradiol led to the suppression of endogenous GST-P expression. These results indicate that C/EBPalpha is a negative regulator of GST-P gene expression in the normal liver [18].

To explain the molecular mechanism underlying its specific expression concomitant with the malignant transformation, several analyses regarding the regulatory element of the GST-P gene and the transcription factor that binds to this element are timely. From the extensive analyses by the establishment of the transgenic rat lines having various regions of GST-P gene, it would be interesting to know if the GPE1 is an essential enhancer element for specific GST-P 7-7 expression. Moreover, an examination of the transcription factor that binds and activates the GPE1, specifically in the early stage of hepatocarcinogenesis and in the HCC could contribute to better understanding of the role of GSTP gene in carcinogenesis process. Such analysis indicates that the Nrf2/MafK heterodimer binds and activates GPE1 element in pre-neoplastic lesions and HCC but not in the normal liver cells [18].

Glutathione S-transferases (GST) are also involved in cellular protection against xenobiotics, oxidative stress as well as in resistance against chemotherapeutic compounds such as doxorubicin. Levels of human placental type GSTP1-1 are known to be increased in many tumors and hematopoietic diseases. Transient transfection assays are used to show that different GST-promoter reporter constructs that generates cell-type specific levels of luciferase activity. In expressing cells, transcriptional activity is strongly dependent on AP-1 binding elements within the -65 to -75 bp region of the GSTP1 gene as shown by site-directed mutagenesis. Electrophoretic mobility shift assays show that DNA binding activity is exclusively observed in GSTP1-1-expressing cells and is increased after stimulation with hydrogen peroxide, TPA, tert-butylhydroquinone and doxorubicin. Non-expressing cells present neither constitutive nor inducible AP-1 binding. Taken together, such findings provide evidence for the induction of the GSTP1 gene via AP-1 binding activity in leukemia cells and contribute to a better understanding of the molecular events regulating genes involved in drug resistance mechanisms [20].

Vanadium (V) has recently been found to possess potent anti-carcinogenic activity in rat colon carcinogenesis. Some researchers have revealed the expression of the number and size of aberrant crypt foci (ACF) positive for placental glutathione S-transferase (GST-P 7-7) during 1,2-dimethylhydrazine (DMH)-induced rat colon carcinogenesis. Vanadium-treated groups significantly showed a reduced ($P < 0.001$) in the expression of GST-P 7-7-positive ACF cells (by 71.13%) for the entire period of the study. Moreover the histopathological

examination also showed that vanadium could minimize the development of ACF ($P < 0.001$). Furthermore, vanadium supplementation also elevated SOD activities in both liver and colon ($P < 0.01$, $P < 0.02$ and $P < 0.01$, $P < 0.02$ respectively) when compared to their carcinogen counterparts. The results confirm that vanadium is particularly effective in limiting the action of the DMH, thereby establishing its anticarcinogenicity in chemically-induced rat colon carcinogenesis (Kanna et al. 2003).

Conclusion

In this review, we have highlighted recent advances on the GST-P 7-7 expression following experimental carcinogenesis. Herein, *in vivo* studies combined with high risk situations are welcomed, as for example, with diabetes, use of therapeutic drugs, or even immunological disorders. In addition, the role of the GST-P 7-7 concerning the interference on cellular signal pathways, gene expression profiles, and epigenetic mechanisms is fundamental to elucidate putative interactions with cellular machinery. Therefore, this is an area that warrants investigation, since the estimation of GST-P 7-7 expression from using such important techniques following experimental carcinogenesis, will be added to those already established in the literature as a way to improve health status and prevention of several chronicle degenerative diseases.

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In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter XI

Roles of Glutathione S-Transferases in Cancer and Aging: A Friend or Foe?

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Abstract

Glutathione S-transferases (GSTs) are well known as phase II detoxification factors that conjugate glutathione to genotoxic electrophiles. Nearly all isoforms are functionally redundant; that is probably because organisms require backup self-defense systems. In addition to the detoxification function, some classes of GSTs participate in scavenging oxidative stress such as reactive oxygen species.

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Since numerous genotoxic compounds are the target substrates for GSTs, it is plausible that dysfunctions of GSTs due to polymorphic mutations, copy number variations, or epigenetic alterations cause tumors and age-associated diseases. Although nearly all isoforms are cytoprotective, certain classes of GSTs have unique cytotoxic properties. Of all the GSTs, GSTT1 possesses bilateral features, including cytoprotection against various toxins and the formation of cytotoxic formaldehyde. Interestingly, although the polymorphic mutations of GSTT1 are a risk factor for certain types of cancer, they reduce the risk of several other types of cancer. The unique character of GSTT1 is thought to be responsible for these opposite phenotypes.

Unlike other GSTs, GSTT1 was found to be upregulated in aged female reproductive cells in our previous reports [1-3]. Although the exact function of GSTT1 in aged cells remains to be clarified, the upregulation of GSTT1 is negatively correlated with the quality of oocytes. Thus, GSTT1 may play an important role in aging as well as tumorigenesis. In this chapter, we focus on the evolution and function of GSTs. The possible roles of GSTs, especially GSTT1, in tumorigenesis and aging will also be discussed.

Abbreviations

ARE	antioxidant-responsive element
C/EBPβ	CCAAT/enhancer binding protein β
CNVs	copy number variations
cRNA	complementary RNA
DCM	dichloromethane
DDP-GSH	cisplatin (CDDP)-glutathione
EGF	epidermal growth factor
GPE	glutathione S-transferase P enhancer 1
GRE	glucocorticoid-responsive element
GST	glutathione S-transferase
GSTO1	glutathione S-transferase omega 1
GSTT1	glutathione S-transferase theta 1
4-HNE	4-hydroxynonenal
HNF1	hepatic nuclear factor 1
JNK	c-Jun N-terminal kinase
KEAP1	kelch-like ECH-associated protein 1
MAPEG	membrane associated proteins in eicosanoid and glutathione metabolism
MAPK	mitogen-activated protein kinase
MEFs	mouse embryonic fibroblasts
MGST1	microsomal glutathione S-transferase 1
MK2	mitogen-activated protein kinase-activated protein kinase 2
MKK1	mitogen-activated protein kinase kinase 1
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B cells
NRF2	nuclear factor-erythroid derived 2-related factor 2
PI3K	phosphatidylinositol 3-kinase
RNAi	RNA interference

ROS	reactive oxygen species
XRE	xenobiotic-responsive element

1. Introduction

Living organisms are exposed to a wide variety of environmental pollutants, including biological and non-biological wastes. Because these waste materials are highly toxic for all organisms, they possess complex detoxification pathways as self-defense systems. Glutathione S-transferases (GSTs), which are found in nearly all prokaryotes and eukaryotes, are such molecules [4]. Through glutathionylation on divergent electrophilic substrates, they very efficiently remove environmental pollutants and endogenous toxic compounds in the phase II detoxification process.

In addition to the detoxification of genotoxins, GSTs have a wide range of functions, such as scavenging oxidative stress, regenerating protein S-thionylation, and catalysis in non-detoxification metabolic pathways [5, 6]. Although GSTs are composed of a number of isoforms, many are considered functionally redundant, probably due to the need for backup self-defense pathways.

Thus, the aberrant expression of GSTs is highly associated with various diseases, especially cancers. In addition, some classes of GSTs are presumed to be associated with aging. In fact, we found that GSTT1 is highly upregulated in aged granulosa cells and may be involved in reproductive aging [3]. Because GSTT1 has unique bilateral features (i.e. the removal of genotoxins such as the other isoforms and the formation of genotoxic formaldehyde), this molecule is considered to play important roles in tumorigenesis and age-related diseases.

In this context, we will review the structural, evolutional, and functional roles of GSTs in organisms, especially mammals, and present our current knowledge of GSTT1 in relation to aging and cancers in this chapter.

2. Structure, Evolution, and Function of GSTs

The environment contains numerous toxins. Non-nutritional foreign chemicals such as artificial chemicals or natural compounds including plant and fungal toxins and reactive oxygen species (ROS) accumulate in organisms and these chemicals are harmful to all organisms [4]. Self-defense systems have thus been developed to protect against these kinds of toxins. These systems are categorized into three distinct phases; phase I involves the oxidation of xenobiotics, phase II involves the conjugation of phase I products, and phase III involves an exclusion process using adenosine-5’-triphosphate (ATP)-dependent export pumps [7]. As discussed above, GSTs are classified into phase II detoxification factors that conjugate reduced glutathione (GSH) to an activated cysteine residue, such as Cys-SOH [8].

GSTs are very ancient proteins that are thought to have evolved from a thioredoxin-like ancestor in response to the development of oxidative stress [4]. They share sequence and

structural similarities with several stress-related proteins [9]. Other GST- and cysteine-binding proteins or non-GST proteins related to GSTs by sequence alignment may have a common ancestor, resulting in the evolution of the thioredoxin fold – the core scaffold of numerous proteins that controls disulfide redox activity in the cell [10].

The GST family is composed of several isoforms that have broad substrate specificities and that exist as homo- and hetero-dimers of approximately 25 kDa polypeptides [11, 12]. GSTs have been classified based on their NH2-terminal amino acid sequences, substrate specificities, sensitivities to inhibitors, and immunological relationships. Cytosolic, mitochondrial, and microsomal GSTs have been found. The largest subgroup is cytosolic GSTs. There exist 12 subgroups, including mammalian GSTs (alpha, mu, omega, pi, sigma, theta, zeta) and non-mammalian GSTs (beta, delta, epsilon, phi, tau). Mitochondrial GST is designated as GST kappa; it shares an evolutional relationship with the cytosolic GSTs [13]. Members belonging to the same subclass of cytosolic GSTs possess greater than 60% amino acid sequence similarity [4]. Across each subclass, proteins have less than 25% sequence similarity.

In humans, at least 18 GSTs have been identified so far [14]. The microsomal GSTs are integral membrane proteins that are designated as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEGs). The MAPEG family consists of four subgroups (I–IV), and protein sequences share 20 to 30% sequence identity among subgroups [15]. In humans, six MAPEG isoenzymes have been identified and categorized into subgroups I, II, and IV [15]. Like cytosolic and mitochondrial GSTs, MAPEGs, such as MGST1, catalyze the conjugation of GSH to a number of electrophilic compounds.

Although cytosolic GSTs are commonly cytoprotective, as described above, the divergent features of GSTs had been added to each GST by a mechanism similar to DNA shuffling [16]. One of the isoforms that has a unique property is GSTT1; it produces formaldehyde, which is very hazardous to DNA, from several halogenated compounds, including dichloromethane (DCM) during its metabolism [17]. In addition, MAPEGs have been reported to possess proinflammatory action and to catalyze reactions in leukotriene and prostaglandin biosynthesis [18]. Moreover, GSTO1 may be involved in the post-translational modification of the inflammatory cytokine interleukin-1β [19, 20]. Further investigation may identify isoform- or subclass-specific functions.

3. Pathways to Induce GSTs

The biological regulation of GSTs is complex as sex-, age-, tissue-, species-, and tumor-specific patterns of expression are evident. In addition, GSTs are regulated by at least 100 chemicals with structural deviations. Because many of these chemicals are found as non-nutrient components in vegetables and citrus fruits, humans seem to be exposed regularly to such compounds. Many inducers, but not all, stimulate the transcriptional activation of GST genes through either the antioxidant-responsive element (ARE), the xenobiotic-responsive element (XRE), the GST P enhancer 1 (GPE), or the glucocorticoid-responsive element (GRE) [21].

3.1. NRF2-ARE Pathway to Induce Phase II Detoxification Factors

The mechanism underlying the induction of phase II detoxification factors by ROS has been studied extensively. A number of antioxidants and phase II detoxification enzymes with the antioxidant response element (ARE) are induced in response to ROS and the other electrophilic agents [22]. The ARE is a *cis*-acting DNA regulatory element with a core nucleotide sequence (5'-GTGACnnnGC-3') found in the 5' flanking region [23]. Among GSTs, GSTA1 (GST Ya), GSTA2, GSTP1, GSTT2, GSTM1, GSTM2, GSTM3, GSTM4, and MGST3 are found to contain the ARE region [24-28].

The central transcription factor associated with ARE is nuclear factor-erythroid derived 2-related factor 2 (NRF2) [29]. The current model to induce these antioxidants is summarized in Figure 1A; oxidative stress induces the dissociation of NRF2 from NRF2-KEAP1 complexes tethered with actin cytoskeleton in the cytoplasm. The released NRF2 translocates into the nucleus, resulting in the transcriptional activation of GSTs (antioxidants) through ARE with the other cofactors [30]. In fact, GSTA1 and GSTP1 are induced by phase II-inducing compounds through an NRF2-ARE-dependent pathway [31, 32]. In addition, NRF2 knockout mice exhibited mild or severe reduction of GSTA1, GSTA2, GSTM1, GSTM2, GSTM4, and GSTM6 [28]. It is thus evident that the NRF2-ARE-dependent pathway plays a pivotal role in GST-associated self-defense.

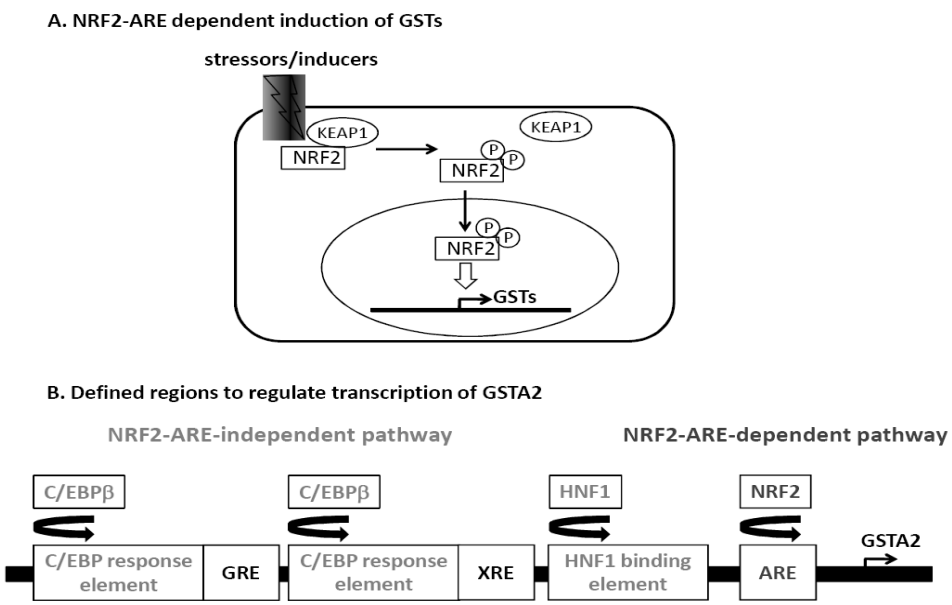

Figure 1. NRF2-ARE-dependent and -independent pathways to transactivate GSTs. Stimuli such as oxidative stress dissociate NRF2 from NRF2-KEAP1 complexes located in the cytoplasm. The released and activated NRF2 is able to translocate into the nucleus (A). In the nucleus, it binds directly to the antioxidant responsive element (ARE) and induces the transcriptional activation of GSTs (B, NRF2-ARE-dependent pathway). The other potent activator is CCAAT/enhancer binding protein β (C/EBPβ). C/EBP binds to the C/EBP response element that is located adjacent to the glucocorticoid response element (GRE) and the xenobiotic response element (XRE) and induces at least GSTA2 in an NRF2-independent manner (B, NRF2-ARE-independent pathway). Another possible activator is hepatic nuclear factor 1 (HNF1). GSTA2 is induced by binding of HNF1 to the HNF1 binding element although this pathway is only active in hepatic cells.

3.2. NRF2-Independent Pathway to Induce GSTs

NRF2-independent pathway in GSTA2 induction was found because PD98059, a specific inhibitor of mitogen-activated protein kinase kinase 1 (MKK1), induced GSTA2, but failed to activate the translocation of NRF2 into the nucleus and subsequent binding to ARE [33]. The upstream of the GSTA2 gene contains several possible promoter regions besides ARE (Figure 1B). One such region is the binding site of CCAAT/enhancer binding protein β (C/EBPβ). Two potential sites are located very near regions called GRE and XRE. PD98059 promoted the nuclear translocation of C/EBPβ and stimulated binding of C/EBPβ to the C/EBP response element in the GSTA2 gene. This process is blocked by wortmannin and LY294002, phosphotidylinositol 3-kinase (PI3K) inhibitors [34]. In addition to these elements, the liver-specific transcription of GSTA2 is mediated by interaction of hepatic nuclear factor 1 (HNF1) with HNF1 binding element [35, 36]. The predicted region is located between the ARE and the C/EBP-response element.

Induction of GSTP1 also seems to be regulated through both NRF2-ARE-dependent and -independent pathways. Although GSTP1 is induced in some cell types depending on the activation of NRF2 [31, 37], the expression of GSTP1 was not affected by the overexpression of NRF2 or KEAP1 in rat liver epithelial cells [38]. Supportively, our data demonstrate that GSTP1 is upregulated in NRF2-deficient mouse embryonic fibroblasts (MEFs) although the expression level of GSTP1 in NRF2-deficient MEF is much lower than that in wild-type MEF. In the same cells, GSTT1 is also induced via the NRF2-independent pathway. However, the exact pathway in which GSTP1 and GSTT1 are induced remains uncertain.

3.3. Involvement of Various Signaling Kinases to Induce GSTs

For the successful transcription of phase II enzymes, mitogen-activated protein kinases (MAPKs) must be required to be activated [39]. However, it is likely that each gene is induced via distinct MAPK pathways.

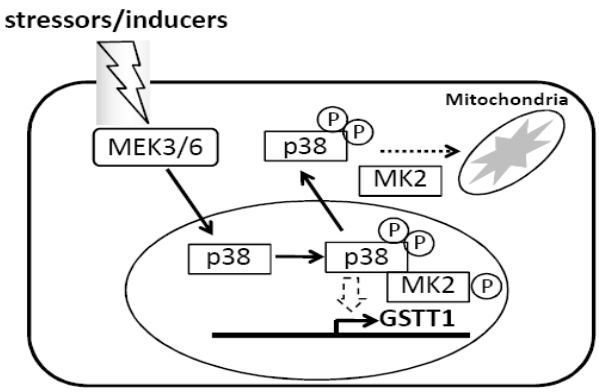

Figure 2. Age (oxidative stress)-associated pathway to induce GSTT1 in human granulosa cells. In response to oxidative stress or other stress inducers, p38, which resides mainly in the nucleus of granulosa cells is activated and conjugated with MK2. Parts of the p38-MK2 complexes formed in the nucleus are exported to the cytoplasm and influence the mitochondrial activity. Other parts of the p38-MK2 complexes may facilitate the transcription of GSTT1.

In hepatocytes, GSTM1 and GSTM2 were induced by geniposide, an irdoid glycoside, through the ERK signal transduction pathway [40]. In Hep3B cells, GSTA1 was upregulated by alcohol through both ERK and p38 pathways [41]. In human granulosa cells, GSTT1 was induced via the p38 pathway in response to oxidative stress [2]. Interestingly, the upregulation of GSTT1 is highly correlated with the activity of MK2, which is a transporter of p38 from nucleus to cytosol (Figure 2, [1]).

In addition to MAPKs, the other kinases are associated with the induction of GSTs. For example, both NF-κB and PI3K signaling pathways play a pivotal role in GSTA1 expression in CaCo-2 cells [42]. Moreover, a specific inhibitor of PI3K blocked EGF-induced GSTA4 in hepatocytes [43]. Similarly, both p38 and PI3K play an essential role in the ARE-mediated GSTA2 in hepatoma cells [44]. A specific protein kinase signaling pathway for each GST may determine a specific activity of GSTs.

4. GSTs Associated with Cancers

Because DNA adducts of various genotoxins cause unexpected genomic mutations, loss-of-function mutations of GSTs are highly correlated with various diseases, especially cancers. For example, null mutation of GSTM1 was demonstrated to be strongly related to p53 mutations and to be a risk factor for non-small cell lung cancer [45]. In addition, null GSTM1 is associated with non-polyposis colorectal cancer, renal cell carcinoma, and prostate cancer [46-48]. Furthermore, the GSTP1 genotype was reported to be a risk factor for lung cancer predisposition and cervical cancer [49, 50]. In females, the GSTM1-null genotype had increased risks of breast cancer, particularly for postmenopausal women [51]. In an animal model, GSTP-deficient mice had a higher susceptibility to skin papillomas [52, 53], although the available data regarding the involvement of GSTs in tumorigenesis using genetically engineered mice are limited.

Focusing on GSTT1, some investigators have reported that mutations of GSTT1 are highly correlated with certain types of cancer. For example, GSTT1 mutants are associated with acute myeloid leukemia, Hodgkin’s lymphoma, and kidney and liver cancers [54-56]. In addition, females with the double null genotype of GSTT1 and GSTM1 appear to have an increased risk of ovarian cancer [57, 58]. Moreover, individuals with GSTT1 genotypes are more susceptible to cutaneous basal cell carcinoma, gastric cancer, and lung cancer [55, 59-61]. GSTT1 and GSTP1 genotypes may also play a role in breast cancer [62], although analyses of Caucasians, African-Americans, and Norwegians failed to show any association between breast cancer and GSTT1 polymorphism [63]. These diseases are likely caused by a lack of cytoprotective GSTT1.

In addition to genetic mutations, the copy number variations (CNVs) of GSTs are associated with cancers. CNVs are defined as a stretch of DNA larger than 1kb that displays the differences in the gene copy number even in the normal population [64]. Because CNVs directly modulate the amount of transcripts, the variations strongly influence the expression of genes that predispose individuals to various diseases [65]. In fact, the mortality rate of patients with colorectal cancer carrying one copy of GSTM1 was significantly lower than that of patients carrying two copies of GSTM1 [66]. In addition, carriers of at least two copies of GSTT1 genes had an increased risk of endometrial cancer [67]. The cytotoxic function of

GSTT1 can be explained for this cancer, although it remains uncertain why the mortality risks found in the CNVs of GSTM1 are reduced in colorectal cancer.

Epigenetic alterations are the other factors that affect the gene expression level. The promoter methylation of some GSTs has been reported. A low expression level of GSTM2 has been detected in lung cancer cells, and a high frequency of promoter GSTM2 hypermethylation was observed in those cells [68]. In addition, the promoter DNA hypermethylation and mRNA downregulation were found in GSTM2, GSTM3, and GSTM5 in Barrett's adenocarcinoma [69]. The epigenetic silencing of the GSTP1 gene is the most common genetic alteration in prostate cancer [70]. These reports provided strong evidence that the downregulation of GSTs as a result of epigenetic alterations results in specific cancers.

Distinct from the roles of GSTs to protect normal cells from tumorigenesis, they guard cancers from anti-cancer drugs and oxidative damages. Out of all of the GSTs, GSTP1 is commonly overexpressed in various cancers and plays an important role in chemoresistance [71]. In fact, GSTP1 was demonstrated to be highly upregulated in cisplatin-treated bladder carcinoma cells [72]. In terms of ovarian cancers, GSTP1 assists in the formation of the cisplatin-glutathione adduct (DDP-GSH) and the acquisition of resistance to cisplatin in ovarian cancer cells [73]. In addition to GSTP1, cells expressing higher levels of MGST1 were shown to be resistant to doxorubicin chemosensitivity in Ewing sarcoma [74]. Similarly, a breast cancer cell line stably overexpressing MGST1 revealed that MGST1 could protect cells against several cytostatic drugs, such as chlorambucil, melphalan, and cisplatin [75]. Furthermore, GSTP1, GSTM1 and GSTT1 are highly upregulated in transitional cell cancers – this may ensure their survival [76, 77]. Throughout our global expression analysis, GSTT2 was highly upregulated in a granulosa cell tumor cell line (KGN) compared to normal granulosa cells (NGC), although GSTM1 and GSTP1 were slightly upregulated (Figure 3). In contrast, GSTA3, GSTA4, GSTT1, and MGST1 were significantly decreased in KGN.

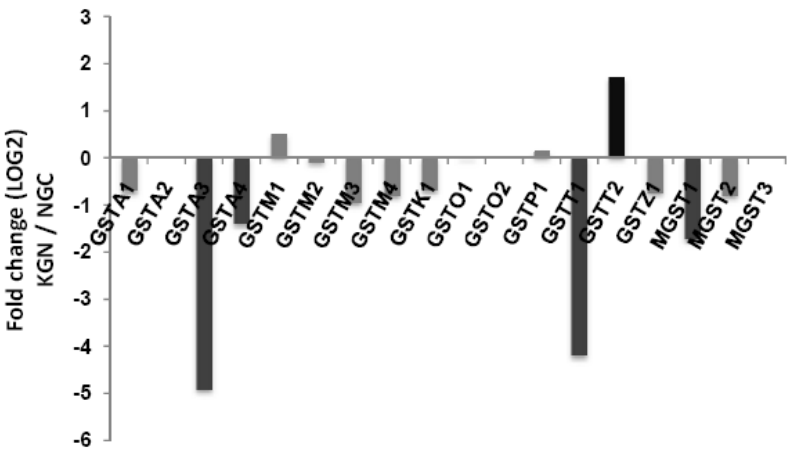

Figure 3. Comparative gene expression profile of GSTs in a granulosa cell line (KGN) and normal granulosa cells (NGC). Total RNA isolated from KGN and NGC was used for templates of cRNA probes to conduct an Affymetrix transcriptome analysis. The y-axis represents the average fold changes of KGN / NGC (LOG2). The dark grey columns indicate the genes with LOG2 (fold change) ≤ –1 (regarded as decreased) and the black column indicates the gene with LOG2 (fold change) ≥ 1 (regarded as increased). GSTT2 is the only gene upregulated in KGN cells whereas GSTA3, GSTA4, GSTT1, and MGST1 are downregulated.

Similar aberrant expression of GSTs was observed in gastric tumors; GSTP was upregulated whereas GSTM and GSTA levels decreased [78]. Thus, certain classes of GSTs, such as GSTP1, are beneficial to the survival of both normal cells and cancer cells.

In addition to susceptibility to cancers, loss-of-function alterations of GSTs are involved in various diseases. GSTT1 null mutation may increase the risk of myelodysplasia [79]. GSTP1 polymorphism is involved in the pathogenesis of malaria [80]. In females, polymorphisms of GSTM1 and GSTT1 may increase the risk of recurrent pregnancy loss [81] and susceptibility to polycystic ovaries [82]. In males with neuronal diseases, a deletion of GSTM1 seems to increase the susceptibility to Parkinson's disease whereas a deletion of GSTT1 increases the susceptibility to motor neuron disease and Alzheimer's disease [83]. In addition, the CNVs of GSTM1 and GSTT1 are highly correlated with gene expression level and play a role in the risk of smoking-associated, xenobiotic-induced lung disease [84]. A combined analysis of the CNVs of GSTM1 and GSTT2 in schizophrenic patients revealed a significant association of non-null genotypes with increased susceptibility to the disease [85].

5. GSTs Involved in Aging

The link between GSTs and aging has been well studied in *Caenorhabditis. Elegans* (*C. elegans*). The increasing activity of GSTs contributes to longevity because of their beneficial functions [86]. Indeed, CeGSTP2-2, which belongs to the pi-class of GSTs, is able to conjugate 4-hydroxynonenal (4-HNE) and prolongs lifespan of *C. elegans* [87, 88]. Supportively, the proteomic analysis of long-lived mutants revealed that the pi-classes of GSTs were upregulated significantly [89].

Although direct evidence that mammalian GSTs are involved in the regulation of lifespan is restricted, some may participate in the process. In an animal model using long-lived Ames dwarf mice GST activities were demonstrated to be elevated in various organs [90]. This suggests that the upregulation of GSTs likely supports cellular survival and prolongs the lifespan. However, unexpectedly, the genetic disruption of GSTA4 in mice resulted in lifespan elongation [91]. GSTA4 has been well studied in regard to the protection of cells against 4-HNE-mediated apoptosis [92]. Overexpression of GSTA4 inhibits the JNK signaling towards apoptosis [93]. Supportively, silencing the GSTA4 genes by RNAi or gene knockout resulted in mitochondrial dysfunction in adipocytes [94]. Although these data are conflicting, NRF2-dependent antioxidant genes are markedly induced in GSTA4-deficient mice because NRF2 was highly activated. Disruption of 4-HNE binding to GSTA4 may elicit a hermetic response to induce NRF2 activation resulting in altered detoxification mechanisms that control life extension. Other unknown mechanisms may, however, be involved in the process.

GSTT1 is a possible candidate to regulate successful aging [95]. The copy numbers of GSTT1 are reported to be associated with a moderate but significant increase in mortality in women, although carrying two copies of GSTM1 is not [96]. We previously reported that GSTT1 is highly upregulated in aged human granulosa cells, although the other GSTs are mostly downregulated during aging [3]. This suggests that the overall antioxidant capacities may be declining in granulosa cells during aging. Interestingly, the expression level of GSTT1 in granulosa cells is highly correlated with the age-related decline in oocyte quality.

The potent involvement of GSTT1 in aging can be explained by the evolution of theta-class of GSTs.

Methylobacterium belonging to α-proteobacterium (a potent ancestor of mitochondria) grows with chloromethane and DCM as their unique carbon and energy sources [97]. Natural organohalogen compounds such as chloromethane are produced in large quantities (an estimated rate of 5 million tons per year) by wood-rotting fungi and a variety of marine algae [98]. To utilize these compounds, bacterial GST (an ancestor of mammalian GSTT) conjugates GSH to them and produces formaldehyde as a central metabolite of methylotrophic growth. Formaldehyde is highly toxic to all organisms from bacteria to humans due to its reactivity with biological macromolecules. However, bacteria utilizing this system have a formaldehyde-activating enzyme that catalyzes the condensation of formaldehyde to methylene tetrahydromethanopterin [99]. In this way, a more efficient energy source is utilized during evolution of creatures, because the evolved organisms require more energy to survive.

The unique characteristic of GSTT remians even after evolution (in mammals) although the functional purposes of these enzymes are completely different. *Methylophilus* lacking endogenous GSTT and expressing mammalian GSTT1 could not grow in a selective DCM medium due to the reduced affinity of mammalian GSTT1 for DCM and the reduced production of formaldehyde [100]. In addition, transgenic *Salmonella typhimurium* expressing mammalian GSTT1 produced much larger amounts of mutagenic metabolites [100].

Thus, mammalian GSTT1 may produce these hazardous compounds from the pollutants accumulated in the body. Supportively, endogenous formaldehyde levels were reported to be elevated during aging in human cells [101]. Furthermore, endogenous formaldehyde in combination with oxidative stress was suggested to decrease cell viability in rat aorta endothelial cells [102]. Therefore, this relic of the evolutionary past may cause various unwanted alterations that lead to aging and diseases in mammals.

Conclusion

A current model of GST-associated aging and tumorigenesis is illustrated in Figure 4. In the young and healthy state, self-defense systems, including GSTs and DNA repair genes are active and bodies can immediately fix mutagenic alterations induced by stressors or toxins. However, some unfixed genomic alterations that promote aging and tumorigenesis accumulate in the body at some time points. Although many GSTs are downregulated during aging, GSTT1 is upregulated and may promote aging because it likely produces formaldehyde, which is damaging to all organisms.

In contrast, the selective upregulation of GSTP1 (or other specific classes of GSTs) in cancer cells may be involved in tumorigenesis because this GST protects cancer cells against anticancer drugs. Although the functional roles of these GSTs in aging and tumorigenesis remain poorly understood, they are attractive targets for the future drug discovery in the field of aging and cancers.

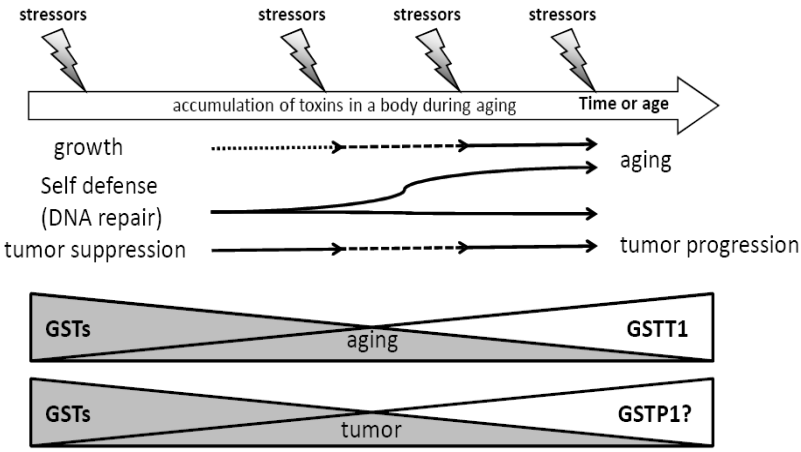

Figure 4. A possible model of GST-associated aging and tumorigenesis. Various stressors including environmental pollutants and endogenous mutagenic toxins, that accumulate in the body activate self-defense molecules, such as GSTs and DNA repair genes. Although these molecules protect cells from deleterious genomic mutations in the young and healthy state, irreversible genomic alterations occur at some time points probably due to the decrease and dysfunction of GSTs and DNA repair genes. These changes accelerate aging and tumorigenesis. Unlike the other classes of GSTs, GSTT1 is unregulated by aging in human granulosa cells and GSTT1 expression is negatively correlated with oocyte quality suggesting that GSTT1 participates in reproductive aging. During tumorigenesis, a certain class of GSTs (commonly GSTP1) is selectively upregulated in cancer cells and protects those cells against anticancer drugs.

Acknowledgments

We thank Ms. Miho Muraki for her technical assistances. This work was supported partly by a grant from Honeybee Research Foundation of Yamada Bee Farm and partly by a Grant-in Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (18591818).

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In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter XII

Glutathione S-Transferase Gene Polymorphisms in Multiple Myeloma

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Abstract

Background: Genetic variations exist in the genes coding for the gluthatione S-transferases (GSTs) that may contribute to the variations in the metabolic breakdown of environmental carcinogenic and chemotherapeutic agents. Such metabolic variations are also considered to contribute to differences in interindividual susceptibility to cancer and disease outcome.

Materials and Methods: In this study the *GSTM1, GSTT1* and *GSTP1* Ile105Val gene polymorphisms have been determined in a study population of one hundred Hungarian myeloma patients and their hospitalized controls.

Results: There was no significant difference in the genotype frequencies between cases and controls, with a notable exception, that in the group of patients with *GSTT1* homozygous deletion (null) genotype, no *GSTP1* 105Val/105Val homozygous variant genotype was detected. For the combined *GSTT1/GSTM1* genotypes, the shortest mean survival was observed in the *GSTT1 null /GSTM1 null* genotype group of patients. The *GSTP1 Val/Val* homozygous variant genotype also appeared to be a risk factor for shortest survival time, however, these trends were not statistically significant.

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Conclusions: *GST* gene polymorphisms may have only a weak, if any, effect in the outcome of multiple myeloma, which could be confirmed by large-scale international molecular epidemiological studies.

Keywords: multiple myeloma, GSTs, predisposition to cancer, impact on survival

Introduction

Multiple myeloma (MM) is a malignant proliferation of plasma cells that is currently an incurable disease. Worldwide incidence of the disease is growing year by year, contributing 1 % to the cancer mortality. The anticipation in onset, the familial occurrence of the disease and the higher cancer risk in families of multiple myeloma patients raise the questions of genetic susceptibility and / or chemical exposure-related initiation of the disease [1, 2, 3]. It has already been shown that exposure to cytotoxic and genotoxic agents, particularly those derived from benzene may be associated with an increased risk for MM [4]. Weber et al. found association between greater myeloma risk and exposure to pesticides, dioxins, benzene, silicone implants and radiation [5].

The glutathione S-transferases (GSTs) are metabolising enzymes that catalyse the conjugation of electrophilic molecules of several chemical agents with glutathione, converting them mostly to less toxic compounds [6,7, 8]. Genetic variations exist in the genes coding for the GSTs that may be responsible for the variations in the metabolic breakdown of carcinogenic environmental agents. Such metabolic variations are considered to contribute to differences in interindividual susceptibility to cancer [9]. The genes encoding GSTM1, GSTT1 and GSTP1 enzymes are polymorphic in human beings. The *GSTP1* Ile105Val genotype variants may alter enzyme function [10]. The null genotypes of *GSTM1* and *GSTT1* that are homozygous gene deletions do not express the enzyme. It would therefore be expected that individuals with *GSTT1* and /or *GSTM1* null genotype who are unable to properly detoxify particular genotoxic agents could be at increased risk for the development of various malignant diseases.

In Caucasian populations, approximately 50% of the population lack GSTM1 and around 20 % lack GSTT1. Therefore, the relationship between these genetic polymorphisms and the incidence of specific cancers has been widely investigated [11, 12, 13, 14]. Conflicting results have been reported which may be due to single gene analysis, or the small size of study population or ethnic differencies between the study populations examined [15].

The aim of the present study was to investigate the *GSTM1*, *GSTT1* and *GSTP1* Ile105Val genotype frequencies and the association with survival in one hundred Hungarian MM patients in comparison to age-matched cancer-free subjects serving as controls.

Materials and Methods

The study population comprised one hundred MM patients and ninety nine hospitalized controls. Subtype distribution of MM patients were as follows: IgG 63, IgA 24, non-secretory 3, biclonal 1, lambda-light chain 4, kappa-light chain 5, IgD 1. Controls were diagnosed for

various unrelated diseases including hypertension, 2nd type diabetes, obliterative arteriosclerosis, metabolic syndromes, such as hyperuricacidaemia or hyperlipoproteinaemia. Exclusion criteria for controls were malignancies and immuno-pathological disorders. Median age was 68 yrs both for the patients and their controls. The male/female ratio was 32/68 for the MM group and 45/55 for the controls.

Blood samples were taken from patients and controls with their informed consent and approval from the scientific ethical committee ETT TUKEB 12236-45/2004-1018EKU. DNA was isolated from peripheral blood according to the standard salting out method. *GSTM1* and *GSTT1* genotyping were carried out by a multiplex PCR essentially according to Lin et al.[16].

GSTP1 Ile105Val genotypes were identified according to the method by Ozawa et al. except that Thermoprime Plus DNA polymerase (Abgene, Epsom, Surrey, UK) was used for catalysing the PCR reaction [17].

Statistical analyses were performed by chi-square/Fisher's exact test and contingency tables, and one-way ANOVA using GraphPad software 4.0 (GraphPad Sowftware Inc, San Diego, CA, USA).

Two-tailed p-values were calculated for the determination of statistical significance. Almost all patients were treated according to protocols, such as, MP (Alkeran and Prednisone) or VAD (Vincristine, Adriablastine and Dexamethasone). In non-responders or on relapse, Thalidomide was introduced in combination with Dexamethasone (ThalDex). All the patients were given in addition the best available supportive care. Survival data could be obtained from 38 patients.

Results

GSTM1 genotype frequencies were similar among cases and controls. There were 48.0 % positive genotypes, i.e. homozygous or heterozygous carriers of the gene, and 52.0 % null genotypes (homozygous gene deletion) in the group of MM patients, and 51.5 % positive and 48.5 % null genotypes among controls, respectively. There was no statistically significant difference between cases and controls for the *GSTT1* polymorphism either, 73.0 % positive and 27.0 % null genotypes among cases, whereas 74.7 % positive and 25.3% null among controls. There was no significant difference in the genotype frequency of *GSTP1* Ile105Val between the cases and the controls [Table 1]. The frequencies of the combined *GST* genotypes were similar in cases and controls [Table 2]. A notable exception was, however, that there was no homozygous variant *GSTP1* 105Val/105Valgenotype in the subgroup of patients with *GSTT1* homozygous deletion (null) genotype.

For the combined *GSTT1/GSTM1* genotypes, the shortest survival was observed in the *GSTT1* null /*GSTM1* null genotype group of patients. There was a great difference in survival between the subgroups of cases with *GSTP1* 105Val/105Val genotypes as compared to *GSTP1* 105 Ile allele carriers. The homozygous variant genotypes had 41 to 49 months shorter mean survival time. Those patients had the longest survival time who carried both *GSTM1* gene and *GSTT1* gene and had wildtype homozygous Ile/Ile genotype for the *GSTP1*. These results, however, were not statistically significant [Table 3].

Discussion

This is the first report on metabolising enzyme gene polymorphism study with respect to multiple myeloma in Hungarian patients. In this study population, the investigated genetic polymorphisms had similar frequencies as in the age-matched controls.

Table 1. Frequency of *GSTM1*, *GSTT1* and *GSTP1* Ile105Val genotypes in a Hungarian study population of MM patients (n=100) and controls (n=99)

	Frequency of Genotypes			
	MM Cases (n=100)		Controls (n=99)	
	Number	%	Number	%
GSTM1 Positive	48	48.0	51	51.5
GSTM1 Null	52	52.0	48	48.5
GSTT1 Positive	73	73.0	74	74.7
GSTT1 Null	27	27.0	25	25.3
GSTP1 Ile/Ile	50	50.0	49	49.5
GSTP1 Ile/Val	44	44.0	42	42.4
GSTP1 Val/Val	6	6.0	8	8.1

The genotype frequency distributions between case and control subgroups were not statistically significantly different, p>0.1

Table 2. Frequency of combined *GSTM1*, *GSTT1* and *GSTP1* Ile105Val genotypes in a Hungarian study population of MM patients (Total number=100) and controls (Total number=99)

Combined genotype	Cases in subgroups		Controls in subgroups	
	N	n - % of N	N	n - % of N
GSTM1 and GSTT1				
GSTM1 + / GSTT1 +	48	34 - 70.8	51	39 - 76.5
GSTM1 + / GSTT1 -		14 - 29.2		12 - 23.5
GSTM1 - / GSTT1 +	52	39 - 75.0	48	35 - 72.9
GSTM1 - / GSTT1 -		13 - 25.0		13 - 27.1
GSTT1 and GSTP1 Ile105Val				
GSTT1 + / GSTP1 Ile/Ile		35 - 47.9		37 - 50.0
GSTT1 + / GSTP1 Ile/Val	73	32 - 43.8	74	32 - 43.2
GSTT1 + / GSTP1 Val/Val		6 - 8.2		5 - 6.8
GSTT1 - / GSTP1 Ile/Ile		15 - 55.6		12 - 48.0
GSTT1 - / GSTP1 Ile/Val	27	12 - 44.4	25	10 - 40.0
GSTT1 - / GSTP1 Val/Val		0 - 0		3 - 12.0
GSTM1 and GSTP1 Ile105Val				
GSTM1 + / GSTP1 Ile/Ile		22 - 45.8		26 - 51.0
GSTM1 + / GSTP1 Ile/Val	48	24 - 50.0	51	21 - 41.2
GSTM1 + / GSTP1 Val/Val		2 - 4.2		4 - 7.9
GSTM1 - / GSTP1 Ile/Ile		28 - 53.8		23 - 47.9
GSTM1 - / GSTP1 Ile/Val	52	20 - 38.5	48	21 - 43.8
GSTM1 - / GSTP1 Val/Val		4 - 7.7		4 - 8.3
GSTM1 and GSTT1 and GSTP1 Ile105Val				
GSTM1 + / GSTT1 + / GSTP1 Ile/Ile	34	16 - 47.1	39	20 - 51.3
GSTM1 + / GSTT1 + / GSTP1 Ile/Val		16 - 47.1		16 - 41.0
GSTM1 + / GSTT1 + / GSTP1 Val/Val		2 - 5.9		3 - 7.7
GSTM1 - / GSTT1 - / GSTP1 Ile/Ile	13	9 - 69.2	13	6 - 46.2
GSTM1 - / GSTT1 - / GSTP1 Ile/Val		4 - 30.8		5 - 38.5
GSTM1 - / GSTT1 - / GSTP1 Val/Val		0 - 0		2 - 15.4

The genotype frequency distributions between case and control subgroups were not statistically significantly different, p>0.1.

Table 3. *GSTP1* Ile105Val, and combined *GSTM1*/*GSTT1* genotypes and survival in a Hungarian study population of multiple myeloma patients

Survival Months	*GSTP1* Ile/Ile (n=18)	*GSTP1* Ile/Val (n=17)	*GSTP1* Val/Val (n=3)	*GSTM1+/ GSTT1+* [a] (n=15)	*GSTM1+/ GSTT1-* [b] or *GSTM1-/ GSTT1+* (n=20)	*GSTM1- / GSTT1-* (n=3)	*GSTM1+/ GSTT1+/ GSTP1* Ile/Ile (n=7)
Mean±SD	61±44	69±50	20±18	54±51	56±45	45±36	86±54
Median	49	37	49	37	44	25	76
Range	1-168	1-202	2-38	1-168	1-202	24-87	27-168

[a] +, homozygous or heterozygous gene carrier (positive genotype); [b] -, homozygous deletion (null genotype).

The differences of survival time between the specified subgroups of patients were not statistically different, p>0.1.

The only notable exception was that in the subgroup of patients with *GSTT1* deletion (null genotype), there was no homozygous variant of *GSTP1* 105Val/105Val genotype. Our results suggest that *GSTM1*, *GSTT1* and *GSTP1* Ile105Val genetic polymorphisms may not influence significantly the risk for MM in the Hungarian population. Literature data are controversial, both positive and negative findings have been reported [14, 18]. There is not either any known agent-specific myeloma-inducing factor or known single genetic polymorphism identified so far that predispose to the development of MM.

In our study, when survival data were compared on the basis of genotypes, the shortest survival was observed in the *GSTP1* 105Val/105Val genotype group of patients. On the contrary, those patients who carried both *GSTM1* gene and *GSTT1* gene and had wildtype homozygous Ile/Ile genotype for the *GSTP1* had the longest survival time. [Table 3]. This finding, although, statistically not significant, calls the attention to the possible differences in response to the therapy which may originate from metabolism polymorphism, which therefore should be further analysed. Recent studies in the literature have revealed interindividual differences to cancer susceptibility and differences in therapeutic response regarding to particular enzyme polymorphisms [19, 20, 21, 22, 23]. Dasgupta et al. investigated the impact of *GSTP1* codon 105 polymorphism in myeloma by comparing the therapeutical effect of standard dose with high-dose melphalan therapy [23]. In the standard dose arm, patients with the variant 105Val allele had an improved progression-free survival (PFS) compared to the 105Ile homozygotes (adjusted hazard ratios for PFS were 0.55 for heterozygotes and 0.52 for 105Val homozygotes, P for trend = 0.04). This had been coupled by a trend of improved overall survival with a greater likelihood of entering plateau and shorter time to reach plateau in patients with the 105Val allele. In this study the genotype had not any impact on the outcome for patients treated with high-dose therapy. However progression-free survival advantage of the high-dose arm was seen only in patients homozygous for 105Ile (P = 0.008). Large-scale international molecular epidemiological studies would be desirable to have sufficient size of study population to estimate how and in what extent *GST* polymorphisms contribute to the development of MM, influence therapeutic effect and survival.

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Part III: Glutathione in Plants

In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter XIII

Glutathione/Ascorbate Cycle and Its Interrelation with Lipoic Acid: Could It Explain Why Plants Do Not Get Cancer?

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Abstract

Human and plant cells are distinguished, among others, by the following features: (1) plant cells contain, besides mitochondria, the chloroplast, an important additional source of activated oxygen species (AOS) formation due to the presence of electron transport chains; (2) following the exposure to carcinogenic stressors such as cadmium human cells respond by massive cell proliferation, whereas plant cells do not undergo neoplastic transformation. So, what is the different resistance to neoplastic transformation due to? Whereas human cell growth programs are kept under constant environmental conditions and constant redox states, plant cell growth programs are developed under a context of ever-changing environmental conditions that can potentially cause large swings in the redox state. Thus, in plants, cellular redox homeostasis is a strictly controlled buffering mechanism that prevents excessive reduction or oxidation. How can this redox homeostasis be controlled? The presence in plants of the glutathione/ascorbate cycle could make the difference. The specific plant enzymes ascorbate peroxidase, mono-dehydroascorbate reductase and dehydroascorbate reductase, involved in the cycle, allow a continuous regeneration of ascorbate (vitamin C) and α-tocopherol (vitamin E). In this way, plants need lower amounts of lipoic acid than mammals to allow the antioxidant network working efficiently, even in stressful conditions. Mammals, instead, need

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vitamin intake and lipoic acid supplementation to get some health benefits such as against cancer.

Abbreviations

AOS	activated oxygen species;
APX	ascorbate peroxidase;
ASA	ascorbic acid;
CAT	catalase;
DHA	dehydroascorbic acid;
DHAR	dehydroascorbate reductase;
DHLA	dihydrolipoic acid;
EPR	electron paramagnetic resonance;
GPX	glutathione peroxidase;
GR	glutathione reductase;
GSH	reduced glutathione;
GSSG	oxidized glutathione;
GST	glutathione transferase;
H_2O_2	hydrogen peroxide;
LA	lipoic acid;
MDA	monodehydroascorbate radical;
MDHAR	monodehydroascorbate reductase;
MMP	matrix metalloproteinase;
$O_2^{\cdot-}$	superoxide radical;
$^{\cdot}OH$	hydroxyl radical;
$^1O_2$	singlet oxygen;
PSI	photosystem I;
PSII	photosystem II;
SOD	superoxide dismutase;
$TO^{\cdot}$	tocopheroxyl radical

1. Introduction

It is known that mammalian cells exposed to carcinogenic stressors such as cadmium respond by massive cell proliferation [1]. Cadmium is also toxic for plants but they are usually resistant to neoplastic transformation, and plant-specific tumors arise only as result of an interaction with pathogens or gall-forming insects [1]. Plants, in contrast with mammals, are subjected to drastic environmental changes. They include freezing, dehydration, soil salinization, excess light, UV radiation etc. Whereas human cells are kept constantly to optimum temperature and pH, and osmotic fluxes are minimized, in the plant kingdom we can find “resurrection” plants, which can experience drought for long periods and completely recover when water is available again [2]. An important factor involved in neoplastic transformation is oxidative stress and all environmental changes which affect plants are

initially generated by activated oxygen species (AOS) which, eventually, lead to lipid peroxidation, oxidation of sulphydryl groups as well as DNA and membrane damages [3]. Moreover, plant cells contain, besides the mitochondrion, the chloroplast, both of them powerful sources of AOS as they possess electron transport chains. What is the different resistance to neoplastic transformation due to? From just only few decades it is known that chloroplasts defend themselves from AOS generation by the glutathione/ascorbate cycle which is involved in the detoxification of hydrogen peroxide [4]. The glutathione/ascorbate cycle was also discovered in mitochondria, peroxisomes and in the cytosol of plant cells [5-7] and only lately it has been hypothesized its connection with dihydrolipoic acid, which is involved in ascorbate and glutathione regeneration [8]. Lipoic acid is called “the universal antioxidant” because of its solubility in aqueous and lipid phases; it is ubiquitous in all eukaryotes being the cofactor of pyruvate and α-ketoglutarate dehydrogenese, even if only some plant organs like potatoes are particularly rich in this antioxidant [9].

Lipoic acid is unique among antioxidant molecules in that it retains protective functions in both its reduced (dihydrolipoic acid, DHLA) and oxidized (lipoic acid, LA) form, although DHLA is a more effective antioxidant [9]. In fact, DHLA acts directly by destroying AOS such as superoxide, hydroperoxyl and hydroxyl radicals. Moreover, DHLA can donate an electron to the oxidized forms of glutathione and ascorbate. Because of its solubility in both water and lipids, DHLA connects in plants the activity of antioxidants in the cell membrane (α-tocopherol) with that of antioxidants in the cytoplasm (ascorbate and glutathione), strengthening the antioxidant network. Lipoic acid has many benefits also for humans. Indeed, beneficial effects of the intake of LA has been shown, in particular, for individuals affected by diabetes and cataracts, with high cholesterol levels and with cardiovascular problems [9]. The question is: could it work also for cancer therapy?

2. Cancer and Oxidative Stress

Oxidative stress is involved in many aspects of neoplastic transformation. Indeed, the exposure of mouse fibroblasts to AOS led to their transformation [10-11]. This could be due to the fact the AOS may have a pro-cancer action promoting proliferation, invasiveness, angiogenesis, metastasis, and suppressing apoptosis [3]. Moreover, during inflammation there is an increased production of AOS which also likely bring to cancer.

2.1. Generation of Activated Oxygen Species (AOS)

Although oxygen is essential for life it can give rise to activated oxygen species (AOS), toxic for the cell. Two are the main mechanisms involved in AOS formation. The reduction of oxygen to form superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) is the principle mechanism of oxygen activation in most biological systems. However, in photosynthetic plants, the formation of singlet oxygen ($^1O_2$) by the photosystems is also of importance.

Activated oxygen is often formed as a component of metabolism to enable "complex" chemical reactions such as the oxidation of xenobiotics or the polymerization of lignin but, in

other instances, activated oxygen is formed by the dysfunctioning of enzymes or electron transport systems, as a result of perturbations in the metabolism caused by chemical or environmental stresses. Main sources of AOS can be the electron transport chains as those we can find in mitochondria or in chloroplasts, as it is in the case of plants [12]. The univalent reduction of oxygen brings to the formation of $O_2^{\cdot-}$, from which the other AOS involved in the progression of oxidative stress are generated (Figure 1). Indeed, subsequent univalent reduction of $O_2^{\cdot-}$ brings to the formation of H_2O_2 and $^{\cdot}OH$. In particular, the chloroplast is the main organelle responsible for the production of AOS. Besides the possibility of electron leakage from chlorophyll *a* of the reaction centers of photosystem II (PSII) and photosystem I (PSI) [12-13], chloroplast could be responsible for the generation of $^1O_2$ following the reversal of the electron spin of oxygen induced by energy transfer from excited chlorophyll (Figure 1).

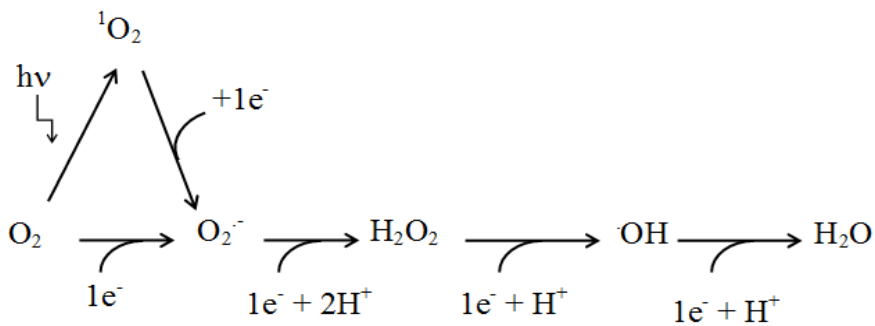


Figure 1. Singlet oxygen formation by illuminated chlorophyll and univalent reductive chain reactions of oxygen. $O_2^{\cdot-}$, superoxide radical; H_2O_2, hydrogen peroxide; $^{\cdot}OH$, hydroxyl radical.

The presence of metals could also bring to the generation of AOS. The most studied metals involved in the reaction are iron [14], copper [15], cadmium [16] and mercury [17]. In the case of iron, the reactions (1) and (2) bring to (3) as schematized below:

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2 \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \quad (2)$$

$$O_2^{\bullet-} + H_2O_2 \xrightarrow[Fe^{3+}]{} OH^- + {}^{\bullet}OH + O_2 \quad (3)$$

The result of the so called “Fenton reaction” is the production of $^{\cdot}OH$, the agent of lipid peroxidation, dangerous for the cell. Fatty acids in the membranes are subjected to the abstraction of hydrogen by hydroxyl radical giving rise to peroxyl radicals and, eventually, to lipid hydroperoxides (Figure 2). The lipid bilayer of membranes is composed of a mixture of phospholipids and glycolipids that have fatty acid chains attached to carbon 1 and 2 of the glycerol backbone by an ester linkage. The peroxidation reactions differ among these fatty acids depending on the number and position of the double bonds on the acyl chain [18].

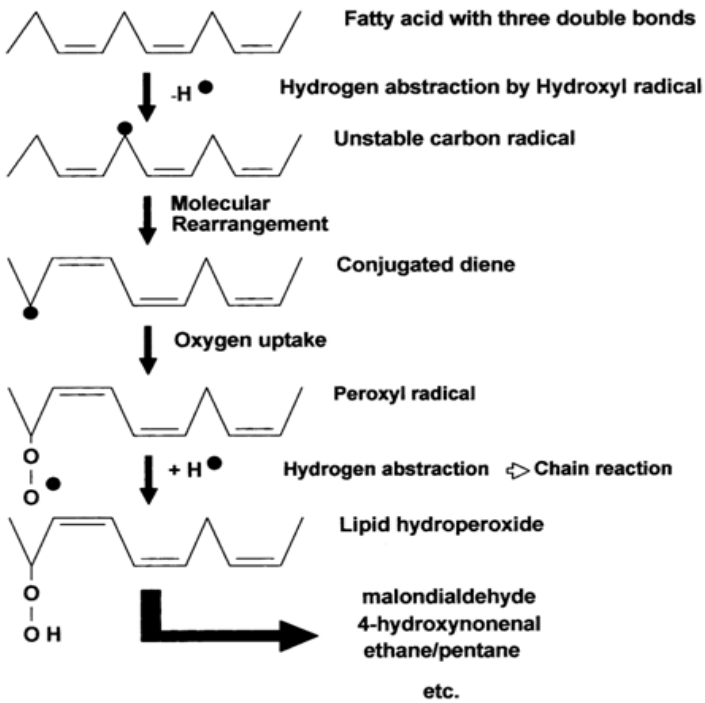


Figure 2. Byproducts of lipid peroxidation following abstraction of hydrogen by hydroxyl radical.

The mechanism of lipid peroxidation can be distinguished in three steps: initiation, peroxidation and termination. The initiation reaction between an unsaturated fatty acid (e.g. linoleate) and the $^{\cdot}$OH radical involves the abstraction of an H atom from the methylvinyl group on the fatty acid; in the case of linoleate this occurs at carbon-11. The remaining carbon-centered radical, forms a resonance structure sharing this unpaired electron among carbons-9 to -13.

In the peroxidation reactions, this resonance structure reacts with triplet oxygen which, being a biradical with two unpaired electrons, reacts readily with other radicals. This reaction forms a peroxyl radical. In the case of linoleate, addition occurs at either carbon-9 or -13. The peroxyl radical then abstracts an H atom from a second fatty acid forming a lipid hydroperoxide and leaving another carbon centered free radical that can participate in a second H abstraction. Therefore, once one $^{\cdot}$OH initiates the peroxidation reaction by abstracting a single H atom, it creates a carbon radical product that is capable of reacting with ground state oxygen in a chain reaction.

The peroxidation reactions in membrane lipids are terminated when the carbon or peroxy radicals cross-link to form conjugated products that are not radicals. Chain breakage and cross-linkage reactions thus occur to produce aldehydes, hydrocarbons, alcohols and cross-linked dimers (Figure 2).

2.2. Involvement of Electron Transport Chains in AOS Production

Main sources of AOS production in eukaryotic cells are represented by mitochondria and chloroplasts, these last as far as concern the plant cell. Responsible is the leakage of electrons

from electron transport chains. Different sites are recognized as responsible for AOS production and the chloroplast represents for plant cell an important additional source for AOS increase compared to mammalian cells. Mitochondria give rise to $O_2^{\cdot -}$ production mainly by complex I. Most oxygen is consumed by the cytochrome oxidase enzyme in the mitochondrial electron transport system, and involves the sequential transfer of four electrons to oxygen, releasing water. When there is a high $NADH/NAD^+$ ratio in the mitochondrial matrix or a reduced production of ATP, due to a higher proton motive force and a more reduced coenzyme Q, there is a univalent reduction of oxygen, which gives rise, eventually, to H_2O_2 by dismutation of superoxide by a MnSOD (superoxide dismutase).

Plant mitochondria have an additional site of oxygen reduction at the alternative oxidase, distinguished from cytochrome oxidase by its resistance to cyanide (Figure 3). It has been demonstrated that isolated mitochondria produce H_2O_2 and $O_2^{\cdot -}$ in the presence of NADH [19-20] and that Antimycin A, which blocks electron flow after ubiquinone, enhances oxygen reduction. Presumably other conditions, which also increase the reduction of ubiquinone, favor the reduction of oxygen in the ubiquinone cytochrome b region of the chain [21]. Various Fe-S proteins and NADH dehydrogenase have also been implicated as possible sites of $O_2^{\cdot -}$ and H_2O_2 formation [22].

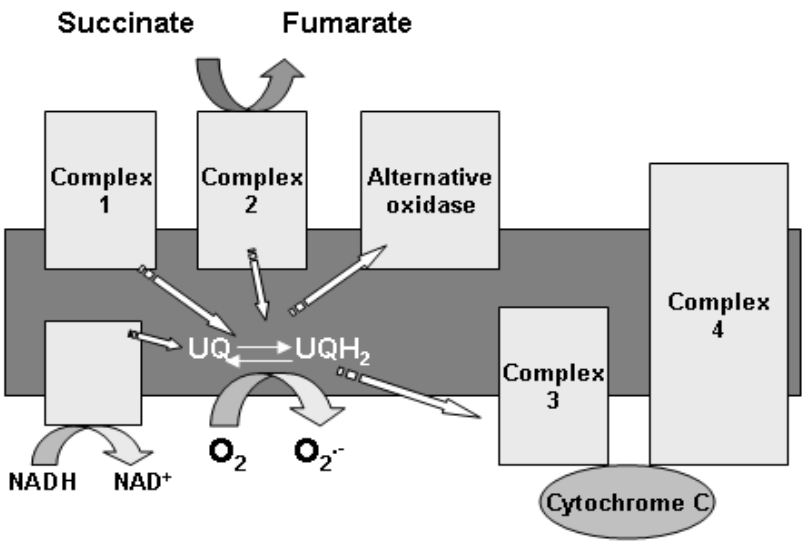


Figure 3. Possible sites of $O_2^{\cdot -}$ production by reduced ubiquinones from electron transport system of the plant mitochondrial membrane.

A lot of oxidative processes including oxidation, hydroxylation, dealkylation, deamination, dehalogenation and desaturation, occur on the smooth endoplasmic reticulum. Mixed function oxygenases, containing a heme moiety, add an oxygen atom into an organic substrate using NAD(P)H as the electron donor. $O_2^{\cdot -}$ is produced by microsomal NAD(P)H dependent electron transport involving cytochrome P_{450} [23]. One possible site at which this may occur is shown in Figure 4. After the univalent reduction of the substrate (RH) and the addition of triplet oxygen to form the P_{450} – RHOO complex, this latter may decompose to P_{450}-RH and release $O_2^{\cdot -}$.

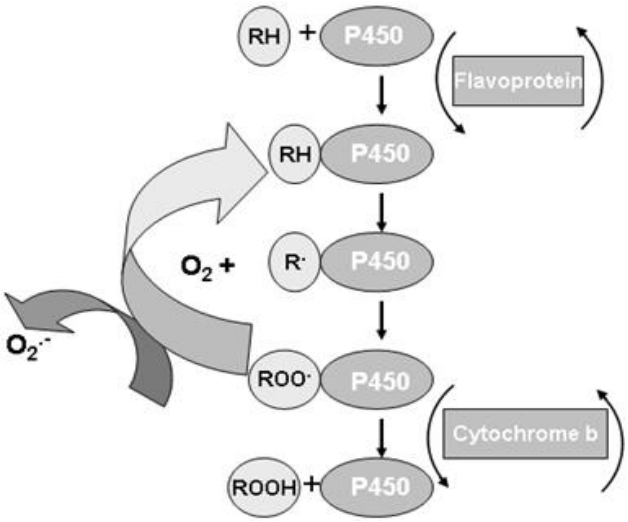


Figure 4. Cytochrome P_{450} electron transport system of the endoplasmic reticulum showing a possible site of $O_2^{\cdot-}$ production.

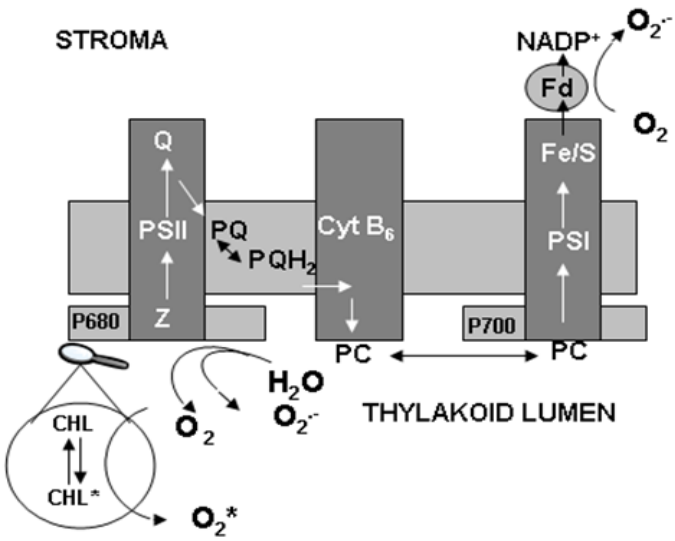


Figure 5. Sites of AOS production in thylakoid membranes of plants.

Although, for several years, it has been generally accepted that the major site of $O_2^{\cdot-}$ production in chloroplasts was the donor side of PSI with the Mehler reaction, Navari-Izzo et al. [13] demonstrated, in agreement with the theoretical analysis of Elstner [24], that also PSII can release $O_2^{\cdot-}$. Thus, there are at least three sites within the chloroplast that can activate oxygen (Figure 5). With the Mehler reaction, the reducing side of PSI is thought to contribute significantly to the monovalent reduction of oxygen under conditions where $NADP^+$ is limiting [12]. This would occur, for example, when the Calvin cycle does not oxidize NADPH as rapidly as PSI supplies electrons. Photoactivated chlorophyll normally transfers its excitation energy to the PS reaction centers, but under conditions that prevent the captured light energy from being utilized in the electron transport systems, this energy can excite

oxygen from the triplet to singlet form. These conditions include stomatal closure caused by drought, damage to the membrane transport systems, lack of specific nutrients, or the presence of xenobiotics such as pollutants or herbicides. The oxidizing side of PSII facilitates four single electron transfers from water to the PSII reaction centre releasing triplet or ground-state oxygen. Leakage of electrons from this site to molecular oxygen, or release of partially reduced oxygen products are thought to give a relatively small contribution to activated oxygen production, but nonetheless, it has been demonstrated that certain alcohols can be reduced by PSII.

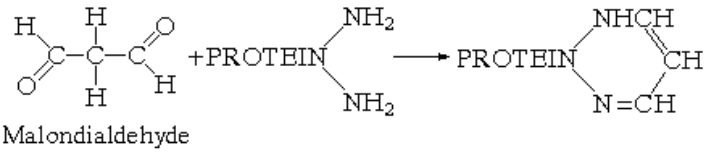
2.3. Damage to Cells

AOS cause oxidative stress, damaging cell. The targets of oxidation are represented by membrane lipids, proteins and DNA. Thus, free radicals ($R^{\cdot}$) as molecules which contain an unpaired electron are some of the most chemically reactive molecules known. Because of the need to pair its single electron, a free radical must abstract a second electron from a neighboring molecule. This cause the formation of another free radical giving rise to a self-propagating chain reaction. Free radicals in human body can arise from fatty food, smoking, alcohol, pollutants, ozone, toxins, ionization, etc. Although free radicals are unavoidable byproducts of living system, being formed by the breakdown of compounds through the process of metabolism, under certain conditions their formation overcomes their degradation bringing to cell damage [25].

Lipid peroxidation (Figure 2) alters structure and functionality of the membranes. Alterations in the unsaturation of fatty acid acyl chains change fluidity of membranes [26] affecting permeability and transport. Indeed, a suitable membrane function requires mobility of protein components and redox carrier, and this mobility would be restricted by changes in the motion of lipid acyl chains. Thus, changes in the physical properties of bulk membrane lipids can alter the behavior of integral membrane proteins affecting activities of some membrane enzymes [27]. In this way the damaged membranes lose their ability to transport oxygen, nutrients or water to the cell. This kind of alterations can be evidenced adopting Electron Paramagnetic Resonance (EPR) technique which employs suitable spin labels. Indeed, the EPR spectra reflect microscopic properties that may be related to other macroscopic properties [27]. Moreover, damages to lysosomal membranes bring to the leakage of degenerative enzymes which start the digestion of the cell itself, spreading thereafter to nearby cell and causing a destructive chain reaction which, eventually, lower the immune system resistance [25].

Oxidation of amino acids by Fenton systems (reactions 1-3) and the consequent protein aggregation and fragmentation are well-known phenomena. During oxidation amino acids can give rise to carbonyls such as oxo acids and aldehydes with the same or less carbon atom than the parent amino acid. For example, glycine can give rise to glyoxal and glyoxylic acid or formaldehyde and formic acid. This general scheme is valid for most of amino acids including the aromatic ones [28]. In particular, for tyrosine, when reductants such as thiols or vitamin E (tocopherols) are absent, we assist to the formation of phenoxyl radicals and their conversion into dityrosine and further products. Hydroxylation of phenylalanine, tyrosine and tryptophan is also a characteristic reaction of hydroxyl radicals [25].

Proteins could be also damaged by lipid derived species (lipid radicals). Indeed, end-products of lipid peroxidation such as malondialdehyde and 4-hydroxynonenal are inactivating agents, possibly via Schiff base formation. For example, malondyaldeyde from lipid peroxidation reacts with proteins bringing to protein byproducts as schematized in reaction (4).



(4)

Thus, in systems that contain lipid hydroperoxides and soluble proteins, metal ion-catalyzed reactions appear to be central. In fact, metal ion-catalyzed damage to membranes affects at the same time lipids and proteins even if it can be restricted by α-tocopherol.

Free radicals also attack the nucleic acid, whose action is to regulate the normal cell function and growth, repairing the damaged tissues. Thus, damage to chromosomes might initiate the growth of abnormal cells, which is the first step in cancer development. The chemistry of oxidation of DNA is quite complex and it is extensively reported in Cooke et al. [29].

In particular, the highly reactive hydroxyl radical ($^{\cdot}OH$) reacts with DNA by addition to double bonds of DNA bases and by abstraction of an H atom from the methyl group of thymine and from each of the C-H bonds of 2'-deoxyribose. Its addition to double bonds of DNA bases occurs with rate constants of 3-10 x 10^9 M^{-1} s^{-1}, whereas the rate constant of H abstraction amounts to 2 x 10^9 M^{-1} s^{-1} [29].

Addition to the C5-C6 double bond of pyrimidines leads to C5-OH and C6-OH adduct radicals, and H atom abstraction from thymine results in the allyl radical. Adduct radicals differ in terms of their redox properties, with C5-OH adduct radicals being reducing and C6-OH adduct radicals oxidizing [29]. In the absence of oxygen, the oxidation of C5-OH adduct radicals followed by addition of OH^-, leads to cytosine glycol and thyamine glycol. The allyl radical yields 5-hydroxymethyl uracil. Products of cytosine may deaminate and dehydrate. C6-OH adduct radicals of pyrimidines may lead to 6-hydroxy-5-hydropyrimidines in anoxic conditions because oxygen inhibits their formation by reacting with OH adduct radicals, whereas pyrimidine glycols and 5-hydroxymethyluracil are formed under both oxic and anoxic conditions.

In the case of purines, hydroxyl radical adds to the C4, C5 and C8 positions generating OH adduct radicals. From adenine at least two OH adducts are formed: the C4-OH and C8-OH adduct radicals.

C4-OH adduct radicals possess oxidizing properties whereas C5-OH and C8-OH adduct radicals are primarily reductants even if different mesomeric structures of these radicals may be oxidizing or reducing, a phenomenon called "redox ambivalence". Oxidative mechanisms have been demonstrated to possess a potential role in the initiation, promotion and malignant conversion (progression) stages of carcinogenesis and, given that cumulative cancer risk increases with the fourth power of age and it is associated with an accumulation of DNA damage, oxidative DNA damage has been investigated in cancer [29].

3. Plants Do Not Get Cancer

In mammals extra cells are dangerous because they usually turn into tumors, but in plants any extra cells can simply be incorporated into normal tissues. In fact, plants are remarkably resistant to neoplastic transformation [30] and common plant tumors arise only as a result of complex interactions with specialized pathogens such as *Agrobacterium* or gall-forming insects [1]. It is also known that mammalian cells exposed to carcinogenic stressors such as cadmium respond by massive cell proliferation. Cadmium is also toxic for plants even if no neoplastic transformation occurs[1].

In the plant kingdom every environmental stress determines an oxidative stress and an oxidative burst is the response of the plant cell to each environmental change. It is generally accepted that the mechanism which underlies the response of the plant cell to each environmental stressor is the same. First, plants respond with an increased production of AOS which can determine a decrease or an increase of antioxidants and antioxidative enzymes.

The antioxidative response of the plant depends, first of all, on stress intensity. For example, when sunflower seedlings were subjected to mild drought antioxidative defenses showed a decrease, whereas following a moderate stress intensity they reacted increasing defenses and attenuating AOS production; eventually, when they were subjected to severe stress, a collapse of defenses and a stronger increase in AOS occurred [31-32]. The relationship between the antioxidative response and the stress intensity is very strict and should be taken always into account to correctly interpret the huge amount of data reported in the literature. In fact, many differences in the levels of antioxidants and in the activity of antioxidative enzymes are reported even when the same plant is subjected to the same kind of stress. Nevertheless, when different plants are subjected to different stressors ranging from metals to drought, from UV radiation to freezing, a different behavior can be expected. In fact, synthesis of antioxidants can be activated or depressed, and antioxidant enzyme activities can be induced or inhibited.

3.1. The Case of “Resurrection” Plants

An interesting group of plants is represented by “resurrection” plants which, after dehydration to extremely low moisture contents, can revive resuming completely all metabolic activities. Their cells are subjected to changes in osmotic pressure with alternate shrinkage and swelling. In these desiccation-tolerant species there are definite limits of tolerance beyond which tissue damage or death occurs. In *Boea hygroscopica* the resurrection process seems to be linked to the rapidity of the dehydration process. In fact, it has been observed that slowly dried leaves are able to survive air dryness in a physiological state called anabiosis where all metabolic activities are reduced to the minimum; on the contrary, rapidly dried leaves are not able to revive. In the resurrection of this plant, when slowly dehydrated, glutathione plays a major role. Since during dehydration metabolic activities are reduced, AOS production decrease but, at the same time, increases of two and 50-fold times can be observed in total ascorbate and glutathione contents, respectively. In particular, the recovery of the plant has been associated with the oxidation of reduced glutathione (GSH) [2]. Indeed, during rapid dehydration almost total glutathione remained in the reduced state and leaves

died. It seems that the oxidation of GSH with the production of GSSG (the oxidized form) was even more important during rehydration when damages should be repaired and AOS increase, as photosynthesis and all physiological processes resume. In fact, following rehydration oxidative processes were intensified with increases in the levels of H_2O_2 and oxidation of ascorbate (ASA) and GSH as well as with an induction of the GSH/ASA cycle [2]. Desiccation tolerance may depend on the plant's ability to process AOS, and this capacity to control the increase in the production of AOS by GSH may be one of the most important mechanisms in the resurrection of *B. hygroscopica*. Indeed, antioxidants such as ASA and GSH, which accumulate during drying, might constitute a reserve which allows *B. hygroscopica* to tolerate oxidative damage during desiccation and, above all, during rehydration when the injury caused by desiccation must be repaired.

3.2. More Tolerant Plants Activate More Defenses

Both ASA and GSH are water soluble antioxidants involved in the removal of H_2O_2 through the enzymic NADPH/GSH/ASA cycle. ASA may also directly reduce $O_2^{\cdot -}$, quench $^1O_2$ and regenerate reduced α-tocopherol. At this regard, Chowdhury and Choudhuri [33] found a correlation between H_2O_2 metabolism and water stress tolerance of jute plants. They observed higher activities of catalase (CAT) and superoxide dismutase (SOD) and lower H_2O_2 contents in *Corchorus capsularis* than in *Corchorus olitorius*, a jute more sensitive to water stress in comparison with the former.

In the same way, the difference in drought susceptibility of two wheat cultivars (cv. Adamello, drought-sensitive; cv. Ofanto, drought-tolerant) was related to the higher $O_2^{\cdot -}$ production by illuminated thylakoids in the sensitive wheat. Changes in the lipid composition of thylakoid membranes as well as oxidation of sulphydryl groups could be related with the fact that antioxidants (carotenoids and tocopherols), more present in the thylakoid membranes of the drought-tolerant cultivar, might have scavenged more efficiently $O_2^{\cdot -}$ and limited its level under stress [34]. In fact, water stress conditions may trigger an increased formation of $O_2^{\cdot -}$ and H_2O_2, which can directly attack membrane lipids and inactivate SH-containing enzymes. This formation is a consequence of the Mehler reaction, which provides a pathway for the removal of excess electrochemical energy determined by drought stress [35]. When cv. Ofanto was subjected to a water deficit program, with two periods of stress of the same intensity followed by re-watering, the first period was characterized by an increased $O_2^{\cdot -}$ production whereas the second one by a decrease [35]. However, notwithstanding after the first period thylakoid membranes showed a higher capacity to leak electrons towards oxygen, an accumulation of H_2O_2 did not occur. Indeed, the enzyme activities of ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) as well as the ASA/dehydroascorbate (DHA) ratio were maintained at the control level whereas the GSH/GSSG ratio showed increases due to the higher GSH levels. Therefore, the greatest portion of the glutathione in the drought-tolerant wheat was maintained in the reduced state, playing an important role in the stabilization of many enzymes and a more general role as an oxidant scavenger, due to the fact that it serves as a substrate for DHAR [4]. GSH is also able to react directly with free radicals, including hydroxyl radicals, so preventing the inactivation of enzymes by oxidation of essential thiol groups. A good functionality of the GSH/ASA

cycle could explain the drought-resistance, indicating the capacity of cv. Ofanto to withstand water depletion. Indeed, during the second period of stress, when a decreased superoxide production was monitored, glutathione and the GSH/GSSG ratio decreased as well as the activities of the enzymes involved in the GSH/ASA cycle [35].

In plant physiology literature it is generally accepted that in more tolerant plants antioxidative defenses, which metabolize AOS, are more induced. Taking a cue from this, geneticists have tried to transform plants by overexpressing genes which encode for detoxifying enzymes such as SOD or CAT. However, efforts were largely unsuccessful [36] because plants respond to stress with a complex machinery of antioxidants that cooperate with each other. We can say that tolerance to stress can be established when antioxidants and antioxidative enzymes work all together but, with the enrichment of just one molecule, the system fails.

3.3. Plants and Metals

Roots of plants, grown in hydroponics in the presence of excess transition metals such as copper (Cu), are affected by a higher stress intensity than leaves [37]. In fact, roots are in direct contact with the toxic metal whereas in leaves the concentration of the metal depends on the translocation rate from the root to the shoot, which can increase even more the difference in metal concentration between the two organs. In wheat plants treated with 150 μM Cu for 168 h the metal content of leaves remained at the same level of the control (15.1 μg g^{-1} dry weight) during the whole treatment, whereas in roots it continuously increased to a value 100-fold higher (2.9 mg g^{-1} dry weight) than the control (25.9 μg g^{-1} dry weight) [37]. As a consequence, roots showed a decrease in NADPH contents as well as an oxidation of GSH and ASA. Notwithstanding roots suffer more oxidative stress than leaves, neither roots nor leaves undergo neoplastic transformation. To counteract AOS formation, likely due to the "Fenton reaction", root cells oxidized antioxidants such as GSH and ASA and utilized NADPH in the GSH/ASA cycle, which was activated during the treatment [37]. Utilization of GSH during copper treatment was also shown in *Raphanus sativus* grown for 10 days in hydroponic culture containing 0.12 (control), 5, 10 and 15 μM copper. Since *R. sativus* is a plant endowed with a good translocation system of the metal from roots to shoots, a 5-fold accumulation of Cu in the leaves of the plants treated with the highest dose occurred. An utilization of GSH against oxidative stress was thus observed also for leaves [38].

Cu is an essential micronutrient for plants and plays a pivotal role in a wide range of biochemical and physiological processes [39]. However, when present at high levels, it becomes strongly phytotoxic and causes inhibition of plant growth [40]. Reduction in root growth is the first response of the plant to the presence of heavy metals, and exclusion processes from roots might represent a first line of defense. When roots accumulate heavy metals, these can be [38] or not [37] translocated to shoots. Even in the case they are not translocated to the leaves, there are a lot of physiological processes, among which photosynthesis [41], that can be influenced, and intracellular signaling mechanisms may occur. Among the molecules involved in signal transduction during oxidative stress we can find AOS [42], ASA and GSH [43]. It is widely accepted that redox signals are key regulators of plant metabolism, morphology and development, and it may even happen that all intermediates and possible other systems of signal transduction arose around this central core

[43]. A dual signaling mechanism involving GSH may be envisaged. On the one hand GSH and GSH/GSSG ratio may operate as an intracellular signaling mechanism for the onset of DNA replication (inward signaling) in response to stress, and on the other hand GSH level may be involved in transport of signals out of the cell (outward signaling). Since GSH is transported throughout the plant, possibilities also exist for long-range signaling via GSH [44]. To evidence a signaling mechanism by GSH it is necessary to monitor an early effect of the metal in a short-term treatment [42]. In *Brassica napus* grown in the presence of 100 μM Cu [45] the decrease in roots of both GSH+GSSG and GSH amounts, monitored within 10 min from the start of the treatment, was in part attributed to the translocation of GSH to leaves. The increase in leaves of GR activity could thus be interpreted as a response of the plant which precedes leaf Cu accumulation. Moreover, it was supposed that a root to shoot signal, through the production of lipid signals at the root level, might be involved in the response of *Brassica napus* to oxidative stress, making the leaf sensitive to stress before translocation of the metal [45].

Among plants which can translocate metals to shoots in huge amounts, there is a group of plants called hyperaccumulators. A group of approximately 400 species that can both tolerate and accumulate high levels of metals in the shoot has been identified. These plants exhibit very high metal concentrations in their above-ground tissues, in some cases containing the metals at a level 1,000 times higher than the soil in which they grow [46]. For this property, hyperaccumulators have been used as a remediation technique for soils polluted by metals. Evidences that antioxidative systems are involved also in the tolerance mechanisms of hyperaccumulators are present. The higher activity measured for both SOD and GR in *Alyssum argenteum*, the hyperaccumulator species, compared with *A. maritimum*, the non-hyperaccumulator one, could explain *A. argenteum* better resistance to high Cd concentrations [46]. Transformed *Brassica juncea* overexpressing either glutathione synthetase or γ-glutamyl cysteine synthetase increased the biosynthesis of GSH and phytochelatins, more than non-transformed plants, and in turn enhanced Cd tolerance and accumulation [46]. In fact, varying responses are also likely related to concentration of sulphydryl (-SH) groups present in the plants or induced by metal treatment. GSH, besides being itself an antioxidant and a metal chelator, is also a precursor for the synthesis of metal-binding phytochelatins. In addition, the oxidation of GSH in response to oxidative damage is also important for protection of plasma membrane from lipid peroxidation. [47]. In various *Thlaspi* species, the ability to hyperaccumulate Ni has been related to high GSH levels in shoot tissues, driven by constitutive activation of enzymes of sulfur assimilation pathway and GSH metabolism [48]. This enhanced GSH synthesis suggests an involvement of GSH antioxidant activity in the hyperaccumulation phenomenon.

4. Antioxidant Defenses and Re-Generation of α-Tocopherol in Plants

4.1. Vitamin E

Tocopherols (vitamin E), specifically α-tocopherol, have been studied as membrane stabilizers and multifaceted antioxidants that scavenge oxygen free radicals, lipid peroxy

radicals, and $^1O_2$ [49]. Tocopherols are actually a family of antioxidants [50] that includes four tocols with a phytyl chain and analogous tocotrienols with a geranylgeranyl chain. α-Tocopherol is generally considered to be the most active form of the tocols whereas the other forms may be biosynthetic precursors. The relative synthesis of the tocols and tocotrienols may depend on the relative availability of phytyl and geranylgeranyl precursors. α-Tocopherol synthesis occurs in plastids with the aromatic ring formed by the shikimic acid pathway and the phytyl chain synthesized from geranylgeranyl pyrophosphate through the terpenoid pathway in the plastid envelope [51]. This pathway forms a common link among the synthesis of chlorophyll, tocopherol, and carotenoids.

The occurrence of tocopherols in plant tissues, and in the chloroplast in particular, is well documented. Among the many reviews, following an early report by Lichtenthaler [52], it was Munné-Bosch and Falk [53] who summarized the state of tocopherol physiology and molecular biology. Accordingly, α-tocopherol is found predominantly in leaves and γ-tocopherol in seeds, where overexpressed enzymes of tocopherol biosynthesis are localized [54-55]. Tocopherol appears to be present in all phototrophic organisms, except for some cyanobacteria. The antioxidative role of tocopherols in plants, as well as of vitamin E in humans, was recognized soon after its discovery [51, 56], but a precise and detailed functional description for tocopherol in plants was possible only very recently. Investigations of mutants and biochemical studies have specified its role in the antioxidant network of the chloroplast and as a $^1O_2$ scavenger in the PSII reaction centre. In fact, it is considered the major membrane-bound antioxidant employed by the cell [57], its main antioxidant function being the protection against lipid peroxidation [58].

During antioxidative reactions, α-tocopherol is converted to an α-tocopheroxyl radical by the donation of a labile hydrogen to a lipid or lipid peroxyl radical. The tocopheroxyl radical is stabilized by the fully substituted benzoquinone ring and, therefore, does not propagate the radical reaction. This is, as a matter of fact, a termination reaction making tocopherol an effective free radical trap. Since the active oxygen of α-tocopherol is located near the surface of the bilayer and because it readily diffuses laterally in the plane of the bilayer, tocopherol can react with peroxyl radicals formed in the bilayer as they diffuse to the aqueous phase. This position also allows the tocopheroxyl radical to be reduced to the original form by ASA and GSH in the aqueous phase [59].

4.2. Glutathione/Ascorbate (Asada-Halliwell-Foyer) Cycle

In the latest 1970s, Foyer and Halliwell [4] demonstrated that chloroplasts defend themselves from AOS generation by ASA and GSH (GSH/ASA cycle or Asada-Halliwell-Foyer cycle), being these molecules involved in the detoxification of H_2O_2. The scavenging of H_2O_2 by APX is the first step of the GSH/ASA cycle [4, 60] (Figure 6). APX activity was first detected in chloroplasts by Kelly and Latzko [61] in a soluble form and by Groden and Beck [62] in a thylakoid-bound form. A cytosolic isoform has then been found [63-64], as well as a form that is associated with the membranes of both mitochondria and leaf peroxisomes [7]. The isoforms differ in size, specificity for electron donor and sensitivity to inactivation. The chloroplast enzyme, which was found both in the stroma, as soluble form, and in thylakoids, as membrane-bound form, is rapidly inactivated if the concentration of ASA is below 20 μM [65]. In contrast, the non-chloroplastic isoforms are more stable in the

absence of ASA. In the case of APXs, two molecules of ASA are used as the preferred substrate. This activity yields to the monodehydroascorbate radical (MDA). Being a radical, MDA can be converted (reduced) to ASA by monodehydroascorbate reductase (MDHAR) [66] or disproportionated to ASA and DHA non-enzymatically. The reduction of DHA to ASA by the enzyme DHAR, at the expense of GSH, results in the production of GSSG. The final step of this cycle is the reduction of GSSG by GR using NADPH as electron donor. Hence, it can be concluded that ASA and GSH are not consumed and the net electron flow is from NADPH to H_2O_2 [67]. Notwithstanding the complete GSH/ASA cycle was first detected in chloroplasts, it was subsequently discovered in mitochondria, peroxisomes and in the cytosol [5-7]. The finding of the GSH/ASA cycle in almost all cellular compartments of the plant cell, as well as the high affinity of APX for H_2O_2, suggests that this cycle plays a crucial role in controlling the level of AOS in plants.

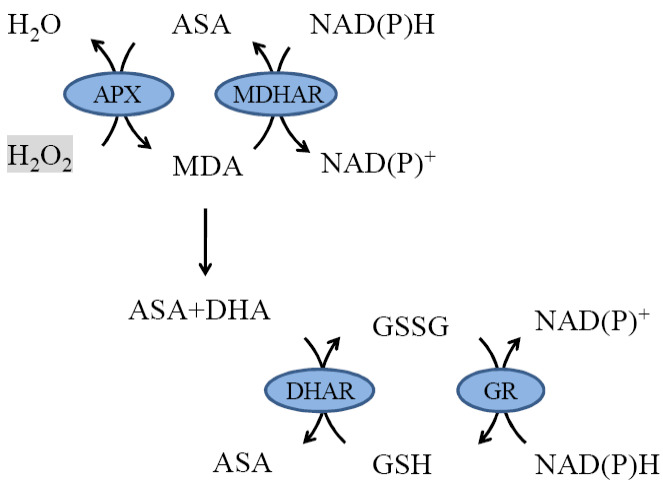


Figure 6. The glutathione/ascorbate cycle. ASA, ascorbate; APX, ascorbate peroxidase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, monodehydroascorbate radical; MDHAR, monorehydroascorbate reductase.

As a substrate of APX, ASA is an important and powerful antioxidant that works in aqueous environments of the plant cell. ASA is a major primary antioxidant [68] reacting directly with $^{\cdot}OH$, $O_2^{\cdot-}$, and $^1O_2$ [69]. In addition, ASA plays an important role in preserving the activities of enzymes that contain prosthetic transition metal ions [70]. ASA is also a powerful secondary antioxidant, reducing the oxidized form of α-tocopherol [70]. Regarding GSH, it has been demonstrated that it functions as an antioxidant in many ways. Besides regenerating ASA via reduction of DHA, it can react chemically with $^1O_2$ as well as with $O_2^{\cdot-}$ and tocopheroxyl radicals, functioning directly as a free radical scavenger [71-72]. Both ASA and GSH have the characteristics to be abundant, to be stable antioxidants, to possess appropriate redox potentials that make them able to interact with numerous cell components, and to be generally in the reduced state. In fact, in compartments where the pools are at or close to the thermodynamic equilibrium and assuming a NADP(H) redox potential of -300 mV or lower, glutathione is expected to be almost completely in the reduced form (GSH). Where they are present together with the enzymes MDHAR and DHAR, the reducing actions of both NAD(P)H and GSH should mean that oxidized ascorbate is present at only trace

concentrations under optimal conditions. Within this highly reducing intracellular context, even the most stable AOS, H_2O_2, has a relatively short lifetime [73]. AOS removal by the GSH/ASA cycle can cause transient or sustained adjustments in all or some of the components of this system. A key theme is that such adjustments have physiological significance,as they can be sensed and transduced to influence multiple signaling pathways. Accumulating evidences suggest that modifications in glutathione and/or in ascorbate status modulate the signaling cascade that governs genetically controlled suicide programs within the cell. Cytosolic APX has been reported as a key regulator of the Programme Cell Death [74] and the glutathione redox potential has been suggested to act as a key determinant of the plant cell death [75]. Thus, an increase in the glutathione redox potential above a threshold value would cause death and/or growth arrest.

4.3. Could the Glutathione/Ascorbate Cycle Be Improved by Lipoic Acid?

Recently, it has been hypothesized that the GSH/ASA cycle could be connected with DHLA, which is involved in ASA and GSH regeneration, reinforcing this cycle and strengthening the antioxidant network [8].

Lipoic acid, a widely occurring coenzyme found in prokariotic and eukariotic microorganisms [76] as well as in animals and plants [77], was first isolated by Reed and co-workers [78]. However, its existence was known since the 1930s when it was discovered that a so-called ‘potato growth factor’ was necessary for the growth of certain bacteria. Lipoic acid (LA) is a sulfur-containing cofactor, and in its reduced form, the dihydrolipoic acid (DHLA), two thiol groups per molecule are present. LA plays a pivotal role in energy metabolism being involved in different multienzyme complexes such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched-chain α-keto acid dehydrogenase, and glycine decarboxilase [79-80]. Its carboxylic group is covalently attached, by an amide linkage of the ε-amino group of a specific lysine, to the dihydrolipoamide acyltransferase subunit (E2) of the pyruvate dehydrogenase complex, as well as to the H protein of the glycine decarboxylase complex [79-81]. LA binds acyl groups and transfers them from one part of the enzyme complex to another one. In this process LA is reduced to DHLA, which is subsequently reoxidized by lipoamide dehydrogenase with the formation of NADH. Overall, LA and DHLA act as a redox couple carrying electrons from the substrate of the dehydrogenase to NAD^+, and for this property, the capacity to act also as antioxidants has recently been highlighted [9].

Most studies on LA were performed on humans suggesting that this molecule is localized, besides in cell membranes [82] and mitochondria [83], in the cytosol [84] and the nucleus [85], with mitochondria-rich tissues being the main source of LA.

So far, little is known about the distribution of LA in plant cells as well as on its action as antioxidant. Sgherri et al. [37] demonstrated the presence of lipoic acid in the stroma of wheat both as free and protein-bound forms. The prevalence of the bound form has been explained with the finding that in wheat the pyruvate dehydrogenase complex is mainly present in chloroplasts (85%) and, to a lesser extent, in mitochondria (15%) [86]. Its presence in chloroplasts certainly represents a significant contribution to the defense system of these organelles, which are the most exposed to oxidative stress [12] and, therefore, strongly need the regeneration of soluble antioxidants.

Usually, antioxidant compounds possess antioxidant properties in their reduced form. LA is unique, among antioxidant molecules, because it retains protective functions in both its reduced and oxidized forms [87], although DHLA is the more effective one in performing antioxidant functions. In addition, the relatively low molecular mass of LA, larger than ASA but much smaller than tocopherol, makes it soluble in both water and fat. Another reason for its dual solubility resides in the chemical structure of the molecule which, containing a carboxylic acid end-group, is more water-soluble than tocopherol. At the same time, LA and DHLA contain more carbon atoms than ASA and this makes them more soluble in lipophylic membrane compartments. Thus, LA, due to its solubility in both water and lipid phases, connects the activity of antioxidants in the cell membrane (α-tocopherol) with antioxidants in the cytoplasm (ASA and GSH), strengthening the antioxidant network. As powerful antioxidants [88], their antioxidant functions involve: (i) quenching of AOS such as $O_2^{\cdot-}$, hydroperoxyl and hydroxyl radicals; (ii) regeneration of endogenous and exogenous antioxidants involving vitamins C (ASA) and E (α-tocopherol) as well as GSH; (iii) chelation of redox metals including Cu(II) and Fe(II); (iv) repair of oxidized proteins. LA is a possible chelator for Cd(II), but much less effective than DHLA. The DHLA/LA couple has a redox potential of −320 mV as DHLA possesses one of the highest antioxidant potential known in biological systems [89]. DHLA also acts synergistically with other antioxidants, indicating that it is capable of regenerating them from their radical or inactive forms, and has the ability to regenerate oxidized thiols of proteins. Indeed, endogenously supplied LA influences the overall redox state of proteins, such as thioredoxin and transport proteins.

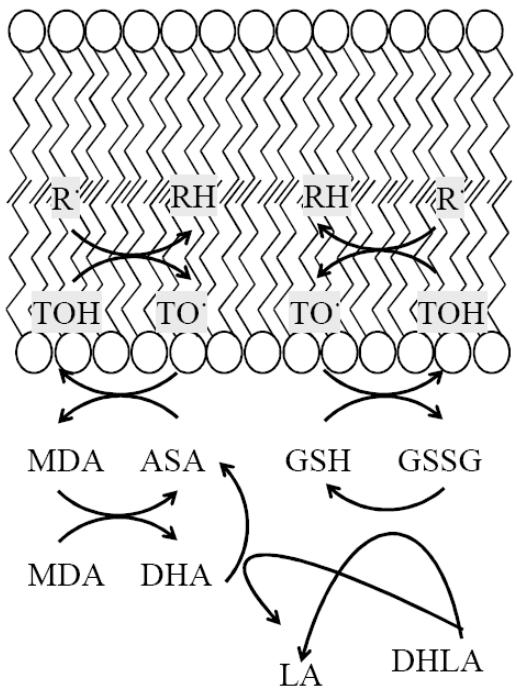


Figure 7. Regeneration of tocopherol (TOH) from tocopheroxyl radical (TO·) by reduced ascorbate (ASA) and glutathione (GSH) and regeneration of ASA and GSH by lipoic acid (DHLA, dihydrolipoic acid; LA, lipoic acid).

Some experiments where DHLA was added exogenously showed that DHLA exerts directly a radical scavenging activity because it interacts with both water-soluble and lipid-soluble peroxyl radicals in the aqueous phase or in hydrophobic domains of the lipid bilayer of liposomes or microsomal membranes [90]. Under oxidative stress conditions, lipoic acid could be released from its protein-bound reserves towards lipid bilayers, thus enhancing the antioxidant activity of membranes. In this compartment lipoic acid is known to synergistically enhance antioxidant potency of tocopherol, regenerating it by ASA or GSH mediated reactions (Figure 7) [90]. This property becomes of particular importance when, in stress conditions, ASA and/or GSH are involved in the reduction of α-tocopheroryl radical and the functioning of GSH/ASA cycle is compromised by the low NADPH availability. In fact, other ways to regenerate and reinforce the GSH/ASA cycle are necessary.

In a study of Sgherri et al. [37] wheat leaves treated with excess copper showed increases in DHLA amounts and the oxidation of its free form to LA. This was explained, in part, with the reduction in GSSG levels and the maintenance of ASA amounts. In fact, due to its redox potential, DHLA can restore ASA levels via reduction of GSSG, without requiring reducing equivalents from NADPH as GR does. Moreover, D'Amico et al. [91] demonstrated that the presence of both LA and DHLA in roots and leaves of wheat contributed to the regulation of the redox state of cells and that DHLA could contribute to the non-enzymatic regeneration of GSH. Since, following sea water treatment, DHLA showed at the same time a decrease in shoots and an increase in roots, it was also invested in the role of signal molecule for the recognition of the need of antioxidant regeneration in that part of the plant directly exposed to oxidative stress. As elevated contents of antioxidant molecules and their de novo synthesis are regarded as positive responses to counteract oxidative stress, Pérez-López et al. [92], with a study performed on two barley cultivars differently sensitive to salinity, related the higher tolerance to salt stress with the higher constitutive amounts of DHLA [8]. Thus, in the salt-tolerant cultivar DHA, besides being regenerated by DHAR, could be reduced non-enzymatically via DHLA owing to the redox potential of the LA/DHLA pair, which could have also contributed to the regeneration of GSSG [8].

4.4. Plants Are Not Particularly Enriched in Lipoic Acid

Up to now, there are few quantitative data on lipoic acid contents in biological and food samples and, due to the different detecting methods employed, a lot of contrasting data are reported in the literature. LA contents have been found to be high (ranging from 0.55 to 2.36 $\mu g\ g^{-1}$) in animal foods derived from tissues with a high metabolic activity [93], but very low (0.09 $\mu g\ g^{-1}$) or not detectable in plant foods [93]. Indeed, Kataoka et al. [93] reported that in asparagus LA amounted to 0.09 $\mu g\ g^{-1}$ whereas in garlic, soybean and welsh onion it was not detectable. However, more recently, LA has been listed among the main potato antioxidants [94]. Low lipoic acid contents were also detected in spinach, broccoli, tomato, garden pea, brussel sprouts and rice bran [95]. Pyruvate dehydrogenase complex was found both in mitochondria and in chloroplasts of barley, pea, wheat and spinach with different subcellular distribution between pea on the one hand, and barley, wheat and spinach on the other. The presence of the enzyme in both organelles may suggest that LA is located in chloroplasts as well as in mithocondria, even if Yasuno and Wada [96] found that in 4-week-old *Arabidopsis thaliana* LA synthase is located only in mitochondria. The activity of the enzyme resulted

higher in flowers and leaves than in roots, in agreement with the trend showed by LA amounts detected by the microbiological assay [77]. In fact, LA levels on a fresh weight basis amounted to 0.17, 0.09 and 0.29 μg g^{-1} in leaves, roots and flowers, respectively. The hypothesis that also DHLA can be found in plants, as the pyruvate dehydrogenase complex is present in their cells, has been recently demonstrated by Navari-Izzo et al. in wheat [9]. Based on determinations with HPLC coupled with an EC detector, they reported that leaves and roots from 15-d-old wheat contained LA and DHLA both in the free and bound forms. Thylakoid and microsomal membranes of wheat did not contain either LA or DHLA although free LA (84.9 ng mg^{-1} Chl) could be detected in the stroma, which, after acidic hydrolysis, showed the presence of both LA (340 ng mg^{-1} Chl) and DHLA (910 ng mg^{-1} Chl). The presence of both LA and DHLA in leaves was also demonstrated by Pérez-López et al. [8] in one-month-old barley. According to these authors their contents changed with the severity of the salt stress applied.

Summarizing the above findings, even if lipoic acid is present in plants in lower amounts than in mammal tissues, it could be enough to allow the antioxidant network working efficiently, even in stressful conditions. In fact, the plant-specific MDHAR and DHAR involved in the GSH/ASA cycle can allow a continuous regeneration of ASA and α-tocopherol utilizing DHLA only when NADPH is missing or GR is inactivated or just to reinforce the cycle.

5. Detoxification in Mammals

5.1. Hydrogen Peroxide Detoxification

As in the case of plants, in mammals detoxification of H_2O_2 occurs both enzymatically and non-enzymatically (by antioxidants) but with the difference that antioxidants come from the diet (ascorbate and tocopherols are vitamins and lipoic acid needs to be enriched in the body to monitor health benefits); however, plant enzymes cannot be absorbed from the gut as they are with the result that APX, MDHAR and DHAR are absent in the body.

$O_2^{\cdot -}$, produced in the different organelles of cells, is rapidly converted to H_2O_2 by the action of SOD. While this enzyme was isolated as early as in 1939, it was only in 1969 that McCord and Fridovich proved its antioxidant activity [97]. SOD exists in several isoforms differing in the nature of the active metal centre and in amino acid composition, as well as in their number of subunits, cofactors and other features. In humans there are three forms of SOD: the cytosolic Cu, Zn-SOD, the mitochondrial Mn-SOD, and the extracellular SOD (EC-SOD) [98]. On the contrary, in plants cytosolic and chloroplastic Cu, Zn-SODs, mitochondrial Mn-SOD and chloroplastic Fe-SOD can be detected [99].

As it has frequently been pointed out, dismutation of $O_2^{\cdot -}$ simply serves to convert one destructive AOS into another one. Since H_2O_2 is a strong oxidant that rapidly oxidizes thiol groups, its accumulation in organelles cannot be allowed. Catalases and peroxidases function in the scavenging of H_2O_2. CAT is an enzyme present in the cells of plants, animals and aerobic (oxygen requiring) bacteria [100]. CAT is located in the peroxisome and promotes very efficiently the conversion of H_2O_2 to water and molecular oxygen. CAT has one of the highest turnover rates for all enzymes: one molecule of CAT can convert around 6 million

molecules of H_2O_2 to water and oxygen each minute. This enzyme has extremely high catalytic rates but a low substrate affinity, since the reaction requires the simultaneous access of two H_2O_2 molecules to the active site. Moreover, it needs that H_2O_2 diffuses from the other compartments of the cell into the peroxisome.

An alternative mode of H_2O_2 destruction is via peroxidases, which are found throughout the cell and which have a much higher affinity for H_2O_2 than CAT. Peroxidases, however, require a reductant since they reduce H_2O_2 to H_2O [71]. In mammals H_2O_2-scavenging peroxidases use GSH as the electron donor and, thus, are glutathione peroxidases (GPX). Humans have four different Se-dependent GPX [100]. All GPX enzymes are known to add two electrons to reduce peroxides by forming selenoles (Se-OH). The antioxidant properties of these selenoenzymes allow them to eliminate peroxides as potential substrates for the Fenton reaction. GPX acts in conjunction with the tripeptide GSH, which is present in cells in high (micromolar) concentrations. As it occurs in plants, in human cells GSSG is reduced back to GSH by the enzyme GR, but chloroplasts are also endowed with APX and phospholipid hydroperoxide-scavenging glutathione peroxidase, which is not a selenoenzyme [101-103].

5.2. Interaction of Lipoic Acid with Other Antioxidants

DHLA appears to be able to regenerate other antioxidants such as ASA and, indirectly, vitamin E (α-tocopherol), from their radical forms. Vitamin E is the major chain-breaking antioxidant that protects membrane from lipid peroxidation and a “vitamin E recycling” is necessary since it exists in biological membranes in a low molar ratio to unsaturated phospholipids (ca. one molecule per 1000-2000 membrane phospholipid molecules). Thus, it is necessary that the antioxidant ability of vitamin E is continuously restored by the action of other antioxidants. These antioxidants are represented by ASA, ubiquinols, and GSH [83]. In particular, ASA can be regenerated through the reaction with thiols such as GSH or DHLA. These can be returned to their reduced forms by various mechanisms, drawing on the reducing power of NADH or NADPH [83]. There are some evidences about the existence of a weak direct interaction between DHLA and the tocopheroxyl radical, as it was found that the presence of DHLA reduced the tocopheroxyl radical EPR signal in liposomes exposed to UV light [83]. However, it seems likely that the major recycling of vitamin E by DHLA in biological systems occurs through other antioxidants. Bast and Haenen [104] have first proposed that DHLA prevents lipid peroxidation by reducing GSSG to GSH, which in turn recycles vitamin E. Alternatively, Kagan et al. [90] proposed that DHLA protects membranes against oxidation by recycling ASA, which in turn recycles vitamin E. In fact, in the studies of Rosenberg and Culik [105] α-lipoic acid was found to prevent symptoms of both vitamin E and vitamin C deficiencies.

5.3. Importance of Lipoic Acid for Humans

Lipoic acid is a naturally-occurring compound. It is produced in our body and is also derived from the foods we eat. Lipoic acid is now recognized as an important component of

metabolic systems in all organisms from bacteria to humans. Once it was thought to be a vitamin (a substance the body needs but that usually it cannot make on its own), but it was later discovered that the body synthesizes lipoic acid. Lipoic acid has two important functions in our body. First, it serves as a coenzyme (i.e. facilitating the action of enzymes) in several metabolic pathways. Second, it is an important antioxidant [87].

Most of the therapeutic effects of lipoic acid are attributable to its antioxidant properties [87]. The ranking of an antioxidant is dependent upon its redox potential, i.e. its ability to be oxidized and reduced. The redox potential of lipoic acid is higher than that of vitamin C or glutathione and this makes it a very potent antioxidant.

Red meat is a relatively good source of lipoic acid. Since the function of lipoic acid in the body is mostly confined to the energy-producing mitochondria in the cells, tissues and organs with high energy demands are good sources. Heart, muscle and liver are good examples. Among the other main sources we can found spinach, brewer's yeast and wheat germ. There is no established requirement in RDA (Recommended Dietary Allowance) for lipoic acid because it is synthesized in the body. For the same reason it is not considered a dietary essential although it is metabolically essential. However, under conditions of oxidative stress, what is produced in the body may not be sufficient to prevent free radical damage and lipoic acid supplementation would certainly be of benefit. At this regard, it may be considered a "conditionally-essential" nutrient like coenzyme Q10 and carnitine. Today, lipoic acid is available in the form of capsules and tablets and, recently, a new hydrosoluble form has been introduced as a soft gelatin capsule formulation with a superior bioavailability.

Supplementation with lipoic acid has been found to have some therapeutic applications in the case of diabetes. Some evidences suggest that the increased production of AOS with the occurrence of oxidative stress is associated with type 1 and type 2 diabetes mellitus as well as with insulin resistance. In patients with type 2 diabetes, lipoic acid supplementation has been shown to improve insulin-sensitivity and oral glucose tolerance [106]. Lipoic acid has been used extensively in Germany for the treatment of diabetes particularly with respect to diabetic peripheral neuropathy [107]. In fact, if blood sugar is not controlled properly, it can lead to an increased protein glycation associated with complications such as neurologic damages. Lipoic acid can block this process and potentially prevent peripheral neuropathy in diabetics or, if the patients already have the condition, lipoic acid has been shown to afford significant relief from the painful condition. There are also some evidences that show how lipoic acid may help diabetics with cardiac autonomic neuropathy [108]. Other benefits of LA oral supplementation were demonstrated for atherosclerosis since lipoic acid has been shown to protect LDL exposed to oxidative stress [109]. Diabetics are known to be at higher risk for atherosclerosis. One reason is the glycation of LDL due to high blood glucose. Increased glycation is associated with increased production of free radicals. Thus, glycated LDL increases the risk for atherosclerosis in diabetics. Based upon laboratory data on the ability of lipoic acid to block protein glycation [110], it appears that lipoic acid may help in reducing the risk for atherosclerosis by more than one mechanism. Other benefits were shown for neurodegenerative disorders [111], hearing loss [112-113], liver diseases [87], cataracts [114] and glaucoma [115]. Among the other conditions where lipoic acid has been found to have health benefits there are radiation injury, smoking, heavy metal poisoning and Chagas disease [87].

6. Cancer and Antioxidative Defenses

6.1. Enzymatic Defenses

Mn-SOD is one of the most effective antioxidant enzymes that has anti-tumor activity. A set of studies on different cell lines has confirmed that overexpression of Mn-SOD leads to tumor growth retardation [116]. However, the general assumption that Mn-SOD is a tumor suppressor protein is far from being elucidated. Though Mn-SOD activity was found to be rather low in many cancers, some experiments showed a markedly increased Mn-SOD expression [116]. The decreased activity of Mn-SOD in some kind of tumors was related to a defect in gene expression rather than to its deletion. In line with this hypothesis there is the finding that the concentration of transition metal ions was significantly reduced in some tumors. For certain tumor cells also the activity of total SOD (Cu, Zn-SOD and Mn-SOD) was found to be reduced [117]. Interestingly, a marked overexpression of Mn-SOD was detected in cancers of the gastrointestinal tract. In this case, high Mn-SOD expression was correlated with poor prognosis, advanced stages of progression and an invasive and metastatic phenotype indicating that abnormally high levels of Mn-SOD, while suppressing cell growth, increase the invasive potential of cancer cells.

Of interest, there are findings that overexpression of Mn-SOD determines the activation of enzymes of the zinc-dependent matrix metalloproteinase family (MMP), namely MMP-1 and -2. The MMPs play various roles in cellular remodeling processes, and some members of the MMP family have been reported to play a critical role in tumor invasion. The activation of MMPs occurs, most probably, via activation of the redox-sensitive transcription factor AP-1 and the nuclear factor NF-$_k$B by elevated levels of H_2O_2 produced by Mn-SOD activity [118]. Thus, it may be hypothesized that an imbalance between $O_2^{\cdot -}$ formation and H_2O_2 degradation, occurring in cells with overexpressed Mn-SOD, might activate the metastatic potential of cancer cells.

6.2. Non-Enzymatic Antioxidants

It is acknowledged fact that vitamin A (carotenoids derived from plants), vitamin C and vitamin E protect membranes against oxidation working in the antioxidant network. These antioxidant molecules, including GSH and lipoic acid, have been the subject of numerous research studies and clinical trials in order to find their effectiveness to counteract cancer. These researches showed that vitamin A, carotenoids and retinoic acid may also regulate transcription factors [119]. For example, retinoic acid is able to inhibit cell proliferation and enhance cell differentiation. Cells, exposed to oxidative stress and simultaneously treated with β-carotene, exhibited a suppressed activation of NF-$_k$B and a production of interleukin-6 (IL-6) and TNF-alpha inflammatory cytokines, suggesting a protective effect of β-carotene. Data on carotenoids have suggested that they may influence the process of apoptosis in healthy cells, whereas the proapoptotic BAX protein is downregulated after induction of external stimuli, and the anti-apoptotic protein Bcl-2 is upregulated by β-carotene supplementation [120]. Thus, carotenoids have an antiproliferative effect on various cancer cell lines and lycopene has been shown to inhibit cell cycle progression in breast, lung and

prostate cell lines. In addition, β-carotene exhibits a pro-apoptotic effect in colon and leukemic cancer cells by a redox-dependent mechanism, where increased levels of AOS and GSSG/GSH ratios are related to an increased NF-$_k$B binding ability, inhibition of cell growth and enhanced pro-apoptotic activity [121].

Several epidemiological trials report the effects of the intake of vitamin E supplements. It has been demonstrated that the intake of vitamin E [200 IU (international units)/day] reduced the incidence of colorectal cancer triggering apoptosis of cancer cells by inducing p21wafi/cip1, a powerful cell cycle inhibitor [122]. Generally, the protective effect of vitamin E is the result of the inhibition of free radical formation and of the activation of endonucleases. However, other studies where vitamin E was supplied in combination with vitamin C and β-carotene showed negative results in the prevention of colorectal cancer adenoma over a period of 4 years [123]. Since vitamin C regenerates vitamin E, it has been proposed that the addition of vitamin E might have hidden the protective effect of vitamin C against oxidative damage [124].

Glutathione has been reported to regulate redox signaling by alterations in both the level of its total (GSH + GSSG) amount and in the ratio of its oxidized (GSSG) to reduced (GSH) form [125]. Cellular GSH depletion has been found to be associated with decreased cell proliferation in vascular endothelial cells and with increased proliferation of fibroblasts. It has been shown that autophosphorylation of the platelet-derived growth factor receptor was inhibited by low cellular GSH levels. GSH is involved in regulating the activation of various transcription factors, including the nuclear factor NF-$_k$B and the activator protein AP-1. In the nucleus, the GSH redox couple appears to regulate DNA binding of the Sp-1 transcription factor. Thus, GSH protects cells against apoptotis by multifactorial mechanisms that involve detoxification and modulation of the cellular redox state as well as the subsequent redox-sensitive cell-signaling pathways and the interaction with pro- and anti-apoptotic signals [126]. Since the level of GSH is an important factor in the protection against apoptosis, the efficacy of anticancer drug-induced apoptosis requires depletion of GSH, thus facilitating tumor treatment. A large number of studies have established an association between cancer incidence and various disorders of GSH-related enzyme functions, alterations of glutathione *S*-transferases (GSTs) being most frequently reported. GSTs are a family of enzymes that utilize GSH in reactions contributing to the transformation of a wide range of compounds, including carcinogens, therapeutic drugs, and products of oxidative stress. GSTs are separated into five classes (α, μ, π, σ and θ) of which the μ class is comprised of five different isoenzymes termed GST-M1 to -M5. Most frequently reported links between cancer and mutations in GSTs concern predominantly GST-M1. The GSH/GSSG ratio measured in the blood of patients with colon and breast cancer has been found to be significantly decreased compared to the control [127]. This has been explained by an increased level of GSSG, especially in advanced stages of cancer progression. These findings are the consequence of an increased generation of peroxides, which causes an increased release of GSSG from various tissues within the red blood cells.

The intake of very high doses of vitamin C, suggested initially by Linus Pauling, has been a subject of intense debate for many years [128]. While intake of high doses of vitamin C (up to 2000 mg/day) has not been consistently reported to result in side effects, the benefit of a high intake of vitamin C has never been established. However, it has been reported that low serum levels of vitamin C in high risk population may contribute to the increased risk of gastric metaplasia or chronic gastritis, which are both precancerous lesions [129]. The

positive effect of vitamin C in reducing the incidence of stomach cancer is most probably due to the inhibitory action in the generation of *N*-nitroso compounds by interrupting the reaction between nitrites and amine groups. A consistent protective effect of vitamin C has also been found in lung and colorectal cancer [130]. Some *in vitro* and animal supplementation studies explored the pro-oxidant properties of ASA [131]. The pro-oxidant effect of ASA was attributed to the release of metal ions from damaged cells. In fact, while vitamin C commonly functions as an antioxidant, it can also act as a pro-oxidant by converting free radicals into H_2O_2, a molecule that can damage cell membranes and DNA, if not neutralized by the cellular enzyme CAT. At sufficient concentrations, vitamin C can produce cytotoxic levels of H_2O_2. It is known that tumor cells are often catalase deficient and, therefore, more sensitive than normal ones to H_2O_2 [132]. Moreover, vitamin C accumulates in solid tumors in concentrations higher than those in surrounding normal tissue. Thus, vitamin C may be useful as anti-cancer agent if cytotoxic ASA concentrations can be achieved in tumors [132]. The finding that lipoic acid was also toxic to tumor cells at millimolar concentrations and that it enhanced the cytotoxicity of ASA in a synergistic way [132] raises the idea, still unexplored, that lipoic acid can act as a new cancer fighter.

Conclusions

Under normal conditions the redox state of a biological system is kept within a narrow range similar to the manner in which a biological system regulates its pH. In contrast, under pathological conditions the redox state can be altered to lower or higher values. The intracellular “redox buffering” capacity in human cells is substantiated primarily by GSH and thioredoxin [133], while in plants by GSH and ASA through the GSH/ASA cycle. Extensive evidences have shown that the redox balance is impaired in cancer cells, which may be related to oncogenic stimulation. In plants, accumulating evidences suggest that modifications in glutathione and/or ascorbate status modulate the signaling cascades that govern genetically-controlled suicide programs within the cell [73].

Summarizing, we can conclude the following: (1) whereas GPX activity is found both in human and plant cells, APX activity is present only in plants. These two enzymes have a common function, the H_2O_2 elimination, but are the plant specific APX, MDHAR and DHAR which allow a continuous H_2O_2 detoxification and α-tocopherol regeneration. This redundancy in plants reflects the metabolic importance of such antioxidant activities; (2) plant cells, being able to endogenously synthesize carotenoids, ascorbate and tocopherols, are always endowed with antioxidants in sufficient amounts unlike humans, who have to take them from the diet.

Thus, we can conclude that plant cells possess a higher ability to eliminate H_2O_2 than human cells and that the GSH/ASA cycle, which works efficiently in plants, is not present in humans. So, while in non-stressful conditions both plants and humans are able to counteract natural oxidative stress and to maintain the redox balance, under stressful conditions humans need to intake more vitamins and antioxidants to allow the antioxidant network not to stop.

It is thus obvious that under conditions of oxidative stress, plants and humans react differently. Under oxidative stress plants are able to synthesize endogenously carotenoids, ascorbate, tocopherol, glutathione and lipoic acid, reinforcing their antioxidant network (Figs.

6 and 7), preventing themselves from oxidative stress and, eventually, from cancer. Moreover, the plant cell needs low amounts of DHLA to regenerate α-tocopherol as ASA is continuously regenerated by MDHAR and DHAR. Human cells, on the other hand, are only able to synthesize de novo glutathione and lipoic acid, and their vitamin levels might not be adequate enough to provide optimal protection which leads to oxidative stress and, eventually, to massive cell proliferation. With these ideas in mind, the addition of lipoic acid to our diet, together with supplementation of vitamin C and E, could be of some help against cancer, allowing to maintain the redox state of the cell within a narrow range.

Acknowledgments

Dedicated to the memory of Elisabetta Paoletti whose death from cancer has inspired the ideas to write this chapter.

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In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter XIV

The Use of Highly Specific GSTs towards the Development of Stress Tolerant Transgenic Plants

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Abstract

Glutathione transferases (GSTs) belong to a super-family of multifunctional proteins. GSTs play a key role in cellular detoxification from xenobiotic substances like herbicides, secondary metabolites and toxic degradation products resulting from oxidative stress and cellular metabolism. Furthermore, environmental conditions generate oxidative stress, the products of which have to be detoxified by plants.

It is anticipated that environmental stresses will worsen over the following years due to climate change. Hence, plants must adapt rapidly to the new environmental conditions in order to both survive and satisfy the constantly increasing human demand for agricultural products. Genetic engineering has been successfully used to develop plants resistant to stresses and, having taken all the necessary precautions, could offer a solution as it can help to develop plants with desirable traits in a short period of time.

We present here the use of GST isoenzymes in the development of transgenic plants. Although transgenic plants over-expressing various GSTs have been used for "*in planta*" evaluation of the enzymes used in response to different stresses, the results show that

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GSTs could be of great value for generating stress tolerant plants. However, the literature is limited and more studies should be performed in order to exploit their full potential.

Keywords: Glutathione transferase, GST, transgenic plants, herbicide detoxification, salinity, drought and temperature stresses, heavy metal tolerance

1. Introduction

Biotic stresses (caused by other living organisms, such as bacteria, viruses, fungi, parasites etc.) and abiotic stresses (caused by non-living factors, like temperature, salt, water deficit, heavy metals, UV radiation etc.) affect all forms of life, but since plants are immobile they are particularly sensitive to environmental conditions and thus are forced to develop sophisticated systems to overcome them. These stresses, such as drought, salinity, high and low temperatures, fungal infections, bacteria and weeds are the most harmful factors affecting the growth and productivity of crops worldwide. It has been claimed that abiotic stresses cause the highest crop loss compared to any other factor and can result in major crops being reduced in yield by as much as 50%. It has also been speculated that this yield reduction will only increase in the future due to the dramatic climate changes expected. For example, production of cold weather crops, such as rye, oats, wheat and apples, is expected to decline by about 15% in the next fifty years and that of strawberries will drop as much as 32%, simply because of the projected rise in temperature by a few degrees [1]. Plants are extremely sensitive to such changes and do not generally adapt quickly.

Agriculture is obliged to provide increased plant material for food, feed, fuel, pharmaceuticals, fumes (as from perfumes), and products for entertainment (fun) like coffee, chocolate, tea and spices. All these have to be produced by plants cultivated in a reduced soil area, using decreasing amounts of water and chemical inputs, while the world population will continue to increase as well as the demand for agricultural products. Moreover, environmental constraints are expected to intensify as global warming and climate changes are taking place faster than anticipated. Climate change will result in increased temperatures and water shortages, hence it is expected to cause serious salinization of more than 50% of all arable lands by 2050 [2, 3]. In addition, large soil losses are expected due to the increase of the sea level and desertification [2, 4], thus a new “green revolution” will be necessary in order to feed the world population.

Biotic and abiotic stresses, like drought and temperature, cause oxidative stress in plants which results in cellular damage, yield loss and even death of plants. Plants carry an arsenal of defence mechanisms against these stresses. Some of these are enzymes, like glutathione transferases (GSTs), superoxide dismutase (SOD), catalase (CAT) and ascorbic acid oxidase (AOX), which are responsible for scavenging ROS and removing xenobiotic molecules [5-8] (Figure 1).

Glutathione transferase (GST, EC 2.5.1.18.) isoenzymes have been identified in animals, plants and fungi [9]. The plant GST family of proteins includes six distinct classes (phi, tau, zeta, theta, dehydroascorbate reductases and lamda) [7, 10]. The phi and tau class GSTs are only found in plants. In contrast, the zeta and theta class GSTs are conserved in animals and plants [6, 11].

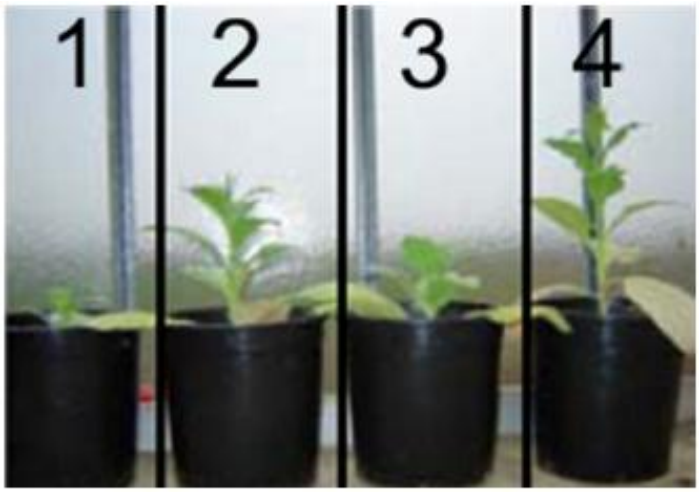

Figure 1. Tobacco over-expressing a soybean GmGSTU4 show increased tolerance against alachlor compared to wt plants 1) wild-type untransformed plants, 2 GST transgenic plants, 3) GST transgenic plants (sprayed with double dosage of alachlor compared to number 2), 4) wild-type untransformed plant (not sprayed) [5].

GSTs are abundant plant proteins which belong to a superfamily of proteins, with 54 members reported in Arabidopsis, over 25 in soybean and 42 in maize [12, 13]. GSTs have multiple functions in stress tolerance, such as the detoxification of xenobiotic compounds and the prevention of oxidative damage [14, 15]. GSTs are thought to play a vital role in plant tolerance to biotic and abiotic stresses.

The mechanism of GST detoxification in the plant involves the conjugation of electrophilic substrates to the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH). Plant GSTs can also reduce the levels of toxic organic hydroperoxides. Furthermore, several plant GSTs can also function as glutathione-dependent peroxidases (GPOX), catalyzing the reduction of oxidative stress products such as organic hydroxyperoxides. Moreover, GST plants are known to function in hormone homeostasis, vacuolar sequestration of anthocyanin, tyrosine metabolism, hydroxyperoxide detoxification and regulation of apoptosis.

Over the past decade, our knowledge of plant adaptation to environmental stress has grown considerably. The problem with stress tolerance is that it is multigene controlled, making it difficult to apply to conventional breeding. It is thus very important and urgent to deploy biotechnological and molecular breeding methods in order to develop stress tolerant plants and continue to supply humans with food [4].

Genetic engineering and plant transformation technologies have played a central role in studying a plethora of biological processes and, in particular, plant stress tolerance. As plant breeding and development of novel characteristics in plants, like tolerance to drought, high and low temperature, would need to be performed in a very short period of time, stress tolerant transgenic plant technology could be the only alternative method [16].

A rapid development of plants tolerant to abiotic stresses might be crucial due to the quick pace of climate changes taking place. Overall, antioxidant enzymes like GSTs are very important enzymes that could have a huge impact on plant tolerance to biotic and abiotic stresses, although there are still missing links in the field of the stress tolerance mechanism. The elucidation of them is of great interest in order to develop stress resistant plants,

including herbicide tolerant plants. Developing transgenic plants and plants adaptive to stresses quickly might be of extreme importance towards this goal. There are five major developing countries cultivating biotech crops: China and India in Asia, Brazil and Argentina in South America and South Africa in Africa, with a combined population of 2.7 billion (40% of global population). In addition, the potential of transgenic technology is emphasized by the decision of the Chinese government to invest around $3.5 billion over 13 years on genetically modified plants (allocated in research, development and public education) [17].

2. The Use of Highly Specific GSTs towards the Development of Stress Tolerant Transgenic Plants

2.1. Transgenic Plants Conferring Tolerance to Herbicides

The most studied group of plant enzymes involved in herbicide metabolism is by far the GSTs, which catalyze the conjugation of the major cellular thiol, GSH, to an electrophilic site of lipophilic substrates. The catalytic mechanism involves the addition of the GSH thiolate anion to the substrate or release of a leaving group (e.g. a halogen or phenolic moiety) from the substrate. GSTs are involved in the detoxification of herbicides, a highly significant function with potential use in agriculture and industry. The first report of GST isoenzymes being involved in detoxification of herbicides was published more than 40 years ago [18], but still the corresponding literature is limited. Edwards and Owen (1986) have described the tolerance conferred by GSTs against the herbicides s-triazine, chloroacetanilide and thiocarbamide in plants [19]. Nevertheless, the role of GSTs in the protection from xenobiotics was proved much later, when expression of the maize GSTIV in tobacco provided protection from metolachlor [20].

Maize GST-27, which belongs to the theta class [21], was introduced into wheat. The transgenic plants became resistant to herbicides belonging to the chloroacetanilide group (alachlor and dimethenamid) and the thiocarbamate herbicide EPTC. In contrast, GST-27 did not provide any tolerance against atrazine or oxyfluorodifen [22]. Although the research of Milligan et al aimed at the development of selectable markers, it showed the potential of GSTs for developing herbicide resistant plants.

Using an antisense approach, Deng et al [23] have transformed Lemont rice (indica×japonica) with the OsGST III subunit from rice in an antisense orientation. Transgenic lines were sensitive to pretilachlor. GST activity utilizing cinnamic acid, 1-chloro-2,4-dinitrobenzene (CDNB), and pretilachlor as substrates was reduced in transformed rice, suggesting that the OsGST III gene plays an important role in the detoxification of the herbicide pretilachlor and the metabolism of phenolic compounds.

Tobacco transgenic plants over-expressing a cotton GST gene, which had been shown to have both GST and GPX (Glutathione Peroxidase) activities, developed enhanced tolerance to methyl viologen (0.03 mmol/L) [24]. Unfortunately, no other herbicide has been tested, while, at the same time, the authors did not mention the number of transgenic copies in their

lines, thus it is not possible to determine if the tolerance is the result of a "gene dosage" effect.

The maize GST I isoenzyme (belonging to the theta class), shows significant catalytic activity for the chloroacetanilide herbicide alachlor and appears to be involved in its detoxification. The gene encoding GST I was introduced into tobacco plants. The transgenic plants showed substantially higher tolerance to alachlor compared to non-transgenic plants in terms of root, leaf and vigorous development when grown on MS medium supplemented with alachlor [8]. Thus, if GSTs are involved in the detoxification of herbicides, they could be of great use in the development of a phytoremediation system for the degradation of herbicide pollutants in agricultural fields. Tobacco plants which are susceptible to fomesafen were transformed with a soya GST gene from the U class and with a dual construct (hGSH and GST). Plants carrying the dual construct were significantly more tolerant to fomesafen compared to wild-type tobacco plants, while plants carrying only hGSH did not exhibit higher protection compared to the wild-type, suggesting that protection requires not only the GST gene but also hGSH in order to become resistant. In contrast, wild-type tobacco was highly susceptible to the herbicide fomesafen [25].

OsGSTL1 is a glutathione transferase lambda class gene from rice (*Oryza sativa* L.). Over-expression of OsGSTL1 in rice enhanced tolerance to chlorsulfuron and glyphosate. [26].

Benekos et al [5] have over-expressed a soybean GmGSTU4 in tobacco and found that the transgenic plants had increased tolerance to the diphenyl ether herbicides fluorodifen and oxyfluorfen (200μM) and the chloroacetanilide alachlor (7.5 mg/L). The tolerance was expressed as reduced electrolyte leakage when compared to wild -type plants.

As described above, GSTs are enzymes able to enhance plant tolerance to herbicides. The first transgenic herbicide crops were introduced in the United States in 1996. These were soybean plants resistant to glyphosate. Later, cotton and maize transgenic herbicide resistant plants were developed. Currently, almost 74 million hectares in 13 countries are being cultivated with five different genetically engineered herbicide tolerant crop species [27]. In order to develop herbicide tolerant plants, safe for both the consumer and the environment, the genes introduced into the plants must lead to a complete detoxification of the herbicide. However, the formation of herbicide conjugates with GSH fundamentally alters their physicochemical properties, rendering the compounds more polar and unable to freely diffuse across cellular compartments or translocate throughout the symplast. Furthermore, GSH-herbicide conjugates are usually the target of further metabolism and can be accepted by energized carriers for their transport out of the cytosol. In order to develop and commercialise herbicide resistant plant species, toxicity studies must be performed [28].

2.2. Transgenic Plants Conferring Tolerance to Abiotic Stresses

Environmental stresses, such as extreme temperatures, are among the major factors resulting in crop losses. GSTs have been shown to be involved in many abiotic interactions of plants with the environment and in stress tolerance mechanisms. Specific GSTs have been engineered in order to investigate their *in planta* function and the possibility of their enhancing plant stress tolerance.

Qi et al [29] have introduced a GST gene from *Suaeda salsa* into Arabidopsis. The specific gene, which was shown to have both GST and GPX activity [30], provided the transgenic plants with enhanced tolerance to salinity up to 200mM NaCl. They also showed almost normal root growth when compared to the wild-type plants despite their 1.5 fold lower photosynthetic rates. The plants also exhibited reduced lipid peroxidation and a high metabolic rate [29].

Another GST, which has dual glutathione transferase and glutathione peroxidase activity, was over-expressed in tobacco. The transgenic seedlings showed enhanced seedling growth when they were exposed to low temperatures or high salinity [31].

Later, Roxas et al [32] also over-expressed a tobacco GST in tobacco and found that the transgenic seedlings grew faster than wild-type, even under different stress conditions such as chilling, heating or salt stress and also showed reduced lipid peroxidation. These results suggest that plants over-expressing GSTs will have adaptive capability in a fast changing environment.

Takesawa et al [33] have developed transgenic rice over-expressing a rice zeta (ζ) class GST gene under the control of the ubiquitin promoter. Interestingly, the transgenic lines show increased germination and growth at low temperatures and, most importantly, even under submergence, which enables the direct sowing of rice in cooler regions, thus reducing the production cost.

Dianthus superbus transgenic plants over-expressing a *Nicotiana tabacum* GST showed enhanced acclimatization compared to the wild-type plants. The transgenic plants showed enhanced tolerance to high light intensity and increased photosynthetic rates under high light and in drought conditions. These characteristics can be extremely useful in the impending climate change conditions. In addition, the transgenic plants showed increased copper accumulation [34].

Tau class GST (GSTU) genes are plant specific, induced by different abiotic stresses, and important for protecting plants against oxidative damage. A GSTU (SbGST) gene, isolated from an extreme halophyte *Salicornia brachiata*, was over-expressed in transgenic tobacco plants, leading to enhanced seed germination and growth under salt stress compared to wild-type [35].

The GST from *Suaeda salsa* has been co-expressed, along with CAT, in rice. The co–expression resulted in a reduced generation of H_2O_2. The plants showed significantly less oxidative damage under salt and paraquat stress conditions. It is interesting to note that GST activity increased only under the herbicide stress. The enhanced tolerance might be the result of the synergistic effect of the two enzymes and the increased SOD activity as well [36]. This suggests that a complex mechanism must be operating in plants which, depending on the diverse stresses, regulates the differential expression of GSTs. [37]. It is thus important to acquire the missing knowledge on GST regulation under different stresses in order to understand the underlying mechanisms and breed/improve plants to withstand extreme climates.

Zhao et al [38] found later that the transgenic rice plants proved to be tolerant to cold, heat and their combinations. The authors also state that the increased tolerance of transgenic plants might be attributed not only to the high levels of expression of the transgenes but also to the coordinated action of the ascorbate-glutathione cycle in the transgenic plants, probably due to differences in H_2O_2, but this has yet to be proven.

Thus far there has been only one report on transgenic plants over-expressing a GST gene that failed to show enhanced tolerance [39]. Light et al [39] have reported that transgenic cotton seedlings over-expressing the tobacco GST (Nt107) failed to provide enhanced tolerance to salt, cold, or herbicides (atrazine and imazethapyr), even though they exhibited five to ten fold higher GST activity compared to wild-type plants. Yet, we should take into consideration that the authors have used 200 mM NaCl, while 100 to 150 mM NaCl is the usual dosage and also that they do not refer to any preliminary results on the NaCl dosage. A phi class GST from Arabidopsis (AtGST10) proved to confer tolerance to salt and disturbed redox status when over-expressed in Arabidopsis transgenic plants. Moreover, it should be mentioned that down-regulation of AtGSTF10 via RNA interference caused reduced tolerance to abiotic stress [40].

Prosopis juliflora is a drought-tolerant tree species of Fabaceae, which is used as a model plant system in order to identify genes involved in abiotic stress tolerance, like the PjGSTU1 gene. Transgenic tobacco lines carrying a PjGSTU1 gene survived under conditions of 15% PEG stress, which acts as a drought stress, compared to wild-type plants [41].

In another study, a novel GST gene from *Limonium bicolor* (LbGST1) has been cloned and over-expressed in tobacco. Transgenic LbGST1 tobacco lines exhibited both GST and glutathione peroxidase activities. In addition, superoxide dismutase, peroxidase (POD), and catalase activities in transgenic plants were significantly higher compared to wild-type plants, particularly when grown under salt stress. LbGST1 was found to be localised in the nucleus, suggesting a possible role in mediating certain physiological pathways or protecting the DNA from oxidative damage [42].

Figure 2. Transgenic tomato plants over-expressing a specific soybean GmGSTU4 isoenzyme show enhanced tolerance to drought stress (unpublished results).

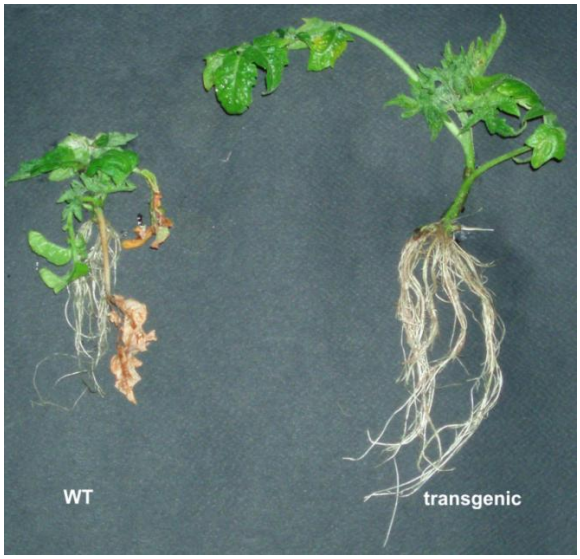

Figure 3. Transgenic tomato plants over-expressing a specific soybean GmGSTU4 isoenzyme show enhanced tolerance to salt stress (200mM NaCl, unpublished results).

Tobacco plants over-expressing the GsGST gene form *Glycin soja* (a soybean species which can survive in high salt and drought conditions) showed enhanced dehydration tolerance and T_2 seedlings showed higher tolerance to salt and mannitol than wild-type plants, during the seedling stage, as measured by longer root length and less growth retardation [43].

Arabidopsis thaliana has been sequenced [44] and thus far 54 GST genes have been identified [45]. Gong et al [46] investigated the "*in vivo*" function of the mustard gene BjGSTF2. The transgenic plants were tolerant to $HgCl_2$ and paraquat and flowered two days earlier compared to wild–type. Moreover, transgenic plants over-expressing the GST gene were highly regenerative, suggesting that GST plays a role in plant growth and development *in vivo* and shoot regeneration *in vitro*.

Finally, transgenic tobacco and tomato plants over-expressing a specific soybean GmGSTU4 isoenzyme have been created in our laboratory and show enhanced tolerance to abiotic stresses such as drought and salt (unpublished results) (Figures 2 and 3).

2.3. Transgenic Plants Conferring Tolerance to Heavy Metals

Transgenic tobacco plants have been engineered to express a GST gene from the fungus *Trichoderma virens.* Transgenic plants with fungal GST showed enhanced tolerance to anthracene (a recalcitrant polyaromatic hydrocarbon) compared to control plants. Remediation of ^{14-}C uniformly labeled anthracene from solutions and soil by transgenic tobacco plants was higher compared to wild-type plants. Transgenic plants (T_0 and T_1) degraded anthracene to naphthalene derivatives, while no such degradation was observed in wild-type plants. The present work has shown that *in planta* expression of a fungal GST in tobacco imparted enhanced tolerance, as well as higher remediation potential of anthracene, compared to wild-type plants. [49]

The same group has performed another study using the glutathione transferase gene from this fungus. When the GST gene was introduced into tobacco plants, the transgenic plants became tolerant to different concentrations of Cadmium (Cd) compared to wild-type plants, with transgenic plants showing lower levels of lipid peroxidation. Interestingly, the levels of different antioxidant enzymes, such as glutathione transferase, superoxide dismutase, ascorbate peroxidase and catalase, showed enhanced levels in transgenic plants compared to control plants. It is also very important to note that Cd accumulation in the plant biomass in transgenic plants was similar to, or even lower than, wild-type plants [50].

Two Aluminium (Al) induced genes from Arabidopis, AtGST1 and AtGST11, were fused to GUS and over-expressed back in Arabidopsis. Transgenic lines showed GST induction by Al treatment as well as by cold stress, heat stress, metal toxicity and oxidative damage, suggesting a common induction mechanism in response to the tested stresses but unfortunately the authors did not investigate the levels of plant tolerance against the stresses mentioned above [51].

2.4. Engineering GSTs Directly into the Chloroplast for Enhanced Plant Stress Tolerance

In an interesting approach, Martret et al [48] expressed GST and DHAR (dehydroascorbate reductase) in tobacco chloroplasts, either alone or in combination with DHAR and glutathione reductase. Although a GST gene has been expressed directly in the chloroplast before [47], the authors here have studied the effect of GST and the combinations used, on plant stress tolerance. Furthermore, methyl viologen treatment had the same effect in wild-type and transgenic plants (transplastomic in the case of genes introduced in the chloroplast) with the single gene, but the lines expressing the double constructs were significantly tolerant as measured by chlorophyll and malondialdehyde levels, membrane damage and electrolyte leakage. Moreover, all transplastomic plants were cold tolerant as their seedlings germinated even at 4^0C, in contrast to wild-type. Transplastomic plants were also tolerant to high salt concentrations (200mM NaCl). It is very interesting that transplastomic plants are resistant to stresses. The chloroplast is the organelle where photosynthesis takes place and thus a place where reactive oxygen species are generated. In order to achieve high rates of photosynthesis, an efficient detoxifying mechanism needs to operate. Hence, plants which over-express ROS scavenging enzymes should perform better under stress conditions.

Conclusion

Plant adaptation and tolerance to stresses is of high importance for farmers, the agricultural industry and the economy, as they are the main constraints for high yield. Some of the most important factors that influence plant yield and quality are the abiotic and biotic stresses and weeds.

Reduction in plant productivity due to abiotic and biotic stresses, and even weeds, is expected to worsen in the following years due to climate changes and global warming. It is of

paramount importance to understand the mechanism behind stress tolerance and develop stress resistant plants. In particular, because of the rate of changes taking place, new plant varieties will have to be produced in a very short period of time. Genetic engineering technology offers a quick alternative to classical plant breeding, although we should not exclude the latter from such an effort. The area cultivated with genetically engineered plants has increased from 1.7 million hectares in 1996 to 148 million hectares in 2010, showing the potential of genetically engineered plants and making biotech crops the fastest adopted crop technology in the history of modern agriculture. The GST family of proteins is part of the xenobiotic detoxification mechanism and is involved in biotic and abiotic stress tolerance in plants. It is noteworthy that, although there is vast literature on GSTs spanning several years, the exploitation of GST isoenzymes aimed at developing stress tolerant plants is limited, as reviewed here.

Hence, studies using genetic engineering to over-express GST isoenzymes in different plants show the potential of GSTs for the development of stress tolerant plants. Moreover, GSTs could offer increased tolerance to herbicides, cold, heat, drought and salt stresses, thereby facilitating agricultural practice and preparing for the forthcoming climate change.

Acknowledgments

We would like to thank General Secretariat for Research and Technology Greece and the grant THALIS for financing our research. The action THALIS.-“Gluathione transferases: multifunctional molecular tools in red and green biotechnology” falls under the Operational Programme "Education and Lifelong Learning (EdLL)" and is co-financed by the European Social Fund (ESF) and National Resources.

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In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter XV

Glutathione: An Important Player in Plant-Microbe Interactions

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Abstract

Plants are exposed to hundreds, thousands and—in the leaf spot diseases of trees—maybe to hundreds of thousands eukaryotic and prokaryotic microbes. Some of them represent dangerous pathogens, while others posses the unique ability to from mutual beneficial symbiotic interactions with terrestrial plants. Both plant pathogens and microsymbionts have developed different infection strategies to overcome the plant defense mechanisms and colonise plant tissues for growth, survival, and reproduction. Plants respond to the attackers with a highly sophisticated defense system that includes passive and active defenses directed against the invader. The core of plant defense system is the signalling network that triggers the plants to respond to the threat of invasion and orchestrate defense against the pathogens by mediating the cross communication between the various defense mechanisms. Glutathione is considered to have one of the central roles in the defense-signalling network, mainly by influencing the cellular redox homeostasis that directly or indirectly affects the regulation of many cellular processes. At the opposite site, the successful establishment and function of symbiotic trade deals between terrestrial plants and microbes are depended on coordinated global changes and reprogramming of many complex physiological, biochemical and molecular processes in both symbiotic partners. These include nutrient transport, nutrient exchange and assimilation, the modulation of plant defence responses, redox modulation and reactive oxygen species detoxification, processes in which glutathione plays a central role. This

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chapter summarizes the recent advances in understanding the complex role of glutathione in both pathogenic and symbiotic plant microbe interactions.

Abbreviations

PAMP: Pathogen Associated Molecular Patterns
ROS: Reactive Oxygen Species
RNS: Reactive Nitrogen Species
NO: Nitric Oxide
SA: Salicylic Acid
JA: Jasmonic Acid
ET: Ethylene
HR: Hypersensitive Response
AM: Arbuscular Mycorrhiza
SNF: Symbiotic Nitrogen Fixation

1. Glutathione in Plant-Pathogen Interactions

In nature, plants are continuously threatened by a wide range of harmful pathogens and pests, including viruses, bacteria, fungi, oomycetes, nematodes and insect herbivores [1]. To defend themselves against all these different types of pathogens, plants have developed perception systems that activate various defense mechanisms. Two main defense pathways are described in plant innate immunity [2], recently reviewed by Dubreuil-Maurizi et al. [3]. The first is the PAMP-triggered immunity, which is based on the recognition of pathogen-associated molecular patterns (PAMP), such as flagellin, chitin, glycoproteins and lipopolysaccharides, by pattern recognition receptors [4]. The second immune response, called effector triggered immunity, is activated when plant disease-resistance gene products detect the presence of pathogen effectors. The perception of the pathogen is triggering a cascade of changes in plants [5]. At the cellular level, many signaling events are rapidly detected, such as ion fluxes (Ca^{2+}, K^{+}, NO^{-}, Cl^{-}) and enhanced production of reactive oxygen species (ROS) [6, 7, 8]. This oxidative burst contributes, together with nitric oxide (NO) production, redox state changes, and mitogen-activated protein kinase cascade activation, to a transcriptional reprogramming leading to the activation of defense responses [9, 10, 8, 11, 12]. Changes in hormone concentration or sensitivity, particularly salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), are recognized as key events in the activation and fine-tuning of plant immunity upon pathogen attack, leading to the production of antimicrobial compounds such as phytoalexins [13], pathogenesis-related (PR) proteins [14], and cell wall strengthening. It is well accepted that plant resistance to biotrophic pathogens is mediated through SA signalling [15]. By contrast, resistance to nectrotrophic pathogens is controlled by JA, ET–signalling pathways, and genetically, SA and JA/ET defence pathways interact antagonistically [15-17]. However, it has been shown that disease resistance of *Arabidopsis thaliana* plants against the oomycete pathogen *Phytophthora brassicae* is SA, JA, and ET independent but needs the indole glucosinolate/camalexin pathways [18, 19]. In

the case of biotrophic pathogens, like *P. brassicae*, a form of plant programmed cell death, named the hypersensitive response (HR), can be detected during the incompatible (resistant plant reaction to the pathogen) interaction that may restrict pathogen growth [18, 20].

Several studies over the past decades have pointed out the role of glutathione in attenuating plant defence responses [21-25]. As well as potential roles in the regulation of the cell cycle, cell death, and light signalling, glutathione status has been clearly implicated in signalling through both SA and JA pathways [26]. Interestingly, significant increases in glutathione were measured in incompatible but not compatible (susceptible plant reaction to the pathogen) interactions [23, 25]. In compatible barley-barley powdery mildew interactions, the ascorbate-glutathione cycle and other antioxidative enzymes (e.g., glutathione S-transferase) are activated, and these processes might diminish the damaging effects of oxidative stress. However, in incompatible interactions, these antioxidative reactions are not or are only slightly activated [27, 28]. A significant inverse correlation between glutathione content and disease incidence was also observed in chemically induced plants [26, 27, 28]. A number of studies support a growing evidence for a connection between glutathione and the accumulation of PR proteins, even if the mechanisms of how glutathione levels influence *PR1* gene expression are not known [29]. *PR* gene expression was induced by overexpression of *GSH1* gene (encodes γ-glutamylcysteine synthetase that catalyzes the first step of glutathione synthesis) and by glutathione feeding, while inhibition of glutathione synthesis suppressed PR gene expression in tobacco [30, 31]. In addition, SA-accumulating mutants with constitutive *PR1* gene expression were shown to have an increased glutathione pool size [32].

The SA-dependent defense responses are characterized by the coordinate activation of a set of PR genes that encode for proteins, many of which have antimicrobial activity [33]. SA signaling is mediated by at least two mechanisms, one requiring the *NON-EXPRESSOR OF PR1* (*NPR1*) protein and a second that is independent of NPR1 [16, 34]. *NPR1* is a central positive regulator of plant defense and functions in multiple nodes of SA-signaling network [28]. It has been shown that overexpression of *NPR1* enhances resistance in Arabidopsis and rice [35, 36]. Application of SA or its analogs stimulates the translocation of *NPR1* into the nucleus [37], which is required for the activation of PR genes. In the uninduced state, *NPR1* exists as an oligomer, but after SA induction, NPR1 is reduced to monomer, i.e., the active form [28]. Increase in SA triggers the reduction of disulphide bonds located on both the regulatory protein *NPR1* and on certain TGA transcription factors (TGA1/TGA4) with which *NPR1* interacts [24, 38]. Additionally, it has also been demonstrated that the SA-induced NPR1 oligomer-to-monomer reaction is regulated by thioredoxin through reduction or oxidation of its intermolecular disulphide bonds [39]. This reduction stimulates both the translocation of NPR1 from the cytosol to the nucleus and the physical interaction of NPR1 - TGA1/TGA4 that is necessary for the activation of PR gene transcription [24, 38]. Furthermore, it is known that SA influences the cellular glutathione concentration that in turn affects the redox potential of the cell [32].

In parallel to the role of glutathione in SA-dependent defense responses, recent reports implicate glutathione-associated components in JA signaling [32, 40, 41]. The JA signal pathway involves several signal transduction events: the perception of the primary wound or stress stimulus and transduction of the signal locally and systemically; the perception of this signal and induction of JA biosynthesis; the perception of JA and induction of defense-related genes such as *PLANT DEFENSIN 1.2 (PDF1.2)* and *Thionin 2.1(Thi2.1)*; and finally, integration of JA signaling with outputs from the SA, ET, and other signaling pathways [42].

Jasmonate synthesis occurs through the octadecanoid pathway and begins with the release of linolenic acid from the chloroplast membrane, a process thought to be catalyzed by a phospholipase [43]. After biosynthesis of JA, the signal is most likely perceived and transduced by specific receptors for JA; however, to date, such receptors have not been discovered [43]. Nevertheless, mutants impaired in signalling downstream of JA biosynthesis have been identified in several genetic screens that have made use of JA as a potent inhibitor of root growth to select mutants that are unresponsive to JA or coronatine, a biologically active structural analogue of JA [44, 45]. These screens have identified mutant alleles of the genes *COI1* (coronatine insensitive 1) and *JAR1* (jasmonate resistant 1). Mutant coi1 plants are insensitive to coronatine- and JA-induced inhibition of root growth, fail to express JA-responsive genes, and are highly susceptible to insect infestation and pathogen infection, indicating that *COI1* is a central positive regulator of JA signaling [45, 46, 47]. *COI1* encodes a 66-kD protein containing an N-terminal F-box motif and 16 leucine-rich repeats [48]. F-box proteins function as receptors that specifically recruit regulatory proteins as substrates for the ubiquitin-proteasome-mediated protein degradation pathway. Because *COI1* is a positive regulator of JA signalling, it is thought to act as a selective derepressor of JA-responsive genes by targeting repressor proteins for degradation. Recruitment of such repressors is mediated by SCF complexes, consisting of SKP1, cullin, and an F-box protein that provides substrate selectivity [43].

Several antioxidative genes are induced by JA, including genes encoding enzymes of glutathione synthesis [49, 50]. It has been shown that a mutation in *gr1* gene (encodes glutathione reductase, which is responsible along with *gr2* for maintaining glutathione in the reduced state) in wt and *cat2*, catalase deficient, Arabidopsis plants affected a suite of JA-associated genes in a day length-dependent manner [51]. In the same study, it was observed induction of JA genes in *cat2* and *gr1* single mutants in short days (SD) but repression in both mutants in long days (LD) [51]. According to the authors of the study, this may indicate (1) an optimum glutathione redox status for induction of JA genes and (2) day length-dependent signals that operate to modulate the impact of changes in glutathione status on JA signaling [51]. Furthermore, the observed day length and *gr1*-modulated expression of several glutaredoxin (GRX) and glutathione S-transferases (GSTs) components may functionally link glutathione and JA signaling (51). Such components could include GRX480 [40], GSTU8-dependent formation of glutathione-oxylipin conjugates [52], and GSTU6, which is among the JA-dependent genes that are rapidly induced by wounding [53].

It is conceivable that activation of plant defense mechanisms is associated with ecological fitness costs [54]. Therefore, plants need regulatory mechanisms to effectively and efficiently adapt to changes in their environment [1]. Crosstalk between hormonal signaling pathways provides the plant with such a powerful regulatory potential and may allow the plant to tailor its defense response to the invaders encountered [55-58]. One of the best-studied examples of defense-related signal crosstalk is the antagonistic interaction between the SA and JA response pathways [1].

Many cases of tradeoffs between SA-dependent resistance against biotrophic pathogens and JA-dependent defense against necrotrophic pathogens and insect herbivory have been documented [58, 59]. For example, induction of the SA pathway in Arabidopsis by the biotrophic oomycete pathogen *Hyaloperonospora parasitica* strongly suppressed JA-mediated defenses that were activated upon feeding by caterpillars of the small cabbage white *Pieris rapae* [18]. Activation of the SA pathway by *Pseudomonas syringae* similarly

suppressed JA signaling and rendered infected leaves more susceptible to the necrotrophic fungus *Alternaria brassicicola* [60]. Pharmacological experiments with Arabidopsis revealed that JA-responsive marker genes, such as *PDF1.2* and *VEGETATIVE STORAGE PROTEIN 2 (VSP2*), are highly sensitive to suppression by exogenous application of SA [18, 61]. The transient nature of the antagonistic effect of SA on JA signaling might be associated with changes in the cellular redox state, influenced by the concentration of the total glutathione pool and the ratio between reduced and oxidized glutathione [61] that activate NPR1. The SA-activated NPR1, except for having a central role in SA dependent defense responses, is reckoned to be essential in mediating the antagonism between SA- and JA-dependent signaling [62].

NPR1 shows striking structural similarity to the animal inhibitory protein IκB [63, 64]. Like NPR1, IκB in animals functions in the cytosol, where it prevents nuclear localization of the transcription factor NF-κB, thereby regulating synthesis of prostaglandins, the structural analogues of JA, in animal innate immunity [65-67]. It is likely that NPR1 is also involved in the inhibition of a positive direct or indirect transcriptional regulator of JA signaling, possibly by inhibiting its nuclear localization; for instance, NPR1 may interfere with the removal of repressors of JA signalling by the SCF-COI1 complex [43] associated with redox effects due to glutathione on the F-box protein COI1.

Koornef et al. [32] have shown that SA treatment of A. thaliana plants resulted in a transient increase in the level of glutathione that returned to baseline levels after 30 h. A combined treatment with SA and MeJA did not alter this pattern. Interestingly, the change in glutathione levels coincided with the window of opportunity in which SA was able to suppress MeJA induced *PDF1.2* transcription. In the same study, a causal relationship between changes in glutathione levels and the downregulation of JA-responsive gene expression by SA was demonstrated by manipulating the glutathione content of the cells and monitoring the effect on PDF1.2 suppression. Inclusion of L-buthionine-sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase (the first enzyme of GSH synthesis), in the growth medium of Arabidopsis seedlings resulted in a strong reduction in SA-induced glutathione levels. To assess the effect of BSO on the ability of SA to suppress JA signaling, BSO was included in the medium either during the whole growth period (two weeks) or only during the last 48 h prior to harvest. Inclusion of BSO in the growth medium for two days reduced the antagonistic effect of SA on MeJA-induced *PDF1.2* marker gene expression. This effect was even more pronounced when BSO was present in the medium for two weeks. Therefore, the fact that glutathione biosynthesis inhibitor BSO affects SA-induced suppression of JA signaling strengthens the hypothesis that this type of SA-JA signal interaction is redox modulated. The involvement of redox modulation is further supported by the observation that overexpression of the SA-regulated glutaredoxin GRX480 antagonizes JA-responsive transcription of *PDF1.2* [40]. This antagonism may arise from the transient interaction of glutaredoxin with TGA transcription regulatory factors that in turn inhibit the expression of JA induced genes [3].

In the case of host-pathogen incompatible interaction, the HR is triggered, causing plant cell death, and hence preventing further pathogen infection [68]. At the cellular level, HR involves many signaling events such as ion fluxes and enhanced production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ion, and nitric oxide (NO), mainly catalysed by plasma membrane NADPH oxidases, encoded by *Respiratory Burst Oxidase Homolog* (*Rboh*) genes [6-8, 69]. The rapid generation of ROS is considered a

characteristic and common feature of HR leading to programmed cell death [68, 69, 71-74]. Glutathione is known to play a central role in antioxidant processes as an efficient scavenger of ROS through its cysteinyl thiol group [75], although its direct role is still disputed [76].

Accumulating evidence suggests that modifications in glutathione and/or ascorbate status modulate the signaling cascades that govern genetically controlled suicide programs within the cell [14]. The balance between the reduced and oxidized forms of glutathione is involved in cellular redox homeostasis and influences, directly or indirectly, the regulation of many cellular processes at the gene and/or protein levels [77, 78].

The putative links between glutathione, the cellular redox potential, ROS production, and HR have been thoroughly investigated in pad2-1 *A. thaliana* mutant plants [3]. The pad2-1 mutants are deficient in camalexin production, the main phytoalexin of Arabidopsis; therefore, they display susceptibility to a broad range of pathogens and pests. This camalexin deficiency is due to glutathione depletion, since glutathione is required for glutathione S-transferase (GSTF6)-catalyzed synthesis of glutathione-indole-3-acetonitrile, a precursor of camalexin biosynthesis [79].

In pad2-1 plants, it was observed that most of the glutathione content was in an oxidized state, most probably due to a less reducing cytosolic glutathione redox potential (E_{GSH} 2275 mV) caused by the diminished glutathione levels [3]. Similarly, the *gr1* Arabidopsis mutant plants, deficient in reduced glutathione, show a low capacity to maintain a reduced glutathione pool after H_2O_2 treatment and display a similar E_{GSH} (2270 mV) to pad2-1 [80]. Also, in the glutathione-deficient cad2-1 mutant, containing 30% of wild-type glutathione, an increased fraction of glutathione is found in the oxidized state. All the above-mentioned mutants, pad2-1, gr1 and cad2-1, having higher E_{GSH} than wt, are more sensitive to pathogens attack [3, 39, 81]. These data collectively indicate that a shift in the E_{GSH} in the cytosol is sufficient to reduce plant disease resistance [82]. Modulation of the cellular redox state is known to be one of the components acting on the regulation of gene expression through oxidation or a reduction of transcription factors [83, 84]. Interestingly, during *P. brassicae* infection, the expression of marker genes of oxidative stress, such as *GR1*, *GLUTATHIONE-S-TRANSFERASE (GSTF6)*, and *RbohD*, was significantly higher in pad2-1 than wt, suggesting the perception of a more oxidized environment during biotic stresses; however, H_2O_2 production was drastically decreased in pad2-1 after oligogalacturonide (OG) elicitation or *P. brassicae* infection [3]. As there is no difference in the ability to degrade H_2O_2 between pad2-1 and the wt plants, this striking discrepancy highlights a central role of glutathione in the regulation of ROS production [3].

Furthermore, pad2-1 cells exhibit a strong decrease in plasma membrane depolarization, a key event in the development of HR, in response to OG elicitation [3]. In the wild type, the use of the anionic channel inhibitor niflumic acid has shown that channel activity is involved in plasma membrane depolarization and acts upstream of ROS production during elicitation. In accordance with their lower plasma membrane depolarization, pad2-1 plants display reduced NO and H_2O_2 production in response to elicitor treatment or *P. brassicae* infection. More generally, the impairment of early signaling events observed in pad2-1 might explain, at least partly, the strong reduction of HR in response to pathogens [3]. Plasma membrane depolarization and ROS and NO production have been reported to mediate pathogen- or PAMP-triggered defense gene expression and cell death [10, 20, 85-87]. In mammals, recent studies indicate that some channels could be regulated via S-glutathionylation [88]. Thus,

glutathione depletion could disturb ion fluxes across the plasma membrane, impairing its normal depolarization [3].

2. Glutathione in Intracellular Symbiotic Plant-Microbe Interactions

A unique characteristic of higher plants is their ability to form ergonomically important intracellular symbiotic interactions with both fungi and bacterial species resulting in the formation of arbuscular mycorrhiza and nitrogen fixing root nodules, respectively. Arbuscular Mycorrhiza (AM) is a widespread symbiotic interaction, established between most terrestrial plant species and zygomycete fungi belonging in the order of Glomales [89]. Plants benefit from AM mainly through the enhanced uptake of soil phosphate, which is the second, after nitrogen, limiting nutrient for plant growth [90]. Apart from phosphorus uptake, AM has been reported to enhance the uptake of several other important macro- and micro-elements, including nitrogen and sulfur [91]. Upon AM establishment and formation, soil fungal hyphae grows towards and penetrates the plant root epidermal cells and continues to grow within the root cortex apoplast, where it finally penetrates individual cortical cells forming highly branched structures called arbuscules, separated from the cytoplasm by a plant derived membrane. Arbuscules are considered to be the main site for nutrient exchange between the plant and the fungus.

In contrast to arbuscular mycorrhiza, the ability to establish Symbiotic Nitrogen Fixation (SNF) interactions is restricted to plant species belonging to the orders Fabales, Fagales, Cucurbitales and Rosales and is typically considered to be a property of the legume plants. This discrepancy reflects the early evolution of arbuscular mycorrhiza, while SNF appeared much later in the evolutionary history of land plants [92]. Legumes are traditionally considered to be an important constituent of the sustainable agriculture concept, as they are able to supply their own nitrogen by the direct fixation (reduction) of atmospheric nitrogen (N_2) to ammonia (NH_3), through their symbiotic association with a group of soil bacteria collectively called rhizobia. Currently, rhizobia are classified within the *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Rhizobium* and *Azorhizobium* genera of the Rhizobiaceae. This mutually beneficial symbiotic association provides legumes, and the subsequent rotating crops, with a free and sustainable source of usable nitrogen. It is estimated that between 40 and 60 million tons of nitrogen are fixed annually by cultivated legumes [93]. Important food legumes accounting for up to one third of the world's primary crop production include common bean (*Phaseolus vulgaris*), broad bean (*Vicia faba*), soybean (*Glycine max*), pigeon pea (*Cajanus cajan*), pea (*Pisum sativum*), chickpea (*Cicer arietinum*), cowpea (*Vigna unguiculata*) lentil (*Lens esculenta*), Alfalfa (*Medicago sativa*), clover (*Trifolium* spp.), and several other forage legumes [94]. SNF in legumes is typically carried out in specialized new root organs called root nodules, by the differentiated forms of rhizobia that are completely surrounded by a plant membrane called the symbiosome membrane (SM), which forms a nitrogen fixing organelle, the symbiosome, within the plant cytoplasm. Infected cortical cells of nodules harbor thousands of symbiosomes, each containing one or a few bacteria. Infected plant cells, interspersed with non-infected cells, constitute the central tissue of nodules, which is surrounded by uninfected parenchyma cells that regulate gas exchange with the soil and

vascular tissues that mediate the exchange of nutrients between the nodule and the root, respectively.

Two major types of nodules have been described: a) Determinate nodules formed by tropical legumes (soybean, common bean, etc.), which exhibit time-limited meristematic activity during their early stages of development, resulting in round-shaped nodules, and b) Indeterminate nodules formed by temperate legumes (alfalfa, trefoil, etc.), which retain tip meristematic activity throughout their development and function, resulting in elongated rod-shaped nodules.

In exchange for ammonia and amino acids released to the plant, rhizobia receive reduced carbon (principally dicarboxylic acids such as malate) and every other element/nutrient required for bacterial cell growth and maintenance [95].

Recent advances in the study of the biochemical and molecular basis of both intracellular symbiotic interactions provided solid evidence that the successful establishment and function of these symbiotic interactions is depended on coordinated global changes both in plant and microbial gene expression, responsible for the reprogramming of many complex physiological, biochemical and molecular processes in both symbionts. These include nutrient transport, nutrient exchange and assimilation, the modulation of plant defence responses, redox modulation and reactive oxygen species detoxification, etc.

This work has been greatly enhanced by the introduction of the model legumes *Medicago truncatula* and *Lotus japonicus*, which have become the focus of genome sequencing and the development of extensive functional genomic resources during the past years. As thiols and particularly glutathione and homoglutathione are key-players involved in these processes, they have attracted much research attention over the past years. Especially homoglutathione (γ-glutamylcystein-β-alanine; hGSH) represents a close homologue of GSH, accumulating at levels higher that GSH in leguminous plants [96]. The following section briefly summarises the recent findings supporting a key-role of glutathione and homoglutathione in the intracellular symbiotic plant-microbe interactions.

2.1. Glutathione and Reactive Oxygen Species Play a Key Role in Symbiotic Nitrogen Fixation

As extensively described during the past years, several reactive oxygen and nitrogen species (ROS and RNS, respectively) are uniformly produced in many organisms as side products of aerobic metabolism and electron transport chains or as a response in a wide range of biotic and abiotic stresses. To this purpose, higher plants have developed an extensive enzymatic and antioxidant molecule (including GSH) repertoire in order to detoxify increased ROS and RNS levels produced under these physiological conditions. Apart from their role in stress conditions, accumulating evidence points towards a role of ROS and RNS in many biochemical and molecular mechanisms governing plant growth and development under normal growth conditions throughout the whole plant lifespan. The symbiotic interaction between rhizobia and legumes, apart from being a sophisticated plant-microbe interaction, represents also a unique and complex developmental process resulting in the formation of a novel plant organ. This complex interaction relays on a fine-tuned succession of molecular recognition, infection and developmental events, triggered and control by a chemical crosstalk between the symbiotic partners [97].

As part of this chemical crosstalk, there is increasing evidence that ROS, and as a consequence, GSH/hGSH, could play an important role in several aspects of legume-rhizobia interaction and the efficiency of the SNF process [98, 99]. In addition, the involvement of ROS and their detoxification mechanisms in the establishment and regulation of legume-rhizobia symbiosis has brought forward the stimulating hypothesis that symbiosis and pathogenesis in plants could share several common molecular regulatory networks [97].

Symbiotic root nodules have higher antioxidant scavenging capacity than roots, including ascorbate and low molecular weight thiols content (GSH and hGSH), and antioxidant enzyme activities including superoxide dismutase, catalase, peroxidases, and enzymes participating in the ascorbate/GSH cycle (GSH reductase, monodehydroascorbate reductase, dehydroascorbate reductase and ascorbate peroxidase [100]. Significant changes in the accumulation of ROS have been detected upon the early stages of nodule development, during the infection process. Increased production of the O_2^- anion has been observed in the developing infection threads carrying the rhizobia towards the developing nodule central tissue. This production of superoxide radicals is possibly mediated by a membrane bound NADPH-oxidase [101]. Interestingly, the production of O_2^- depends on the ability of the rhizobium to produce Nod factors, signal molecules that are responsible for triggering many of the plant responses leading to the establishment of the symbiotic interaction [102]. In contrast, infection with Nod factor producing wild-type rhizobia has been reported to inhibit the production of H_2O_2 [103]. During the later stages of nodule development, increased accumulation of H_2O_2 and O_2^- has been observed in the infected cells harboring the nitrogen-fixing bacteroids, indicating that ROS could also play a role in the later stages of nodule development and function, including nitrogen fixation process [101, 104]. In mature nodules, leghemoglobins together with the exceptionally high rates of plant and bacterial respiration are believed to be the main sources of ROS production. In particular, the participation of leghemoglobins in the production of ROS has been demonstrated by RNAi-induced silencing of the respective genes, resulting in significant lower levels of H_2O_2 in nodules [105].

Coupled to the production and accumulation of ROS, the induction of detoxification apparatus of both symbiotic partners is crucial for the development and function of nitrogen-fixing nodules. In order to cope with the oxidative burst, rhizobial genomes contain multiple copies of superoxide dismutases, converting O_2^- to O_2 and H_2O_2, and catalases for the subsequent scavenging of the H_2O_2. The importance of these enzymatic scavenging mechanisms in the proper establishment and function of SNF has been elegantly demonstrated by the use of rhizobial genetics. *Sinorhizobium meliloti* strains mutant for multiple katalase isoforms (*katB/katC* and *katA/katC* double mutants) have been shown to be defective in nodule formation and nitrogen fixation, respectively [106]. In the case of the *S. meliloti katB/katC* double mutant, although the nodules harbor many infection threads carrying the rhizobia to the nodule central tissue, the rhizobia released into the plant cytoplasm are not differentiating into nitrogen-fixing bacteroids and senescence shortly after. In contrast, *S. meliloti katA/katC* double mutants differentiate into their nitrogen-fixing form upon release into the plant cytoplasm, but they also rapidly undergo senescence. Interestingly, proper nodule development seems to be a fine tune between ROS production and scavenging, as an *S. meliloti* strain overexpressing the katB gene and able to rapidly degrade H_2O_2, exhibited a delayed nodulation phenotype [107].

In addition to the enzymatic detoxification of ROS, GSH and hGSH production in nitrogen-fixing nodules participates in the scavenging of ROS. GSH represents a crucial part of the antioxidant repertoire of the cell, mainly via the ascorbate/GSH cycle.

Both symbiotic partners posses the molecular machinery for the biosynthesis of GSH. The *in silico* identification and characterization of the respective genes have been greatly facilitated by the complete genome sequence available for all the main rhizobia species (http://genome.kazusa.or.jp/rhizobase/), and the extensive genome and transcript sequence databases available for both model and crop legumes (http://www.comparative-legumes.org/). These genomic recourses revealed that all rhizobial species contain both genes coding for the enzymes catalyzing the subsequent steps in GSH biosynthesis, namely γ-glutamylcysteine synthetase (γECS) and glutathione synthetase (GSHS). In the first step, γ- glutamylcysteine (γ-EC) is formed from glutamate and cysteine, and in the second step glycine is added to the C-terminal of γ-EC to form GSH. The importance of the rhizobial GSH biosynthesis pathway during the establishment and function of SNF has been extensively studied using mutant strains deficient in either step of GSH biosynthesis. A *S. meliloti* mutant strain unable to synthesize GSH due to the disruption of the *gshA* gene coding for γECS was unable to grow as free-living under non-stress conditions, precluding any nodulation—an indication that the synthesis of γ-EC is indispensible for bacterial growth. Interestingly, the abolishment of GSHS activity through the disruption of *gshB* gene did not have any detrimental effects in the growth of free-living bacteria, indicating that γ-EC could at least partially substitute for GSH under non-stress conditions. However, the *ΔgshB* strain showed a delayed nodulation phenotype and abnormal nodule development, coupled to a 75% reduction in nitrogen fixation [108]. Thus, rhizobial GSH production has been shown to be important both for nodule development and efficient nitrogen fixation. In addition, both *gshA* and *gshB* mutant strains exhibited higher catalase activity in comparison to wt *S. meliloti* strain, suggesting that both strains had undergone oxidative stress conditions. Similar results were obtained for *Rhizobium tropici*, the microsymbiotic partner of common bean. In that case, inoculation of *P. vulgaris* plants with the *R. tropici* CIAT899 *gshB* mutant strain resulted again in a delayed nodulation phenotype and a reduction in the dry weight of aerial organs of the plants, indicative of a diminished nitrogen-fixation activity [109]. In addition, nodules formed by the *gshB* mutant had undergone early senescence, a phenotype associated with increased levels of superoxide accumulation.

The legume GSH and hGSH biosynthetic pathways and their roles in SNF have also been extensively studied, especially in the case of the model legumes *L. japonicus* and *M. truncatula*. hGSH biosynthesis deviates from the main pathway as β-alanine is added to the C-terminal of γ-EC, a reaction catalyzed by homoglutathione sythetase (hGSHS). In *L. japonicus*, the model legume forming determinate type nodules, three single-copy genes involved in the biosynthesis of GSH and hGSH have been characterized [110]. These include γ-glutamylcysteine synthetase (*γecs*), glutathione synthetase (*gshs*) and homoglutathione sythetase (*hgshs*). In addition, the gshs gene has been found to be transcribed into two alternative transcripts, representing cytoplasmic and plastid isoforms of glutathione synthetase. Determination of transcript levels revealed that *γecs* and *hgshs* are expressed in both symbiotic and non-symbiotic plant organs. In contrast, *gshs* was found to be significantly upregulated in nitrogen-fixing nodules, indicating an important role for GSH in this legume. Unfortunately, the demonstration of GSH significance during symbiosis in the determinate nodules of *L. japonicus* by reverse genetic approaches is still pending.

Significantly more is known about the physiological role of plant derived thiol tripeptides in the indeterminate nodules formed by the model legume *M. truncatula*. Early studies used pharmacological inhibition of GSH and hGSH biosynthesis by L-buthionine-[S-R]-sulfoximine (BSO), a specific inhibitor of γECS [111]. The presence of 0.1 mM of BSO resulted in an 85% reduction in GSH/hGSH content in the roots of the treated plants. GSH-depleted plants formed significantly fewer nodules, whereas the addition of 0.5 mM GSH completely reversed this effect. In order to unambiguously address the significance of plant-derived GSH and hGSH in this phenotype, the authors also inhibited GSH/hGSH synthesis by expressing GSHS and hGSHS in the antisense orientation in transgenic roots of *M. truncatula*.

These treatments also led to the significant decrease in GSH and hGSH contents, coupled with an increase in γ-EC content. Also in this case, transgenic roots inoculated with *S. meliloti* formed significantly fewer nodules, indicating that this phenotype is due to the depletion of plant-derived thiol tripeptides. Interestingly, the reduction in nodule number was not correlated to a reduction of the infection events, indicating that the inhibition of plant GSH/hGSH biosynthesis affects the later stages of nodule development.

Apart from affecting the process of nodule development, GSH/hGSH deficiency has been shown to affect global host legume gene expression patterns during the symbiotic interaction between *M. truncatula* and *S. meliloti* [112].

Using cDNA-amplified fragment length polymorphism analysis (cDNA-AFLP) and RT-qPCR, the authors demonstrated significant differential expression for 181 genes between inoculated control and inoculated (h)GSH depleted roots, among the 6,149 expression tags detected. This analysis revealed that GSH/hGSH depletion results in the downregulation of genes involved in meristem formation and the transcriptional reprogramming of genes involved in stress responses, including the upregulation of salicylic acid-regulated genes.

Recently, the effects of both increased and decreased GSH/hGSH contents in nitrogen fixation in *M. truncatula* nodules have been described in detail [113]. The GSH/hGHS biosynthesis genes showed a tissue-specific expression pattern in nitrogen-fixing nodules, and all genetic manipulations were conducted using the tissue specific promoter from Nodule Cysteine Rich family member 001 (NCR001) gene, in order to modify GSH/hGSH content only in the nodule nitrogen-fixing zone, leaving thus unaffected the early infection and nodule development effects.

RNAi induced silencing of *γecs* gene resulted in a significant reduction in SNF associated with a reduction in the expression of leghemoglobin and thioredoxin S1 genes. In addition, reduced GSH/hGSH content resulted in a reduction in nodule size. In contrast, the increase in GSH content observed after the over expression of the *γecs* gene resulted in an increase in SNF.

An interesting hint about the physiological and molecular roles of GSH in various stages of nodule development came from the study of the *Arachis hypogaea* (peanut)-*Bradyrhizobium* sp. SEMIA 6144 symbiotic interaction. The interaction between these symbiotic partners is distinguishable as the early infection process does not proceed through the formation of cell-to-cell infection threads, as in the *M. truncatula-S. meliloti* paradigm. Instead, infection proceeds through intracellular penetration (so-called crack entry), at places like the point emerging lateral roots [114].

In this unique, simplified mode of infection, the disruption of the rhizobial *gshA* gene, coding for the bacterial γ-ECS, did not result in any visible symbiotic phenotype [115],

suggesting that bacterial-derived GSH may be important only in the cases of complex infection procedures involving the formation of infection threads.

In contrast, BSO-treated peanut plants, inoculated either with the *Bradyrhizobium* wild-type or GSH-deficient strain, showed a significant reduction in the number and dry weight of nodules, suggesting that the plant GSH content could play an important role in the nodulation process [116].

2.2. Glutathione in Arbuscular Mycorrhiza Symbiotic Association

In contrast to the well-studied roles of thiol tripeptides in the legume-*Rhizobium* symbiosis, very little is known about the significance of GSH for the establishment and function arbuscular mycorrhiza endosymbiosis. Although the enhanced P uptake from the soil is traditionally thought to be the major benefit of arbuscular mycorrhiza association, recent studies have pointed towards a more global role of this symbiosis in plant nutrition [117, 118]. Sulfur nutrition and assimilation was found to be significantly enhanced in carrot roots colonized by *Glomus intraradices* mycelium [119]. Apart from the direct uptake of sulfate by the fungus and its transfer to the plant roots, the microsymbiont has been found able to supply the plant with reduced forms of sulfur.

This effect is similar to that observed in ectomycorrhizal oak trees (*Quercus rober*), where labeled GSH has been shown to be imported from the fungal mycelium to the root, at rates comparable to sulfate [120]. Interestingly, the addition of millimolar amounts of GSH to the extraradical mycelia resulted in a suppression of its growth.

The growth effect was coupled with a significant decrease in the transfer of S from the mycelium to the root system and the downregulation of a fungal putative sulfate permease. Thus, GSH seems to modulate mycelium growth and S uptake and partitioning during arbuscular mycorrhiza symbiosis, although the exact biochemical and molecular mechanism remains elusive.

Apart from its participation in the improvement of the nutritional status of land plants, arbuscular mycorrhiza symbiosis has received much attention for its ability to improve plant response to many abiotic stresses, including heavy metal tolerance and water deficit [121, 122].

In the former case, heavy metal tolerance has been, at least partially, assigned either to the selective immobilization of heavy metals within mycorrhiza-colonized root tissues [123] or to the high capacity of the extraradical mycorrhizal mycelium to accumulate metals [124].

In any case, heavy metal tolerance induced in mycorrhiza-colonized plants has been correlated with changes in the expression of both plant and fungal genes presumably involved in this beneficial effect.

The cadmium (Cd)-buffering effect has been clearly demonstrated in mycorrhiza-colonized pea plants [125]. Using a real-time RT-qPCR transcriptomic profiling approach, the authors have demonstrated that although the traditional plant heavy metal chelation pathways contribute to Cd stress responses in pea, they may not make a major contribution to Cd tolerance responses operating in the arbuscular mycorrhizal symbiosis.

Thus, genes coding for γ-ECS and GSHS were upregulated by Cd treatment both in mycorrhizal and non-mycorrhizal plants. Interestingly, transcripts of an hGSHS gene were found to be specifically upregulated in mycorrhiza-colonized roots in the presence of Cd.

Studies on the transcriptional changes in the fungal microsymbiont revealed that in *Glomus intraradices* grown under high concentrations of heavy metals, several abundant ESTs had significant sequence homologies with Glutathione *S*-transferases (GSTs) from other organisms [121].

Recently, proteomic studies revealed that mycorrhization resulted in systemics changes in key components of the oxidative-stress response pathways in the shoots of Cd-treated *M. truncatula* plants [126]. These include the downregulation of a 2,4D inducible GST, S-adenosylmethionime synthetase, DHA reductase class GST, and ascorbate peroxidase. In addition, a mitochondrial voltage-dependent anion channel (VDAC) was found to be upregulated in the shoots of the mycorrhiza-colonized plants, indicating a decrease in the oxidative damage in photosynthetic plant organs, in comparison to non-inoculated plants.

Based on these findings, the authors proposed that the ascorbate-glutathione cycle is less operative in mycorrhizal plants and that mycorrhization helps in reducing oxidative stress in photosynthetic organs. Apart from its role in heavy metal tolerance, arbuscular mycorrhiza reported to also enhance the photosynthetic efficiency and the antioxidative response of plants subjected to drought stress [127, 128].

In rice, the arbuscular mycorrhization enhanced the plant photosynthetic efficiency, induced the accumulation of GSH and reduced the accumulation of H_2O_2 and the oxidative damage to lipids under water stress [129].

Conclusion

Glutathione is considered to have one of the central roles in the defence-signalling network mainly by influencing the cellular redox homeostasis that directly or indirectly affects the regulation of many cellular processes.

It is involved in the SA-dependent defence mechanism by participating in the activation of the NPR protein, while at the same time mediates via NPR the cross talk with JA-dependent defence responses. Furthermore, glutathione can affect directly the expression of JA-responsive genes by influencing the cellular redox state. Similarly, glutathione regulates ROS production and plasma membrane depolarization, key events in the hypersensitive response-related programmed cell death. Glutathione also plays an important role in symbiotic plant-microbe interactions.

During SNF, plant and *Rhizobium* derived glutathione plays a key role, both at the early stages of infection and nodule development and at modulating the efficiency of nitrogen fixation, mainly by participating in the metabolism of ROS generated during these processes. In arbuscular mycorrhiza symbiosis, glutathione metabolism has been implicated in the improved plant response to many abiotic stresses. Future work should aim towards the better understanding of the exact biochemical and molecular mechanism governing glutathione role in plant-microbe interactions. Regardless of being pathogenic or symbiotic, these interactions, apart from their immediate agronomic significance, represent a unique opportunity for plant biologists to study a model that elegantly integrates both infectious and developmental processes.

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Part IV: Glutathione in Microorganisms

In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter XVI

Glutathione Metabolism in Protozoan Parasites: Making the Difference

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Abstract

Generation of reactive oxygen species (ROS) is a common feature of abiotic and biotic stresses. ROS need to be detoxified to avoid the occurrence of deleterious reactions. Parasitic diseases such as sleeping sickness, Chagas disease, and malaria, between others, are major health problems in poverty-stricken areas. Redox metabolism in parasitic protozoa is a potentially relevant target for developing specific drug inhibitors. The design of efficacious and safe drugs is possible after the occurrence of metabolic pathways that are unique and essential to the parasite, but absent in the host. In most organisms, glutathione is the most abundant low-molecular weight thiol acting in cellular redox systems. In those parasites with deficit or lacking the glutathione machinery, other active molecules are functional in mimicking the former reductive compound.

Many of the known glutathione-dependent processes are directly related to specific life-style of the respective parasite. Thus, malaria parasites have a dual antioxidant system based on glutathione and thioredoxin. Proteins involved in glutathione-dependent metabolic pathways include glutaredoxins, glyoxalases, thioredoxins, glutathione reductase and glutathione *S*-transferases. In contrast, in trypanosomatids (*Trypanosoma* and *Leishmania* spp.), the redox network is centered around trypanothione [N^1,N^8-bis(glutathionyl)spermidine], instead of the ubiquitous glutathione. In these parasites,

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trypanothione participates in essential thiol–disulfide exchange reactions as electron donor to different trypanothione-dependent enzymes such as tryparedoxin, glutaredoxin and peroxiredoxins. Since trypanosomatids lack glutathione reductase and thioredoxin reductase, trypanothione is the central node in these microorganisms taking electrons from NADPH to achieve antioxidant enzymes. Concerning some anaerobic parasites such as *Entamoeba histolytica* or *Giardia lamblia*, they are human pathogens that lack the capacity to synthesize glutathione, and they have cysteine as the major low-molecular weight thiol.

In this chapter, we review the glutathione dependent metabolism in protozoan parasitic cells, pointing out its relevance in vital functions of the parasite. We also comparatively analyze differences and similarities between glutathione and alternative metabolisms in the different protozoa. Particular attention is given to the role of glutathione in redox regulation and adaptation to stresses, highlighting the importance of enzymes belonging to redox metabolism, many of them proposed as target of antiparasitic drugs.

Abbreviations

L-Cys, L- cysteine;
L-Glu, L-glutamine;
γ-GC, gamma glutamil cysteine;
γ-GCS, gamma glutamil cysteine synthetase;
L-Gly, L-glycine;
GSH, glutathione;
GR, glutathione reductase;
TRX, thioredoxin;
TRXR, thioredoxin reductase;
TPx, thioredoxin peroxidase,
Glo, glyoxalase;
GPx, glutathione peroxidase;
GRX, glutaredoxin;
GSSG, oxidized glutathione;
GSNO, *S*-nitrosoglutathione;
HNO, nitrosyl acid;
PSH, protein thiol;
PSSG, glutathionylated protein;
PSO, sulfenic acid protein;
PLX, plasmoredoxin;
GST, glutathione *S*-transferase;
RX, electrophilic compound;
RSG, glutathionylated compound;
ROOH, hydroperoxide;
SOD, superoxide dismutase;
TS, trypanothione synthetase;
GspS, glutathionylspermidine synthetase;
GspSH, glutathionylspermidine;

$T(SH)_2$, trypanothione;
TR, trypanothione reductase;
TS_2, oxidized trypanothione;
TXN, tryparedoxin;
RR ribonucleotide reductase;
TXNPx, tryparedoxin peroxidase,
GPxI, glutathione peroxidase I;
MetSOR, methionine sulfoxide reductase;
SeCys, selenocysteine.

1. Introduction

Aerobic life requires the maintenance of an appropriate intracellular redox environment minimizing the generation of reactive oxygen species (ROS) (such as superoxide anions, hydrogen peroxide and hydroxyl radicals) which damage nucleic acids, proteins, lipids and membranes and yet avoids essential metabolic functions [1,2].

In order to critically preserve adequate reducing conditions, aerobic organisms have developed efficient antioxidant systems. The homeostasis of the intracellular redox environment is essential for cell survival [3], and different mechanisms have been identified as involved in overcoming oxidative stress within the cell.

Three primary ways for the maintenance of intracellular reducing environments can be highlighted:

- Antioxidant enzymes (e.g. superoxide dismutase, catalase, glutathione peroxidase)
- Antioxidant molecules (e.g. ascorbate, glutathione, tocopherol)
- Metal chelators (e.g. transferrin for iron, ceruloplasmin for copper)

Reduced glutathione (GSH) is a tripeptide containing a unusual peptidic linkage between the amine group of cysteine (which carboxyl-moiety is forming a normal peptide linkage with a glycine) and the carboxyl group of the glutamate side-chain (γ-glutamyl-cysteinyl-glycine) [4]. The –SH group of the Cys residue is the important reaction center in GSH, allowing its action as a thiol buffer in the cell to maintain Cys residues in proteins at a reduced state [5]. In this process, GSH is oxidized to GSSG:

$$2\,\mathrm{GSH} \rightarrow \mathrm{GSSG} + 2\,\mathrm{e}^- + 2\,\mathrm{H}^+$$

The GSSG/GSH couple has a standard reduction potential ($E^{o\prime}$) of -240 mV, which makes GSH a moderate reducing agent, being the glutathione system one of the most important redox buffers in the cells [5].

GSH pools are maintained by action of the enzyme glutathione reductase (GR), a pyridine nucleotide disulfide reductase that transfers electrons from NADPH to GSSG to generate GSH [6]. Thus, the oxidation of GSH and subsequent reduction of GSSG form a cyclic process. GSH is used as electron donor for both, peroxidases to scavenge H_2O_2 and glutaredoxins (GRX) in the mixed-disulfides reduction of proteins [7-9]. Since the conversion

of GSSG to GSH is very fast, normally concentration of GSSG in the cell is much lower than the reduced form. Typically, GSH in cells ranges from 0.1-10 mM while GSSG is about 5-15 μM, depending on the redox environment of the cell [5]. GSH is the predominant intracellular non-protein thiol and its physiological significance may be framed into two categories: sulfur metabolism and antioxidant defense. GSH is a product of the primary sulfur metabolism acting as a form of transport and storage of reduced sulfur, which is important as antioxidant and redox buffer [4,5].

Parasitic diseases such as amoebiasis, sleeping sickness, Chagas disease, and malaria, between others, are major health problems in poverty-stricken areas. There is priority urgency in public health, which is the development of a new, specific, economic and sure chemotherapeutic drug against these diseases. The design of effective and safe drugs is expected from, for example, inhibition of metabolic pathways that are unique and essential to the parasite, being absent in the host. Redox metabolism is a key target for the design of such parasitic chemicals, and the complete understanding of its occurrence in the different organisms is a critical prerequisite for successfully reach goals in the field. This chapter focuses on redox scenarios emerged from biochemical research on many molecular components characterized in different protozoa having relevant incidence in human health problems.

2. Gluathione-Dependent Metabolism in *Plasmodium falciparum* - Malaria Drug Therapy Problems

Plasmodium falciparum, a unicellular parasite, is one of the species of *Plasmodium* that cause malaria in humans. It is transmitted by the female *Anopheles* mosquito [10]. Tropical malaria constitutes one of the most impoverishing infectious diseases, annually affecting around half a billion people worldwide and causing the death of a million children in Africa. It is more prevalent in sub-Saharan Africa than in other regions of the world, and in most African countries more than 75% of cases are specifically due to *P. falciparum.* About 40% of the world's population is at risk of being infected [6]. The actual disease develops when *P. falciparum* multiplies in human erythrocytes and, consequently, most drugs are being directed against this stage of the parasite life cycle [10]. A major reason for this devastating situation is the emerging resistance of *P. falciparum* to classical and affordable antimalarial drugs [11]. The critical search for new chemicals requires the identification of new potential targets for chemotherapeutic intervention.

2.1. *Plasmodium falciparum* Life Cycle

The life cycle of all *Plasmodium* spp. is complex. Infection in humans begins with the bite of an infected female anopheline mosquito. Sporozoites released from the salivary glands of the mosquito enter the bloodstream during feeding and quickly invade liver cells. At this stage parasites differentiate and undergo asexual multiplication resulting in tens of thousands

of merozoites which burst from the hepatocyte. Individual merozoites invade red blood cells, undergoing an additional round of multiplication to produce merozoites within a schizont. Clinical manifestations of malaria, fever and chills, are associated with the synchronous rupture of the infected erythrocyte. Released merozoites go on to invade additional erythrocytes. Not all of the merozoites divide into schizonts, as some differentiate into sexual forms, male and female gametocytes. These gametocytes are taken up by a new female anopheline mosquito during a blood meal. Within the mosquito midgut, the male gametocyte undergoes a rapid nuclear division, producing flagellated microgametes which fertilize the female macrogamete. The resulting ookinete traverses the mosquito gut wall and encysts on the exterior of the gut wall as an oocyst. Soon oocysts crack, releasing thousands of sporozoites into the mosquito body cavity where they eventually migrate to the mosquito salivary gland [10, 12].

2.2. Oxidative Species Generation in *Plasmodium*-Infected Cells

Reactive species derivved from oxygen and nitrogen, such as hydrogen peroxide, nitric oxide, superoxide radical, peroxynitrite and hydroxyl radical are continuously generated in living organisms. These reactive species are produced as by-products in cell-respiration as well as in xenobiotic detoxification, defense against infection and ultraviolet radiation [1]. The exposure to physiological concentrations of oxidant species induces changes in metabolism that wake up cell responses, such as reparation, adaptation or transformation. Reactive species are able to cause damage on all major groups of biomolecules, including: lipids, proteins and nucleic acids [1,13], thus constituting potentially toxic and mutagenic agents.

Parasitized red blood cells are exposed to high fluxes of ROS and additional prooxidant agents that may inhibit parasite growth [14]. Malaria parasites are particularly vulnerable to oxidative stress during their erythrocytic life stages. The parasites live in a pro-oxidant environment that contains oxygen and iron, key components for ROS formation via the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO\cdot + HO^-$) [6,14]. Hemoglobin is taken up by parasites into their acid food vacuoles, which leads to the spontaneous oxidation of Fe^{2+} to Fe^{3+} with generation of hydrogen peroxide and subsequently hydroxyl radicals, both highly reactive and toxic oxygen intermediates. In addition, toxic haem (ferri/ferroprotoporphyrin IX; FP IX) is released upon hemoglobin digestion and it needs to be detoxified. Most of the free FP IX is transformed in inert hemozoin to prevent membrane damage and parasite death. In these processes, the thiol-dependent redox metabolism is implicated in the resistance to oxidative stress and pathology of malaria [14, 15].

2.3. Redox and Antioxidant Systems in *Plasmodium falciparum*

P. falciparum is repeatedly exposed to oxidative stress during the erythrocytic stages of its life cycle, being highly adapted for surviving in such physiological conditions. The parasite presents a main antioxidant defense based on the functionality of several redox components like NADPH; thioredoxin reductase, TRXR; thioredoxin, TRX; glutathione and

superoxide dismutase. However, the canonical antioxidant enzymes catalase and selenocysteine-containing glutathione peroxidase seem to be absent in the parasite [6]. The malarial parasite exhibits an intense and functional glutathione-dependent metabolism that has been subject of intense investigations. The glutathione pair (GSH/GSSG) is the main intracellular thiol redox buffer in *P. falciparum* [15]. GSH is a cofactor for several enzymes such as glutathione *S*-transferase (conjugates glutathione to non-degradable compounds like, for example, antimalarial drugs), glutaredoxin (GRX) and glyoxalases (detoxifies methylglyoxal, a byproduct of glycolysis). Moreover, GSH supports rapid cell growth providing electrons for deoxyribonucleotide synthesis and it further takes part in detoxifying haem. The vital importance of thioredoxin and glutathione systems in the pathology of malaria has been demonstrated by several works showing that these redox systems are essential for the survival of intraerythrocytic *P. falciparum* [14,15].

Glutathione metabolism in *Plasmodium* presents quite numerous roles, not only limited to its redox and antioxidant functions. Notably, it has been suggested that GSH is involved in drug resistance both as a cofactor for enzymatic reactions and helping to mediate resistance as a source of reductive detoxification of FP IX [16].

2.4. Glutathione Relevance in *Plasmodium falciparum*

GSH is the major low-molecular-weight thiol redox buffer in almost all aerobic cells. Most studies on malarial parasites at the trophozoite stage in human red blood cells showed that the GSH:GSSG ratio is usually between 10:1 and 100:1, which is essential for intracellular redox homeostasis. This is mainly achieved by the action of glutathione reductase (GR) and by *de novo* synthesis of the tripeptide, which contributes to sustaining sufficiently high intracellular GSH levels [6,15]. In addition to its role as a thiol redox buffer, GSH is a cofactor for a variety of enzymes including glutathione-dependent peroxidases [17], glutathione *S*-transferases [16], glutaredoxins [18] and glyoxalases [19].

It can be also involved in antioxidant reactions, for instance, the thiyl radical $GS^{\bullet}$ and the glutathione disulfide radical $GSSG^{\bullet}$ are single electron intermediates in reaction sequences that terminate radical-based chain reactions [6]:

$$R^{\bullet} + GSH \rightarrow RH + GS^{\bullet}$$
$$GS^{\bullet} + GSH \rightarrow GSSG^{\bullet}$$
$$GSSG^{\bullet} + O_2 \rightarrow GSSG + O_2^{\bullet}$$
$$GS^{\bullet} + HO^{\bullet} \rightarrow GSOH \xrightarrow{GSH} GSSG + H_2O$$
$$GS^{\bullet} + NO^{\bullet} \rightarrow GSNO \xrightarrow{GSH} GSSG + HNO$$

Under oxidative conditions, protein thiols (PSHs) are particularly susceptible to oxidative modifications and can undergo a diverse array of redox reactions.

These diversities are largely dependent on the reactive species and concentration of the oxidants they contact [20]. In the presence of increasing ROS concentrations and an oxidative cellular environment, PSH can be oxidized into sulfenic (PSOH), sulfinic (PSO_2H) or sulfonic (PSO_3H) acids [21]. Whereas the two latter species are usually irreversible, the

formation of PSOH can be reversed by GSH through the formation of disulfides between protein sulfenic acid and GSH (PSSG) [21]:

$$\mathrm{PSH} \xrightarrow{\mathrm{ROS}} \mathrm{PSOH}$$
$$\mathrm{PSOH} + \mathrm{GSH} \rightarrow \mathrm{PSSG} + \mathrm{H_2O}$$

In this way, GSH protects cysteine residues in proteins against further irreversible oxidation and then the mixed disulfides must be reduced in order to reconstitute the functional thiol proteins [22]:

$$\mathrm{PSSG} + \mathrm{GSH} \rightarrow \mathrm{PSH} + \mathrm{GSSG}$$

2.5. GSH: De Novo Biosynthesis in *Plamodium falciparum*

Malaria parasites possess a functional pathway for glutathione synthesis, similar to many other organisms. The generation of GSH is constitutive and involves the enzymes γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS), which catalyze the linking between glutamate and cysteine followed by the addition of glycine [15]:

$$\mathrm{L\text{-}glutmate} + \mathrm{L\text{-}cysteine} + \mathrm{ATP} \xrightarrow{\gamma\text{-GCS}}$$
$$\gamma\text{-}\mathrm{L\text{-}glutamyl\text{-}L\text{-}cysteine} + \mathrm{ADP} + \mathrm{Pi}$$

$$\gamma\text{-}\mathrm{L\text{-}glutamyl\text{-}L\text{-}cysteine} + \mathrm{glycine} + \mathrm{ATP} \xrightarrow{\mathrm{GS}}$$
$$\gamma\text{-}\mathrm{L\text{-}glutamyl\text{-}L\text{-}cysteine\text{-}L\text{-}glycine} + \mathrm{ADP} + \mathrm{Pi}$$

Several works indicate that *Pf*γ-GCS is encoded by a single gene copy and it shows three major insertions as well as a C-terminal extension with respect to the human enzyme.

The gene is stage-specifically transcribed, which is well correlated with the intensity of metabolism and hemoglobin digestion (highest in trophozoite but diminished in the schizont stage) [14]. The reaction catalyzed by γ-GCS is rate-limiting, being regulated by GSH via feedback inhibition and nitration [15].

On the other hand, *P. falciparum* glutathione synthetase (*Pf*γ-GS) is a protein of 655 amino acids, which is also encoded by a single exon. Differences between *P. falciparum* and human enzymes include a number of insertions in the *Pf*γ-GS sequence and the multitude of 11 cysteine residues found in the former [15].

Plasmodium cells are killed by the specific γ-GCS inhibitor, D,L-buthionine-S, R-sulphoximine (BSO), presumably because GSH pools decrease significantly making unable the maintenance of the GSH:GSSG ratio. This suggests that the parasite cells are highly dependent on a GSH functional synthesis. As BSO acts specifically on γ-GCS, it can be used as a research tool that efficiently depletes GSH without affecting other metabolic pathways or

proteins. However, the inhibitor not only acts on the parasite enzyme but also inhibits the enzyme from other host cell [6].

2.6. Glutathione Reductase, the Enzyme for GSH Recycling

In many organisms, glutathione reductase (GR) is the enzyme responsible for keeping glutathione in its reduced state [4]. GR belongs to flavo-disulfide oxidoreductase family which also includes thioredoxin reductase, mercuric ion reductase and trypanothione reductase [1]. The enzyme catalyzes the NADPH-dependent reduction of GSSG:

$$\mathrm{NADPH} + \mathrm{H}^{+} + \mathrm{GSSG} \xrightarrow{\mathrm{GR}} \mathrm{NADP}^{+} + 2\,\mathrm{GSH}$$

P. falciparum GR (*Pf*GR) has been characterized at the biochemical and kinetic level. Clinical and epidemiological observations suggest that *Pf*GR could be a potential target for the development of antimalarial drugs [6]. The primary structure of *Pf*GR contains specific insertions in the FAD- (residues 123–134), central- (residues 314–347) and the interface-domain (residues 496–499). Each subunit of *Pf*GR contains a disulfide (Cys39 and Cys44) and a flavin, which are in redox contact [23]. The catalytic mechanism of *Pf*GR has been studied in detail. The enzyme ground state (E_{ox}) is reduced at the expenses of NADPH *via* the flavin, to give the EH_2 state (two-electron reduced enzyme). In EH_2, a charge transfer complex occurs between one of the nascent thiolates (Cys44) and the flavin. The other nascent thiol in EH_2 (Cys39) initiates the dithiol-disulfide interchange with GSSG. The 4-electron reduced species (EH_4) is a labile form of the enzyme and catalytically inactive [24]. The enzyme is a homodimeric protein and its three-dimensional structure was solved. *Pf*GR presents 45% sequence identity with human GR with a very similar overall architecture. However, there are three major features distinguishing *Pf*GR from human GR which might serve for structure-based drug development: (i) the three major insertions in *Pf*GR (related to protein stability and FAD cofactor binding capacity), (ii) the size of intersubunit cavity, and (iii) the pair of interface helices involved in protein folding and dimerization [23]. Differences in the cavity of the dimer interface may permit the design of inhibitors that specifically bind in this area of *Pf*GR. In fact, it has been suggested that tricyclic heteroaromatic compounds such as isoalloxazine derivatives and methylene blue exert their effects through this area of the protein [6,25]. *Pf*GR is encoded by a single copy gene within the Plasmodium genome, but it is present in both cytoplasmatic and apicoplast localization. The apicoplastic isoform is generated by alternative-translation-initiation at a start-codon upstream of the canonical ATG start. The expression of the cytoplasmatic isoform is strictly dependent on the integrity of the downstream ATG triplet as shown by in vitro and in vivo functional analysis of mutants in a recent work [26].

2.7. Glutaredoxins, the Multipurpose GSH-Dependent Proteins

Glutaredoxins (GRX, formerly called thioltransferases) are ubiquitous small thiol-disulfide proteins of the thioredoxin superfamily, GRX possesses the ‘thioredoxin fold’

consisting of a central β-sheet surrounded by α-helices and a CXXC redox active site motif [8]. It is a small protein of 10 to 24 kDa that catalyze the reduction of proteins that are thiolated by GSH (glutathionylated, PSSG) [22]. The reduction of PSSG is carried out by GSH, which is oxidized to GSSG and recycled to GSH via the recycling system of NADPH and GR (NADPH → GR → GSH → GRX → PSSG). It has been established that GRX can reduce protein disulfides (PSSP) using a dithiol mechanism. It can also reduce PSSG, or low molecular weight dithiols (GSSR) via either a monothiol or dithiol mechanism [8]. According to their redox active centre, GRXs are included in two main classes: the CPYC, and the CGFS type. The second class GRXs possesses only one conserved cysteine residue at the active site. These proteins were detected initially in human, 1-Cys GRX which interact with protein kinase C (PICOT proteins, protein kinase C interacting cousin of thioredoxin), but have already been described in many organisms [8,18].

The redox potential of GRXs ranges between -190 and -230 mV [27], these proteins acting as electron carriers in the glutathione-dependent synthesis of deoxyribonucleotides by ribonucleotide reductase. In addition, GRXs participate in antioxidant defense, performing the dehydroascorbic acid reduction and serving as electron donor for several peroxiredoxins [15]. GRX protects cells from oxidative damage and is associated with control of transcription and apoptosis. Additionaly, GRXs were shown to bind iron-sulfur clusters and to deliver the cluster to enzymes on demand [8].

P. falciparum possesses one classical glutaredoxin (*Pf*GRX1) with the typical CPYC redox active site. *Pf*GRX1 was shown to reduce ribonucleotide reductase and also glutathionylated-substrates [28].

In addition, *Pf*GRX1 transfer reducing equivalent to plasmoredoxin (PLX), another member of the thioredoxin superfamily that appears to be unique to *Plasmodium* parasites. PLX itself reduces ribonucleotide reductase, 2 Cys peroxiredoxin 1 (*Pf*TPx1) and GSSG but its precise function in *Plasmodium* remains without a fully characterization [29,30]. *P. falciparum* also possesses a 1-Cys GRX (*Pf*GLP-1, glutaredoxin-like protein), which is involved in the synthesis and delivers of Fe/S-cluster [18]. Moreover, *Pf*GLP1 acts in protein deglutathionylation that is inevitably formed during exposure to oxidative stress and generally lead to a reversible inactivation of protein functions [15]. Apart from *Pf*GLP-1 two more GLPs have been detected in *P. falciparum.* The function of these proteins remains to be revealed [26].

2.8. Protein S-Glutathionylation in Malaria Parasite

Protein *S*-glutathionylation is a widely distributed post-translational modification of thiol groups with glutathione that can function as a redox-sensitive switch to mediate redox regulation and signal transduction [21, 22]. Protein *S*-glutathionylation can occur through several reactions:

1. Direct interaction between partially oxidized (activated) protein sulfhydryls, for example, thiyl radical ($PS^{\bullet}$), sulfenic acid (PSOH), or protein *S*-nitrosothiol (*S*-nitrosated protein, PSNO) and GSH.

2. Thiol/disulfide exchange reactions between protein thiols and GSSG or PSSG; or protein disulfides and GSH.
3. Reaction between protein thiols and intermediate *S*-nitrosothiols such as *S*-nitrosoglutathione (GSNO), which is able to modify PSH by both protein *S*-nitrosation (PSNO) and *S*-glutathionylation (PSSG).
4. Direct interaction between a free protein cysteinyl residue and GSH triggered by many oxidants.

Furthermore, glutathione sulfenic acid (GSOH), glutathione disulfide *S*-monoxide [GS(O)SG] and glutathione thiyl radical ($GS^{\bullet}$) are considered reactive sulfur species and can perform as alternative mediators of *S*-glutathionylation [22].

The malaria parasite *P. falciparum* is exposed to intense oxidative stress and possesses the enzymatic system required to regulate protein *S*-glutathionylation. A recent work has characterized the *Plasmodium* "glutathionylome" based in applied enzymatic deglutathionylation, affinity purification and proteomic methods [31]. About 493 targets of protein *S*-glutathionylation in *Plasmodium* were identified. Functional profiles revealed that the targets are components of central metabolic pathways, such as nitrogen or protein metabolisms. Fifteen identified proteins, such as thioredoxin reductase, thioredoxin, thioredoxin peroxidase 1, glutathione reductase, glutathione *S*-transferase, plasmoredoxin, dihydrolipoamide dehydrogenase, glutamate dehydrogenase, glyoxalase I and II, ornithine δ-aminotransferase, lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase, phosphoglycerate mutase (with important functions in redox and energetic metabolic pathways) presented ability to form mixed disulfides with glutathione *in vivo* and *in vitro*. In addition, previous reports showed that *P. falciparum* glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and ornithine δ-aminotransferase are reversibly inhibited by *S*-glutathionylation and this inhibition is reversed by not only *Pf*GRX, but also thioredoxin 1 and *Pf*Plx [31].

2.9. GSH-Dependent Detoxification Enzymes

2.9.1. Glyoxalases

The glyoxalase system is composted by glyoxalase I (GloI), glyoxalase II (GloII), and GSH [19]. This cyclic enzymatic system removes toxic 2-oxoaldehydes, such as methylglyoxal, by converting them to the corresponding non-toxic 2-hydroxycarboxylic acids like D-lactate:

$$\text{2-oxoaldehyde} + \text{GSH} \rightarrow \text{hemithioacetal}$$

$$\text{hemithioacetal} \xrightarrow{\text{GloI}} \text{S-2-hydroxyacetylglutathione}$$

$$\text{S-2-hydroxyacetylglutathione} \xrightarrow{\text{GloII}} \text{2-hydroxyacid} + \text{GSH}$$

GloI catalyzes the formation of *S*-2-hydroxyacylglutathione (a thiol ester of GSH), and GloII then hydrolyzes the ester, thus producing GSH and a free 2-hydroxycarboxylic acid

[19]. The main physiological substrates of the glyoxalase system are glyoxal and methylglyoxal.

Red blood cells infected with *P. falciparum* present high production rate of D-lactate from methylglyoxal [15]. *Pf*GloI and *Pf*GloII are active as monomeric zinc-containing proteins of 44 kDa and 40 kDa, respectively [19, 32]. The presence of the active system was demonstrated in blood stage parasites. Inhibition of the parasite glyoxalase system by selective drugs can cause parasite cellular toxicity, being suggested as possible chemotherapeutic target. Several *S*-(*N*-hydroxy-*N*-arylcarbamyl) glutathione were tested as *Pf*Glo inhibitors, exhibiting activity in lower nanomolar range [19, 32, 33].

Although *P. falciparum* possesses a cytosolic glyoxalase system (comprising cytosolic *Pf*GloI and *Pf*GloII), the sequences of two additional glyoxalase-like enzymes were identified in the genome of *P. falciparum*: a putative GloI, which is smaller in size than *Pf*GloI (a glyoxalase-1-like-protein, GILP), and a putative second *Pf*GloII with a targeting sequence (tGloII).

By means of localization assays using GFP-fusion, it was determined that both GILP and tGloII present apicoplastic localization. In addition, tGloII was found to localize also in the cytosol. Further studies will reveal the roles of these different enzymes in GSH metabolism and detoxification processes [26,32].

2.9.2. Glutathione-S-Transferase

Glutathione-*S*-transferases (GST) are enzymes that catalyze the nucleophilic attack of sulfur atom of GSH (conjugation) on the electrophilic center from the second substrate [16]:

$$\mathrm{GSH} + \mathrm{R\text{-}X} \xrightarrow{\mathrm{GST}} \mathrm{R\text{-}SG} + \mathrm{X}^- + \mathrm{H}^+$$

These enzymes participate in the intracellular detoxification of mutagens, carcinogens, noxious chemical substances and byproducts of oxidative stress. Multiple isoforms of GST have been revealed in virtually every organism where GST activity was detected [4]. Unlike higher eukaryotes, malaria parasites only possess a single gene encoding GST. The *P. falciparum* GST (*Pf*GST) is a homodimer.

Each subunit has a size of 26 kDa and shares up to 37% sequence identity with known GSTs. The enzyme exhibits a cytoplasmatic localization and represents 1 to 10% of the cellular protein. *Pf*GST catalyzes the GSH-dependent modification of 1-chloro-2, 4-dinitrobenzene (a typical GST substrate). *In vitro Pf*GST shows a slight alkyl hydroperoxide peroxidase activity which might have an essential role as glutathione peroxidases for parasite survival [34,35].

The three-dimensional structure of *Pf*GST has some similarities to μ-class GSTs. However, two marked differences have been found: 1) *Pf*GST has a larger hydrophobic binding pocket than μ-class mammalian GSTs, and 2) the C-terminus of the protein is truncated in comparison to other GSTs [35,36].

These differences cause that the *Pf*GST binding pocket could be more solvent-accessible, because the C-terminal of μ-class GSTs structurally restricts access of substrates into the hydrophobic binding pocket [36]. This could explain why *Pf*GST detoxifies a wide substrate range and why these parasites do not require as many GST isoforms as do other eukaryotes.

GST activity in malaria parasites is directly and positively related to drug pressure. A number of *Pf*GST inhibitors, including FP IX, have already been identified [35]. The protein acts as efficient kidnapper of free FP IX, through the binding to FP IX, and this result in severe uncompetitive inhibition of the GST activity.

Unfortunately, neither of the structural analyses refers to the potential binding site for FP IX. This intriguing phenomenon remains to be investigated in future structural studies.

2.10. Other Antioxidant Enzymes Collaboratingwith the GSH-Dependent System

2.10.1. Superoxide Dismutase

Superoxide dismutases (SODs) are metalloproteins that use copper/zinc, iron or manganese as metal cofactor [1]. The enzyme catalyzes the dismutation of $O_2^{\cdot -}$ into O_2 and H_2O_2, thus representing a relevant antioxidant defense in almost organisms. *In vivo*, SOD constitutes the first line of defense against ROS, protecting to cells and tissues from oxidative destruction.

Eukaryotic cells have several forms of SOD, being Cu/ZnSOD mainly in cytoplasm and MnSOD in mitochondria. Many parasitic protozoa have only FeSOD.

The enzyme SOD is biologically essential because $O_2^{\cdot -}$ reacts even faster with another biological radicals such as NO, generating a most toxic component: $ONOO^-$. SOD has the fastest turnover number of any known enzyme (~10^9 $M^{-1}s^{-1}$), being this reaction only limited by the collision frequency between the enzyme and the substrate $O_2^{\cdot -}$ [37,38].

P. falciparum possesses two distinct genes that encode different SODs. One of them is a cytosolic Fe-SOD (*Pf*SOD-1) that appears to be transcribed and expressed throughout the erythrocytic cycle of the parasite [39,40]. It is unlikely that it acts on superoxide anions produced during hemoglobin digestion. The second *P. falciparum* SOD (*Pf*SOD-2) is a mitochondrial protein.

The presence of a mitochondrial SOD seems to be crucial in this parasite, which have an active respiratory chain which inevitably produces high concentration of superoxide anions [41,42]. These have to be detoxified by a mitochondrial SOD to prevent damage to metabolic functions, nucleic acids, proteins and membranes of the organelle.

2.10.2. Thioredoxin System

The thioredoxin system includes the proteins thioredoxin reductase (TRXR) and thioredoxin (TRX) [13]. TRXs are small redox proteins (~12 kDa) with two cysteine residues in its redox active site within the conserved WCGPC motif [1]. TRX can exist either in reduced [dithiol, TRX-$(SH)_2$] or in oxidized (disulfide, TRX-S_2) forms [13]. The function of TRX as a thiol reductant is based on the fast reaction between reduced TRX and disulfide protein substrates, such as ribonucleotide reductase, methionine sulfoxide reductase, 2-Cys peroxiredoxins, and a number of transcription factors. This reductive capacity is linked to the reductive power of NADPH to disulfide reduction of target proteins, according to the following reduction equivalents transference sequence: NADPH → TRXR → TRX → Target protein. TRXR catalyzes the electrons transfer (NADPH + H^+ + TRX-S_2 → $NADP^+$ + TRX-$(SH)_2$) following a thiol/disulfide exchange mechanism.

P. falciparum thioredoxin reductase (*Pf*TRXR) is a homodimeric (55 kDa per subunit) enzyme with FAD as a prosthetic group, which catalyzes the TRX reduction. Phylogenetic analyses revealed that the protein, similar to dihydrolipoamide dehydrogenase, trypanothione reductase, glutathione reductase and mercuric ion reductase, belong to flavoenzyme disulfide oxidoreductases family [43]. The physiologic substrates of *Pf*TRXR are the *P. falciparum* thioredoxins (*Pf*TRXs) and NADPH, which are reduced/oxidized with high turnover (~10^6-10^7 $M^{-1}s^{-1}$). In addition, *Pf*TRXR showed NADPH-dependent *S*-nitrosoglutathione (GSNO) reductase activity with low efficiency (10^3 $M^{-1}s^{-1}$). However, GSSG was not substrate for the enzyme [43,44]. Despite the protein is encoded by a single copy gene within the Plasmodium genome, *Pf*TRXR has both cytoplasmatic and mitochondrial cellular localization The mitochondrion targeted isoform is generated by an alternative translation initiation (ATI)-site upstream of the canonical translation start ATG codon (which generates a protein with a mitochondrial signal peptide) [26].

The *Pf*TRXR is high molecular mass TRXR like mammalian enzyme, but there are significant differences between the active sites of parasite and host proteins [45]. *Pf*TRXR contains two disulfide catalytic relay centers. In each center, electrons are transferred from NADPH via flavin to the cysteine pair C88/C93, subsequently to the C-terminal cysteine pair C535'/C540´ of the other subunit, and from there to the substrate [45,46]. The C-terminal redox-active centre of the mammalian enzyme comprises a selenocysteine-cysteine pair (UC) whereas the *Pf*TRXR contains a CXXXXC motif instead [46]. This chemical difference has been suggested to be sufficient to allow the design of specific inhibitors against the parasite enzyme. Importantly, the *P. falciparum trxR* gene has been genetically validated as a potential drug target as its disruption proved to be lethal for the parasites [47,48]. Thus, the protein provides good potential for the design of inhibitors that specifically target parasite survival [47,49].

P. falciparum possesses at least two classical thioredoxins that present a typical WCGPC sequence in their redox active site: *Pf*TRX1 and *Pf*TRX2 [26]. *Pf*TRX1 is a soluble cytoplasmatic thioredoxin that in addition to active-cysteines, has one more cysteine at position 43, which would be responsible for dimer formation [43]. Reduced *Pf*TRX1 is an efficient electron donor to cytoplasmatic thioredoxin peroxidase 1 (*Pf*TPx1, ~10^6 $M^{-1}s^{-1}$) [50] and no for SeCys glutathione peroxidase (*Pf*GPx, ~10^4 $M^{-1}s^{-1}$) [17]. Furthermore, it show a significant capacity for reduce GSSG and GSNO at physiologic concentrations [43]. In the presence of NADPH and *Pf*TRXR, *Pt*TRX1 acts as an electron shuttle between reduced TRXR and GSSG:

$$\mathrm{NADPH} + \mathrm{H}^+ + \mathrm{TRX\text{-}S_2} \xrightarrow{\mathrm{TRXR}} \mathrm{NADP}^+ + \mathrm{TRX\text{-}(SH)_2}$$
$$\mathrm{TRX\text{-}(SH)_2} + \mathrm{GSSG} \rightarrow \mathrm{TRX\text{-}S_2} + 2\,\mathrm{GSH}$$

The second-order rate constant for the reaction is 650 $M^{-1}s^{-1}$ [43], which allows rates that can satisfy most metabolic needs. This activity might be particularly relevant for stages of *P. falciparum*, such as the merozoites, which contain glutathione but practically no glutathione reductase [6]. The GSSG reduction by the thioredoxin system plays a role in a variety of physiologic, pharmacologic, and pathologic conditions. In addition, GSNO (that is not reduced by *Pf*GR and is a weak substrate of *Pf*TRXR), can be efficiently reduced *in vivo* by *Pt*TRX1, with a rate constant of 567 $M^{-1}s^{-1}$ [43]. Based on a *Pt*TRX1 mutant

(*Pf*TRX1C33S) affinity chromatography, several target proteins were identified for cytosolic TRX [51]. The proteins identified included those involved in pathways such as the redox cycle, protein biosynthesis, energy metabolism, signal transduction, and *S*-adenosylmethionine metabolism [51,52]. This approach led to the discovery of several putatively redox-regulated proteins and contributed to the understanding of the redox interactome in malarial parasites.

The second TRX (*Pt*TRX2) exhibited a mitochondrial localization when analyzed by green fluorescent protein fusion; however, strong evidence has been provided that *Pt*TRX2 is also targeted to the parasitophorous vacuole [26]. The actual data suggest that this TRX is the natural partner of *Pf*TPx2, a mitochondrial peroxiredoxin. *Pt*TRX2 was found to be a specific reducing substrate for this peroxiredoxin (~10^5 $M^{-1}s^{-1}$), neither GSH nor dihydrolipoamide presented capacity to reduce *Pt*TPx2 [53]. Alternatively, a non-classical third TRX has been described in *P. falciparum*, *Pt*TRX3, which exhibit endoplasmatic reticulum localization. This agreed with the prediction of a transmembrane helix in the N-terminal region of the protein [26].

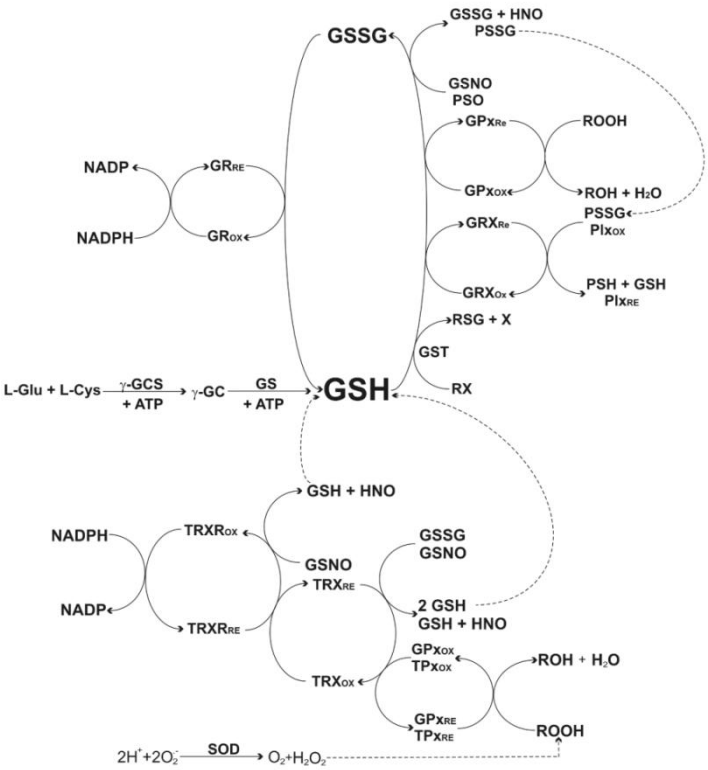

Figure 1. Schematic representation of a redox scenario associated to glutathione and thioredoxin system in *Plasmodium falciparum*. L-Cys, L- cysteine; L-Glu, L-glutamine; γ-GC, gamma glutamil cysteine; γ-GCS, gamma glutamil cysteine synthetase; L-glycine, L-glycine; GSH, glutathione; ; GR, glutathione reductase; TRX, thioredoxin; TRXR, thioredoxin reductase; TPx, thioredoxin peroxidase, GPx, glutathione peroxidase; GRX, glutaredoxin; GSSG, oxidized glutathione; GSNO, S-nitrosoglutathione; HNO, nitrosyl acid; PSSG, glutathionylated protein; PSO, sulfenic acid protein; Plx, plasmoredoxin; GST, glutathione S-transferase; RX, electrophilic compound; RSG, glutathionylated compound; ROOH, hydroperoxide; SOD, superoxide dismutase.

3. A Peculiar Thiol Redox Metabolism Operates in Trypanosomatids

Trypanosomatids are protozoa parasites belonging to the order *Kinetoplastida*. Two genera inside this family are of relevance from the point of view of human and animal health. *Trypanosoma* and *Leishmania*. *T. brucei gambiense* and *T. b. rhodesiense* are causative agents of African sleeping sickness; whereas *T. congolense*, *T. vivax* and *T. b. brucei* are causative agents of Nagana, a disease of cattle and other animals of interest in agriculture.

Another parasite of significance, inside this family, is *T. cruzi*, the etiologic agent of Chagas' disease, while several species of the genus *Leishmania* are responsible for both visceral and cutaneous forms of leishmaniasis [54]. These parasitic illnesses are considered neglected diseases by the World Health Organization.

Most of the trypanosomatids have complex life cycles. They can live in more than one host species, including a bloodsucking insect and the blood and/or tissues of a vertebrate. Trypanosomatids transmitted by hematophagous insects to their hosts have a digenetic life, being necessary to undergo biochemical and morphological adaptations to survive in both vertebrate and invertebrate hosts [55]. *T. cruzi*, for example, spends a part of his life into an insect vector (triatomine bug). When the insect feeds on blood of an infected vertebrate host, *T. cruzi* passes to the insect. In its gut of this vector the parasite become epimastigote, a replicative form of the parasite. Once in the insect rectum, *T. cruzi* changes again its morphology from epimastigote to metacyclic trypomastigote form, a non-replicative but infective form. They can infect both professional and non-professional phagocytic cells, were they live in the replicative amastigote form. From these cells, amastigotes can transform into trypomastigotes, burst out of the cell to infect other cells. Trypomastigotes could infect a mammal host through the insect feces at the place of the sting, wounds, ocular conjunctiva or other mucosal membranes [55-57]. More information about the life cycle, genome and biology of the different human pathogenic trypanosomatids can be obtained from the web site of the TriTrypDB Kinetoplastids Genomics Resources, Wellcome Trust (http://tritrypdb.org/tritrypdb/).

Despite existing differences among species and stages in the life cycles of these parasites, they are physiological, biochemical and genetically similar between them and different to prokaryotes and other eukaryotes.

The biochemical peculiarities are of special interest for in depth analysis and development of new drugs. Thus, for instance, this family of parasites has a specific thiol redox metabolism, which is mostly dependent of trypanothione ([bis (glutathionyl) spermidine]), instead of glutathione as in most organisms [58, 59].

In fact, glutathione is in trypanosomatids one of the key precursors for trypanothione synthesis [60-62]. Other putative functions of glutathione are summarized in this chapter. In contrast to the advances on bacterial infections therapy, treatment of parasitic diseases, particularly infections caused by protozoa, still depend on drugs that act on unspecific targets.

Thus, the treatment of African trypanosomiasis, the different clinical forms of leishmaniasis and Chagas' disease, have numerous side effects due to toxicity of drugs like organometallic derivatives of arsenic (or antimony), nifurtimox or benznidazole [54]. Nowadays, an increasing number of scientific studies focus to the characterization of different metabolic pathways in trypanosomatids. This makes possible the rational design of new

agents for specific therapy. The concretion of the genome projects of representative kinetoplastids has evidenced that trypanosomatids lack genes coding for relevant enzymes in the redox homeostasis of their host cells, such as glutathione reductase, thioredoxin reductase, catalase and selenocystein-containing glutathione peroxidases [63,64].

The absence of these genes, and consequently the enzymes codified by them, has encouraged the exploration of the pathways involved in redox homeostasis in trypanosomatids, resulting in the elucidation of a peculiar thiol-dependent redox metabolism.

Peculiarities of this metabolism are interesting not only from a therapeutic point of view, but also from an evolutionary one, being trypanosomatids an early branch in the evolution of eukaryotic organisms.

3.1. Low Molecular Weight Thiols in Trypanosomatids

Trypanothione [N^1, N^8-bis(glutationyl)spermidine; $T(SH)_2$] is the predominant and most important low molecular weight thiol in trypanosomatids. It is the central thiol in the antioxidant defense, and it replaces glutathione in most redox reaction involving thiols (thiol-disulfide exchange) in these parasites [58,59,65]. Trypanothione is conformed by two glutathione molecules covalently linked to spermidine [66-68]. In fact, the majority of glutathione in trypanosomatids, is used to synthesize trypanothione, remaining in much less extent either as N8-glutationylspermidine (GspSH) or free GSH [58,68].

Table 1. Thiol levels in representative trypanosomatids at the different stages of lyfe cycle

Organism	Stage	Thiol content (nmol/10^8 cells)		
		GSH	GspSH	$T(SH)_2$
L. donovani	Promastigote*	4.8 (± 1)	1.6 (± 0.6)	6.3 (± 0.5)
	Amastigote**	0.24 (± 0.04)	0.04 (± .01)	0.45 (± .01)
T. cruzi	Epimastigote*	2.1 (± 0.2)	0.8 (± 0.1)	6.4 (± 0.2)
	Trypomastigote (cell culture derived)**	0.41 (± 0.3)	0.17 (± 0.1)	0.95(± 0.34)
	Amastigote (in cell culture)** ‡	0.42 (± 0.04)	0.07 (± 0.01)	0.12 (± 0.04)
T. brucei	Procyclic trypomastigote*	2.1 (± 0.2)	0.7 (± 0.3)	3.3 (± 0.5)
	Bloodstream trypomastigote**	1.5 (± 0.1)	0.21 (± 0.06)	0.42 (± 0.2)

Adapted from: [69]. * Insect stages.** Mammalian stages.‡ Reported in: [67].

In *T. cruzi*, *Leishmania* spp. and in *Crithidia fasciculata* (a non-pathogenic kinetoplastid, which have a life cycle completely developed in an insect host) another thiol called ovothiol A (N^1-metyl-4-mercaptohistidin) was found [69], being its functional relevance still not in depth explored. Concentration of each low molecular weight thiol and its respective oxidized form depends on the parasite stage, the cellular redox status, or even on the growth phase in the case of axenic cultures. Some data related to this quantitative aspect are exemplified in Table 1. The reduced form of trypanothione, $T(SH)_2$ is a more powerful reducer than GSH, even when their redox potential are very similar (-242 and -240 mV, respectively) [70,71]. The difference in reducing capacity can be explained by other properties. For example, while cysteines p*K*a for $T(SH)_2$ is 7.4, for GSH is 8.7 [70]. This difference implies that at pH 7.4, which is approximately the trypanosomatid intracellular pH, the thiolate ionization percentage is greater for $T(SH)_2$ than for GSH. The higher ionization percentage increases its reactivity in thiol-disulfide exchange reactions [68], which is maintained in a range of pH from 5.5 to 9.5 [70]. Moreover, the supremacy of this thiol resides in the fact that its dithiolic character favors kinetically the disulfide formation, in this case, an intramolecular disulfide.

3.2. Biosynthesis of Glutathione and Trypanothione in Trypanosomatids

In trypanosomatids, like in other organisms, GSH synthesis is performed from its precursor amino acids L-glutamate, L-cysteine and L-glycine through two catalyzed reactions. The first reaction is mediated by γ-glutamylcysteine synthetase (γ-GCS), and the second one by glutathione synthetase [71]. The first reaction is the limiting step of the synthetic process, being the most studied in these protozoa, particularly in *T. brucei* [72]. Structurally, the amino acidic sequence of *T. brucei* γ-GCS (*Tb*γ-GCS) shows 36 to 45% identity with those γ-GCS from other eukaryotes such as rat, human, *Caenorhabditis elegans* or *Saccharomyces cerevisiae*, for example. Unlike most trypanosomal genes, the gene encoding γ-GCS is a simple copy gene. The protein was obtained in a recombinant way characterized biochemically, being its quaternary structure a monomer with a molecular mass of 75 kDa [72]. It catalyzes the ligation reaction of L-glutamine with L-cysteine, and it displays a specific activity similar to that reported for γ-GCS from rat or from *Ascaris suum.* However, the specificity of substrate for *Tb*γ-GCS has some differences with the enzyme from rat or *A. suum* [72]. These results and other similar have revealed that a regulatory domain is not necessary in trypanosomatids, making an important difference with the mammalian synthetase.

Studies of reverse genetic related to *Tb*γ-GCS have been performed by RNA interference (RNAi) in procyclic form of *T. brucei* [73], showing depletion of *Tb*γ-GCS transcripts, low levels of γ-GCS, growth arrest and death in the parasites. It is indicating univocally the essentiality of this enzyme. The reduction of *Tb*γ-GCS activity not only reduces the intracellular concentration of GSH but also the levels $T(SH)_2$ and GspSH. When parasites deficient in γ-GCS were cultured in the presence of externally supplied GSH, the deleterious effects of this deficiency were reverted, even at the intracellular thiol level. These experiments indirectly show that requirements of GSH in trypanosomatids could be supplied *in vitro*. Thus, it could be speculated with the action of a putative parasite transporter of γ-GC to obtain γ-GC from the mammalian host. Some inhibitors of γ-GCS were analyzed. For

instance, buthionine sulfoximine (BSO), which is a proved strong inhibitor of γ-GCS, showed effectiveness to cure or extend life of mice infected with *T. brucei* [74]. However, it was suggested that γ-GCS could not be the unique target of BSO in trypanosomatids [73] after the external supply of GSH to parasite cultures treated with BSO was not able to restore intracellular parasite levels, unlike the case of cultures lacking γ-GCS after RNAi assays.

There are several pathways for GSSG reduction to recover GSH levels in these parasites. There is neither evidences about functionality of these pathways in every stage or organelle, nor if they operate simultaneously. One of these metabolic possibilities is the non-enzymatic reduction of GSH by $T(SH)_2$ [75]. Lipoic acid [76] could be also involved in thiol disulfide exchange reactions like the reduction of GSSG; however, the viability of this reaction has been disputed because the marginal concentrations of lipoate found in trypanosomatids cells [77].

Recently, as a part of the characterization of a functional dithiolic glutaredoxin in *T. cruzi* (*Tc*GRX), it was evidenced that electronic transference from $T(SH)_2$ to GSSG can be mediated by *Tc*GRX *in vitro* [78]. In *T. brucei* two glutaredoxins, *Tb*GRX1 and *Tb*GRX2, can catalyze GSSG reduction by $T(SH)_2$ [79]. For this reaction activities of *Tb*GRX2 and *Tc*GRX are similar, while *Tb*GRX1 activity is 50-fold higher. Other candidate, a 52 kDa protein having thioltransferase activity was reported to catalyze this reaction too [75]. In general, it has been considered that kinetic constants for direct transference of reduction equivalents from $T(SH)_2$ to GSSG support the feasibility of this reaction. However, the probability of this non catalytic reaction must be reconsidered when ratio $TS_2/T(SH)_2$ is high, meaning under oxidative stress situation.

Spermidine and glutathione pathways of synthesis are connected in the biosynthesis of trypanothione, since both are precursors of this thiol [71]. Trypanothione synthesis occurs in two consecutive steps, catalyzed by trypanothione synthetase (TS) [60,61,66]. In the first step a molecule of GSH is conjugated to spermidine to form GspSH. Then a second molecule of GSH is added to GspSH in a similar way, forming $T(SH)_2$. Both catalytic steps occur with ATP hydrolysis. TS from *C. fasciculata* [60], *T. cruzi* [80], *L. major* [61] and *T. brucei* [62] have been evidenced and quite widely characterized. Appart from TS, in *C. fasciculata* there is a specific glutathionylspermidine synthetase (GspS), which can catalyze the first step of the synthesis, that is the synthesis of GspSH, this step cannot by catalyzed by *C. fasciculata* TS [60]. In other words, both metabolic steps of $T(SH)_2$ synthesis in *C. fasciculata* are catalyzed by different enzymes. *T. cruzi* is the only human pathogen having a gene encoding a GspS in its genome. Until now, this enzyme has been not characterized, so it could be speculated that TS would be not the only enzyme involved in $T(SH)_2$ synthesis in *T. cruzi*.

The kinetic properties of *Tc*TS, *Cf*TS and *Cf*GspS [60,80] are very similar. It was verified that *Tc*TS is able to catalyze the synthesis of a $T(SH)_2$ homolog namely homotrypanothione, using aminopropylcadaverine [81]. However, homotrypanothione was only found *in vivo* when cultures of *T. cruzi* were supplied with cadaverine or spermine. Indeed, when cultures are supplemented with equivalent quantities of spermine and spermidine, dithiol synthesis occurs preferably using the second one. It was speculated that a wide specificity of substrate for this synthesis could be an advantage selected in *T. cruzi* because of its apparent incapacity to synthesize polyamines *de novo*. However, such a hypothesis was not verified yet.

Both TS and GspS have additional amidase activity in absence of ATP. This is associated to the N-terminal domain in each protein and renders $T(SH)_2$ hydrolysis: GspSH, GSH and

spermidine [60,80]. It was hypothesized (in *T. cruzi* and *C. fasciculata*) that the existence of these antagonist activities in the same molecule could have a regulatory function related to maintenance of polyamine levels, as a response to availability, stage or growth phase. Although, some advances in the elucidation of these mechanisms were reached, more studies are necessary to explain these aspects and establish differences among different trypanosomatids [82].

Despite it was initially hypothesized that $T(SH)_2$ localized in the cytoplasm, in *T. cruzi* $T(SH)_2$ biosynthesis could take place in other cellular compartments if we consider the localization of enzyme using this thiol as electron donor. However, this hypothesis was not univocally proved [58]. Validation of TS as a drug target was carried out in *T. brucei* working with bloodstream forms and following the RNAi methodology [83]. In knocked-down parasites, it was possible to diminish but not to suppress *Tb*TS expression. In these parasites $T(SH)_2$ levels were significantly reduced, therefore resulting in cell growth arrest and loss of viability. Moreover, it was established that knocked-down parasites manifested increased susceptibility to peroxides such as H_2O_2. This phenotype evidenced a morphological phenomena associated to damage in plasmatic membrane, that could be related to an impaired hydroperoxides detoxification metabolism, as a consequence of $T(SH)_2$ depletion.

Both $T(SH)_2$ and GspSH are maintained in their reduced state by the activity of the enzyme trypanothione reductase (TR) [84]. This is the first enzyme in every metabolic pathway depending on $T(SH)_2$, taking electron from NADPH. TR is a flavoenzyme that shares several physicochemical and biochemical properties with GR from other organisms, being the substrate specificity the main difference. TS_2 can be reduced only by TR, being this enzyme not able to catalyze the reduction of GSSG.

This substrate specificity could be explained analyzing the amino acids composition in the substrate binding site of TR, which is creating in the TR enzymatic site a wider, more hydrophobic and relatively more negatively charged pocket than that from GR [68]. TR cellular localization could be not univocally determined; in bloodstream procyclic forms of *T. bucei* the enzyme was localized in cytosol, while in *T. cruzi* it was found both in cytosol and mitochondria [85,86].

Studies of down regulation of TR performed by means of partial or conditional knock out [87,88] revealed that TR is an essential enzyme in *L. donovani*. However, it must be highlighted that low levels of TR activity are satisfactory for maintaining intracellular trypanothione in its reduced state, even when these levels are sub-optimal for cell growing. When this phenotype was obtained in *Leishmania* it was possible to reduce TR activity 85%, in other words 15% remaining activity, without detrimental effects on parasite proliferation in axenic conditions of culture, while having an impaired ability to survive inside macrophages. Conditional knock out phenotype in *T. brucei* showed proliferation arrest after 90% depletion of TR activity. However, levels of total thiols were kept constant, which could be indicating a rise in the thiols synthesis. Taken together these results suggest that depletion of TR activity and compensatory up regulation of TS might act simultaneously to maintain thiol levels in trypanosomatids. Metabolic pathways downstream TR could be seriously affected with lowered concentrations of reducing equivalents coming from $T(SH)_2$ (for example, the synthesis of deoxyribonucleotides by ribonucleotide reductase), supporting TR as a new drug target [89].

3.3. Biological Roles of Glutathione and Trypanothione in Trypanosomatids

As mentioned above, redox metabolism in kinetoplastids is highly different to other eukaryotes, since the central role in hydroperoxides (and other ROS) detoxification is performed by trypanothione instead of glutathione. Although the most important function of GSH is as a precursor in $T(SH)_2$ synthesis, it is probable that other specific roles could be played by GSH in trypanosomatids. The compounds involved in the most relevant reactions depending on $T(SH)_2$ and those involving GSH are detailed afterward, being schematized in Figure 2.

Together with TR and $T(SH)_2$ there are other proteins of preponderant functions in the redox metabolism of trypanosomatids. One of them is a dithiolic protein called tryparedoxin (TXN) [90]. This oxidoreductase has a CPPC amino acidic motif in its active site and belongs to the thioredoxin family of proteins. TXN has been studied as a part of a complex system of hydroperoxides detoxification [91], being characterized from *C. fasciculata* [90], *T. cruzi* [92], *T. brucei* [93] and *L. infantum* [94]. TXN can be localized in both cytosol and mitochondria.

It has been characterized exclusively in trypanosomatids, where became as one of the crucial component of this particular redox metabolism. However, TXNs share several functional characteristics with GRXs and TRXs, the former protein being reduced directly by $T(SH)_2$ (Km values from 30 to 150 μM were reported for TXNs of different species). The couple $T(SH)_2$ /TXN [$T(SH)_2$ is in a range of 0.2 to 1.5 mM in the parasitic cells] is the main electron donor for the reduction of mixed disulfides formed between low molecular weight thiols and proteins as well as of thiols in proteins (such as ribonucleotide reductase) and of different peroxidases [such as trypanothione peroxidase (TXNPx) and glutathione peroxidase (GPx1)] [89,95-97]. The proposed action mechanism of TXNs is similar to that for TRX, GRX and other thioltransferases [98], the thiolate anion (formed when N-terminal cysteine in the active site is deprotoned) is nucleophilic and reacts with a specific substrate, forming a mixed disulfide between the TXN sulfhydryl group and that in the molecule target.

Then, the C-terminal cysteine in TXN attacks this mixed disulfide and delivers the target protein in the reduced form and oxidized TXN, which is then reduced non enzymatically by $T(SH)_2$. In other cell types, such as mammalian cells, thioredoxin plays an interesting role in regulation of cellular processes, through inter-conversion of disulfides-thiols in proteins (examples are given in [99,100]).

Some of these processes operate in conditions that not necessarily implicate hydroperoxides detoxification. It has been speculated that most of these roles played by TRX in other cell types could be played by TXN in trypanosomatids, especially considering that even when a TRX is present in the latter organisms [101] its concentrations is low and its physiological reductant action is unknown. *In vivo* pull down assays were originally performed to elucidate *Tc*TXN1 interactome [102]. Following this strategy it was possible to identify several targets for *Tc*TXN1, some of them involved in polyamines metabolism or in the synthesis and degradation of proteins, for example.

The NADPH dependent hydroperoxide detoxification has a relevant peroxiredoxin working together to the $T(SH)_2$/TXN system, the tryparedoxin peroxidase (TXNPx) [58,96].

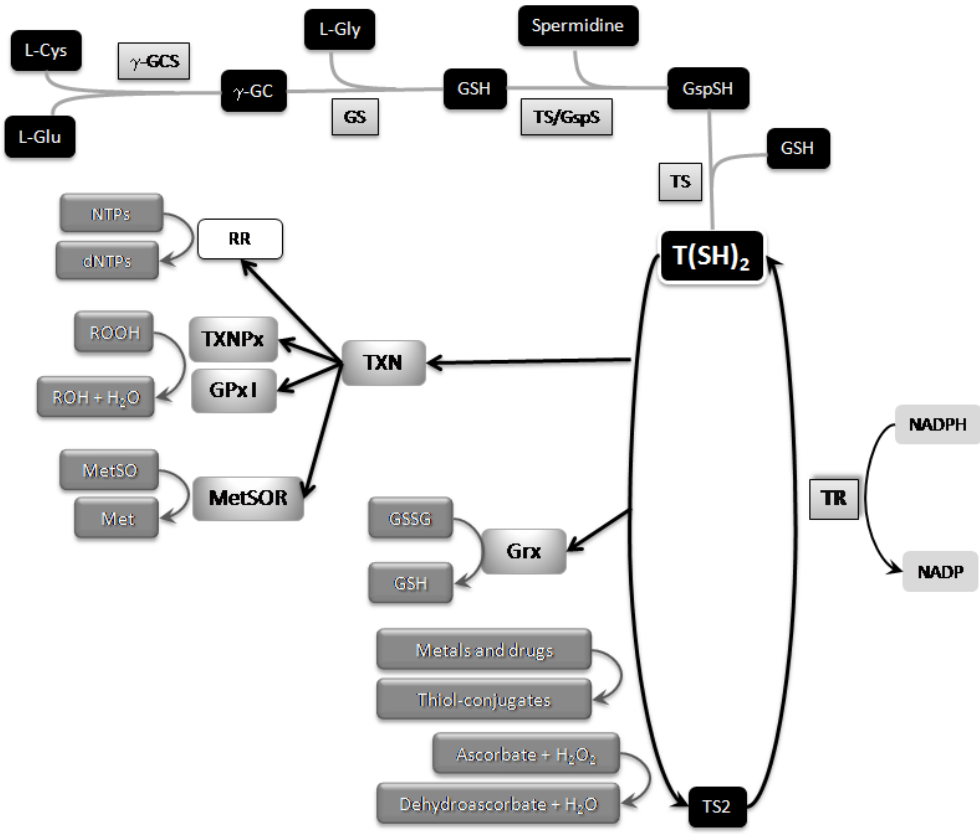

Figure 2. Relevant reactions in trypanothione-based redox metabolism. L-Cys, L- cysteine; L-Glu, L-glutamine; γ-GC, gamma glutamil cysteine; γ-GCS, gamma glutamil cysteine synthetase; L-glycine, L-glycine; GSH, glutathione; TS, trypanothione syntthetase; GspS, glutathionylspermidine synthetase; GspSH, glutathionylspermidine; $T(SH)_2$, trypanothione; TR, trypanothione reductase; TS_2, oxidized trypanothione; TXN, tryparedoxin; RR ribonucleotide reductase; TXNPx, tryparedoxin peroxidase, GPxI, glutathione peroxidase I; MetSOR, methionine sulfoxide reductase; GRX, glutaredoxin; GSSG, oxidized glutathione; ROOH, hydroperoxide.

TXNPx is characterized by the presence of conserved cysteines in VCP conserved motifs in the active site [68]. It was established that trypanosomatids codify en their genomes several identical copies of these TXNPx. Homolog enzymes were characterized in *T. cruzi* [103], *C. fasciculata* [104] [95], *T. brucei* [105], *L. donovani* [96] and *L. infantum* [106], evidencing in most cases the presence of cytoplasmic and mitochondrial forms for this enzyme.

Although TXNPx activities are several orders of magnitude lower than classically efficient glutathione peroxidase (containing selenocysteine), this deficiency is partially balanced with the high concentration of this enzyme in trypanosomatids. For example, abundance of TXNPx in *C. fasciculata* represents approximately 6% of the total protein [95]. However, it is worth to remark that trypanosomatids only tolerate low levels of hydroperoxides. These peroxiredoxins have a low specificity of substrate, they are able to detoxify peroxides such as H_2O_2, t-butyl hydroperoxide, fatty acid hydroperoxides, and they also are effective in peroxinitrite detoxification. It has been established that these proteins,

with ca. 20 kDa in molecular mass, are active exclusively in oligomeric form. Their mechanisms, kinetics properties and structures are object of several studies.

Several non-selenocysteine containing glutathione peroxidases (GPx) are codified in the genome of *T. cruzi* and several homologues have been identified also in *T. brucei* and *L. major* [97] [6]. Two of them, *Tc*GPxI and *Tc*GPxII from *T. cruzi* have been deeply characterized [107] [108]. Both are able to decompose organic peroxides, particularly those derived from phospholipids, but they cannot decompose hydrogen peroxide. However, some differences among them exist in the profiles of the hydroperoxides metabolized, for example short chain alkyl hydroperoxides, such as cumene hydroperoxide or t-butyl hydroperoxide are substrates for *Tc*GPxI, but not for *Tc*GPxII. It was postulated that these GPx should perform functions in preventing damage derived from membrane lipid peroxidation [97]. It has been established that *Tc*GPxI is localized in cytosol and in glycosomes [103,105,109], while *Tc*GPxII is localized in endoplasmic reticulum [108]. The affinity of these GPxs for GSH is quite low (Km is approximately 6 mM) [97]. It has been demonstrated that *Tc*GPxI is active in a system having as electron donor both $T(SH)_2$ /TXN and GSH. *Tc*GPxII can accept electrons from GSH exclusively. Neither *Tc*GPxI, nor *Tc*GPxII are directly reduced by $T(SH)_2$.

Additionally, dithiolic glutaredoxins were characterized both in *T. cruzi* (*Tc*GRX) [78] and *T. brucei* (*Tb*GRX1 and *Tb*GRX2) [79]. Characterization of recombinant *Tc*GRX showed that glutaredoxin was able to catalyze reduction of mixed disulfides formed between hydroxiethyldisulfide (HEDS) and GSH, or between protein thiols and GSH (glutathionylated proteins). *Tc*GRX can use GSH, $T(SH)_2$ or GspSH as electron donors, displaying similar activities in all cases. It is unable to reduce intramolecular disulfides in proteins, such as insulin (as a model disulfide) or trypanosomal specific proteins involved in redox metabolism (TXNPx, GPx, etc). In *T. brucei*, both GRXs were localized in different places and they can also perform deglutathionylation. Further identification of partners for these thioltranferases in trypanosomatids could be important to in depth analyze the involvement of GRXs in regulatory processes of enzymatic activities by glutathionylation-deglutathionylation reactions [110].

4. Entamoeba Histolytica: An Eukaryote without Glutathione

Amoebas are anaerobic unicellular parasites characterized by the presence of pseudopodia that are used for movement and uptake of food. These organisms belong to the phylum Amoebozoa and the class Archamoebae [111]. Several species within the genus *Entamoeba* can infect humans, but only *Entamoeba histolytica* is of medical importance [112]. *E. histolytica* has a simple life cycle that comprises an infectious cyst form and an amoeboid trophozoite stage. The cysts have a thick wall made partly of chitin, which makes them resistant to both environmental stress and the gastric acid in the stomach of the host. Humans and occasionally non-human primates [113] are the only natural hosts of *E. histolytica*, being limited the zoonotic importance of this parasite. Infection occurs when cysts are transmitted by fecal-oral route (ingestion of contaminated food or water) or through person-to-person contact. Excystation occurs in the small intestine, and the trophozoites that

are released migrate to the large intestine, where they reproduce by binary fission. Encystation takes place in the colon, thus completing the life cycle by excretion of cysts in the stool. In cases involving diarrhea, trophozoites can be excreted as well, but they can survive for only a short time outside the body of the host. Infections that remain luminal are usually asymptomatic.

Clinical amoebiasis occurs only when the trophozoites disrupt mucosal barrier and penetrate the colon wall, which causes ulcers that lead to amoebic dysentery. Much less frequently, trophozoites are spread through the portal vein to the liver and, very rarely, they even disseminate to other organs such as lungs and brain.

E. histolytica trophozoites usually reside and multiply within the human gut, which constitutes an anaerobic or microaerophilic (up to 5% oxygen in the gas phase) environment. However, during tissue invasion, the parasites are exposed to an increased oxygen pressure and have to eliminate toxic metabolites such as ROS or reactive nitrogen species (RNS) produced by activated phagocytes during respiratory burst [114].

The optimal antimicrobial activity of neutrophils and other phagocytes is based on the production of superoxide anions ($O_2^{\bullet-}$) by activated NADPH oxidases. This $O_2^{\bullet-}$ can be then processed to give both hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), hypochlorous acid (HOCl), and oxidizing derivatives of nitric oxide (NO) such as peroxynitrite ($ONOO^-$) [115]. To fight against oxidative death caused by ROS generated during inflammation or after been phagocytosed by macrophages, many parasites have developed multilayered defense systems, including DNA repair systems, scavenging substrates, and antioxidant enzyme systems. *E. histolytica* lacks most of the components of the eukaryotic oxidative stress defense systems including catalase, peroxidase, glutathione, and glutathione-recycling enzymes. However, it possesses alternative mechanisms for detoxification of the reactive oxygen and nitrogen species. In these parasites, alternative mechanisms involve enzyme activities like superoxide dismutase (SOD), peroxiredoxin, and flavodiiron proteins (FDPs), and antioxidant agents (thiols), especially L-cysteine [116-118].

In most organisms, the glutathione cycle constitutes one of the primary defense mechanisms against toxic oxygen and nitrogen species. Despite *E. histolytica* lacks glutathione reductase activity, and the inability to synthesize glutathione *de novo*, it has been suggested that in this pathogen protozoon exogenous glutathione can be taken up and conjugated to spermidine to produce trypanothione [119]. This affirmation is still a matter of debate [120] because so far trypanothione was only found at physiologically relevant concentration in trypanosomatids. Most pathways for amino acid biosynthesis have been eliminated in this parasite, except those for serine and cysteine, which are probably retained for the production of cysteine [117], the major intracellular thiol present in its reduced form [121]. Thus, cysteine plays an important role in maintaining the redox balance of thiols. The high levels of cysteine in *E. histolytica* may compensate the lack of glutathione and its associated enzymes.

In addition, cysteine provides an inorganic sulfur atom for the biosynthesis of Fe-S clusters, which are important in various proteins, including pyruvate ferredoxin oxidoreductase, ferredoxin, and hydrogenase [122]. Since *E. histolytica* lacks classical mitochondria and the usual aerobic respiratory pathways, the finding that they can grow without glutathione supports the hypothesis that a primary function of glutathione in eukaryotic cells involves protection against oxygen toxicity associated to mitochondria [121].

Among a number of metabolic peculiarities, metabolism of sulfur-containing amino acids in *E. histolytica* is distinct from that of its mammalian host in a variety of aspects. First, it lacks both forward and reverse trans-sulfuration pathways and thus is unable to interconvert L-methionine and L-cysteine [117]. Second, it possesses methionine glyase (MGL) which degrades L-methionine, L-homocysteine, and L-cysteine [123-125]. Third, *E. histolytica* possesses enzymes for the *de novo* S-methylcysteine/L-cysteine biosynthesis [126-128]. The S-methylcysteine/L-cysteine biosynthetic pathway involves serine acetyltransferase (SAT) that catalyzes acetyl CoA-dependent acetylation of the side chain hydroxyl group of L-serine to form O-acetylserine (OAS) [129]. Subsequently, cysteine synthase (CS) catalyzes the reaction of OAS with methanethiol or sulfide to produce S-methylcysteine or L-cysteine, respectively. Recombinant amoebic CS isotypes possess both S-methylcysteine and L-cysteine synthesizing activities *in vitro*. Cultivation of amoebic trophozoites requires high concentrations of L-cysteine, and this requirement can not be replaced by other thiols [130]. In *E. histolytica*, L-cysteine is required for the growth, attachment, survival, and protection from oxidative stress [130,131]. In most eukaryotes, glutathione is the major thiol, and L-cysteine levels are maintained many fold lower than that of glutathione [132]. *E. histolytica* viability relies on L-cysteine / L-cystine as a major redox buffer [116,117,123]. The L-cystine reduction is necessary for the maintenance of the redox balance. This task can be performed by an atypical NADPH-dependent oxidoreductase (*Eh*NO2) [133] or by the tandem system involving thioredoxin reductase and thioredoxin recently described in this organism [134].

The treatment of amoebiasis with metronidazole is closely related to the redox metabolism in this parasite. Different adverse effects were described for metronidazole including anorexia, nausea, vomiting, diarrhea, abdominal discomfort, disulfiram-like alcohol intolerance, and hypersensitivity [135,136]. In pregnant, the use of metronidazole for the treatment of amoebiasis is not currently recommended [137] after the drug can be mutagenic in bacteria and carcinogenic (and teratogenic) in rodents [138-141], being also able to cross placental barrier. Otherwise, neurological side effects like dizziness, vertigo, paresthesias, and, rarely, encephalopathy or convulsions warrant discontinuation of the treatment [142].

Metronidazole enters the cell and its organelles via passive diffusion. It is relatively inert until its 5-nitro group is reduced. Its activation occurs only under strong reducing conditions. Metronidazole is usually activated through the acceptance of electrons from ferredoxin.

The latter is reduced by an enzyme of the amoebic glycolysis pathway, the pyruvate ferredoxin oxidoreductase [143-147]. The reduction of metronidazole results in a nitro-radical form, which binds transiently to DNA to disrupt or break the nucleotide strands, leading to cell death [148-151].

Cases of therapy failure due to metronidazole resistance are occasionally observed. There are no reports of high levels of resistance to metronidazole in clinical isolates of *E. histolytica*. However, inadequate short-term exposure to metronidazole and exposure to sublethal levels of metronidazole could induce increased drug resistance [152,153]. Indeed, under experimental conditions, a step-wise increase in drug concentration induced metronidazole resistance in two axenic cell lines of *E. histolytica* [154].The 5-nitro group from metronidazole can be activated not only by PFOR or ferredoxin but also by thioredoxin reductase [155].

Glutathione is also absent in *Giardia lambia*, another anaerobic parasite. As previously observed in *Entamoeba hystolytica*, after high-pressure liquid chromatography analysis of *Giardia* whole-cell lysate, cysteine seems to be the major low-molecular-weight thiol in this

organism [156]. *G. lambia* lacks both the sulfur assimilatory *de novo* cysteine biosynthetic and reverse trans-sulfuration pathways, being not clear how *Giardia* acquires sufficient methionine and cysteine in the small intestine [157].

The *Giardia* genome revealed the presence of genes coding for two key enzymes required for glutathione synthesis, glutamate-cysteine ligase and glutathione synthase but it is unknown if they are metabolically active. A glutaredoxin from *G. lambia* has been recently characterized; it is a dimeric protein that has a Fe-S cluster but its exact chemical structure was not determined. For homolog proteins two glutathione molecules are required for the ligation of the Fe-S cluster to the protein. Until now it is not know the origin of that glutathione in the case of *G. lambia* [158].

Conclusion

Glutathione-dependent system plays a role in a variety of physiologic, pharmacologic and pathologic conditions in parasitic organisms. The characterization of this redox metabolism, contributes to the understanding of antioxidant responses activated in the parasite under oxidative stress situations. Most of the components of the glutathione related redox metabolism can be in depth analyzed through reverse genomics.

In this chapter are resumed synthetic and recycling pathways involving glutathione in *Plasmodium falciparum* and trypanosomatids as well as the metabolic ways related to its principal derivate in trypanosomatid parasites, trypanothione. Moreover, we illustrated the importance of these thiols, after analyze some of the roles played by them in the redox metabolism context.

Taking into account the needs of these parasites to survive, the redox metabolism in general, and the thiols dependent pathways in particular, constitutes an ideal source of molecular targets for the rational design of new drugs for pharmacological intervention in the treatment of parasitic diseases.

Finally, glutathione seems to be a ubiquitous metabolite in eukaryotic cells that deals with oxygen and nitrogen reactive species. Some pathogenic anaerobes are an exception and in them, glutathione appears to be dispensable. Unique metabolic pathways that are present in pathogens but absent or divergent in their hosts are always potential rational targets for drug development. The overall picture detailed herein refers that one of those biochemical targets for the development of antiparasite drugs include, in many parasites, the redox metabolism due the remarkable qualitative differences between parasites and their hosts.

Acknowledgments

This work was supported by grants from UNL (CAI+D Orientados and Redes), CONICET (PIP 2519) and ANPCyT (PICT'07 668 and PICT´08 1754). VEM and MSC are fellows from UNL and ANPCyT, respectively. DGA, AAI and SAG are investigator career members from CONICET.

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In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter XVII

Glutathione and Programmed Cell Death in *Kluyveromyces Lactis*

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Abstract

We have investigated programmed cell death (PCD) in the respiratory yeast *Kluyveromyces lactis*, using the drugs staurosporine (STS) and phytosphingosine (PHS), which induce PCD in other organisms. While *K. lactis* seemed to be insensitive to STS, PHS induced PCD. We also investigated whether the effects of these drugs were altered by the deletion of the gene encoding glutathione reductase (GLR).

Our results suggest that glutathione metabolism in *K. lactis* is influenced by culture phase, since GLR depletion causes an accumulation of oxidized glutathione (GSSG) when cells enter the stationary phase, but not in the exponential phase of growth.

We propose that the insensitivity of *K. lactis* to STS might be dependent upon the higher levels of reduced glutathione (GSH) found in cells treated with STS.

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In human cells, PCD induced by STS causes GSH efflux, but GSH exporter proteins are absent in *K. lactis*. In addition, GLR appears to be involved in PHS-triggered PCD because cells lacking this enzyme are more resistant to the drug than the wild type strain and the mutant *rag2*::loxP, which has increased levels of GLR activity.

The addition of GSSG or GSH to the medium partially restores growth of the wild type and *rag2*::loxP strains grown on PHS, pointing to this antioxidant having a role in the avoidance of PHS-induced PCD.

Abbreviations

CFU: colony forming units
CLS: chronological Life Span
DADS: diallyldisulfide
GLR: glutathione reductase
GSH: reduced glutathione
GSSG: oxidized glutathione
OS: oxidative stress
PCD: programmed cell death
PHS: phytosphingosine
PI: propidium iodide
PPP: pentose phosphate pathway
RLS: replicative Life Span
ROS: reactive oxygen species
STS: staurosporine
YO-PRO 1: 4-[(Z)-(3-methyl-1,3-benzoxazol-2(3H)-ylidene)methyl]-1-[3-(trimethylammonio)propyl] quinolinium diiodide

1. Introduction

Apoptosis is a phenomenon through which cells undergo programmed suicide. It is the best characterised type of Programmed Cell Death (PCD), which includes necrosis and autophagy [1]. Identifiable signs of apoptosis include chromatin condensation, DNA fragmentation and exposure of phosphatidylserine on the cell surface [2]. Apoptosis can be triggered by numerous exogenous or endogenous elements [3]. The endogenous elements include mutations and aging, whereas the exogenous stimuli embrace a wide variety of substances, such as H_2O_2, metals and drugs. One outcome of apoptosis is the removal of damaged cells without injury to the remaining tissue, so initially apoptosis was considered as a process exclusive to multicellular organisms. It was not until 1997 that it was discovered that unicellular organisms, in this case the yeast *Saccharomyces cerevisiae* [4], can also undergo PCD. This finding is significant in the study of PCD, since yeasts are good tools for investigation owing to their unicellularity, which makes them simpler model systems and therefore much easier to manipulate than mice, apes or even human cell lines [5].

Interest in aging studies has increased in recent years, mostly in relation to diseases such as neurodegenerative disorders or cancer. Discovering new routes involved in apoptosis or

PCD would provide a better understanding of the mechanisms that cause these diseases. Most studies relating to this subject have been carried out on *S. cerevisiae*, a yeast model with a predominantly fermentative metabolism. Human tissues such as the neuronal network have oxidative metabolism, so we propose *Kluyveromyces lactis* as an alternative model, since this yeast has a predominantly respiratory metabolism. *K lactis* also shows other important physiological differences from *S. cerevisiae* [6]; for example, it has been reported [7] that glucose limitation in *S. cerevisiae* (calorie restriction) promotes a decrease in reactive oxygen species (ROS) formation and an increase in longevity that does not occur in *K. lactis*. This difference was attributed to the fact that *S. cerevisiae* shows a catabolic repression of respiration (alleviated by low glucose levels), whereas *K lactis* does not.

One of the external stimuli that can trigger PCD is H_2O_2. It has been shown that H_2O_2 in low doses induces apoptosis [8]. Specific heavy metals can also cause the same phenotype; for example cadmium leads to a caspase and glutathione synthesis-dependent cell death [9]. Although traditionally ROS production was seen as a consequence of mitochondrial disruption during PCD [11], the idea that accumulation of ROS triggers apoptosis in yeast has become increasingly supported [10].

There are several pathways that lead to ROS accumulation in the cell, causing oxidative stress, but in this study we have focussed on the depletion of glutathione reductase (GLR), the protein responsible for recycling the oxidized form of glutathione (GSSG) into reduced glutathione (GSH). We have previously reported that *K. lactis* cells lacking GLR showed an increase in ROS levels [12]. Interestingly, this mutation did not cause a variation of GSH or GSSG concentration in cells during the exponential phase of growth. Glutathione is the major molecule responsible for maintaining the redox status of the cell. There are many examples of its function in detoxifying ROS, xenobiotics and heavy metals [13, 14] and it seems logical to think that H_2O_2-induced apoptosis could be related to glutathione metabolism.

Studies performed with allicin (a substance extracted from garlic), which oxidizes enzymes *in vitro* through a thiol-disulphide exchange [15], showed that this molecule induces apoptosis in tumor cells [16], and its breakdown product, diallyldisulfide (DADS), induces death in *Candida albicans* cells by causing oxidative stress through the depletion of GSH [17, 18]. The addition of allicin in *S. cerevisiae* cultures produced a change in the global electrochemical potential of the cells, as calculated by the Nernst equation using the global concentration of GSH and GSSG [19], while the addition of glutathione prevented staurosporine-induced cell death in the filamentous fungus *Neurospora crassa* [20]. This evidence suggests that changes in the redox status of the cell are involved in the induction of PCD.

In this study, we provide insight into the mechanisms of PCD in the yeast *K. lactis*, which, as a respiratory yeast, we contend is a more suitable metabolic model for more complex organisms than the conventional fermentative model.

As far as we are aware, there are no previous studies on PCD in *K. lactis*, so we decided to use two drugs, staurosporine (STS) and phytosphingosine (PHS), that trigger PCD in human and fungal cells, respectively [21, 22]. Using a wild type *K. lactis* strain (NRRL-Y1140), a glycolytic mutant *rag2*::loxP, and their respective knock-out mutants for GLR (NRRL-Y1140*ΔKlglr1* and *rag2*::loxP*ΔKlglr1*), we tested whether the effects of these drugs were altered by the Δ*Klglr1* mutation, and thus determined whether glutathione and/or GLR are involved in PCD. As aging is considered a stimulus that can trigger PCD, we also investigated the effect of the Δ*Klglr1* mutation on life span of these strains.

2. Materials and Methods

2.1. Strains, Media and Culture Conditions

The following *K. lactis* strains were used: wild type NRRL-Y1140 (CBS2359), phosphoglucose isomerase mutant *rag2*::loxP [23], NRRL-Y1140Δ*Klglr1* [12] and *rag2*::loxP*ΔKlglr1* [24].

Yeasts were handled and grown using standard procedures [25]. The cells were cultivated, unless otherwise stated, in Erlenmeyer flasks at 30ºC and 250 rpm in synthetic complete medium CM [26] containing 0.1-2% glucose. Standard YPD medium (2% bactopeptone, 1% yeast extract and 2% glucose) was also used. The flasks were filled to 20% volume with culture medium. Solid growth media also contained 1.5% agar.

To test the possible effects of the PCD drugs PHS and STS, cells were grown until they reached stationary phase, the OD_{600} was recorded and an aliquot was taken, the OD_{600} was adjusted to 0.1 and serial dilutions were spotted on to CM plates with the corresponding concentrations of the drugs (10, 20 or 30 µg/mL of PHS and 15 µM or 30 µM of STS). All plates were incubated for 48h at 30ºC.

For GSH and GSSG measurements, 15 µM STS or 10 µg/mL PHS were added to overnight cultures in CM, which were then incubated for 1h at 30ºC and 200 rpm.

2.2. Apoptotic Marker Determination

Cells from the wild type strain in the stationary phase of growth were diluted in CM to an OD_{600} of 0.1. After incubating for 4h at 30°C and 250 rpm, PHS at 30 µg/mL was added. Permeability to YO-PRO 1 (an apoptotic marker [27]) and to propidium iodide (PI, a necrotic marker) were assessed by incubation of a 0.2 mL cell culture aliquot with 0.1 µM YO-PRO 1 for 20 min at 4°C, followed by addition of 2 µg/mL PI. Annexin V binding was determined in spheroplasts prepared from cells previously incubated with 15 µg/mL PHS for 30 min (total PHS incubation time at the end of spheroplast formation was approximately 60 min). Spheroplasts were prepared by digestion with 8 mg/mL lysing enzymes (Sigma) in 35 mM phosphate buffer pH 6.8 with 0.5 mM $MgCl_2$ and 1.2 M sorbitol, and incubated with annexin V-FITC (Immuno Tools) according to the manufacturer's instructions. Annexin V binding was quantified by flow cytometry. Flow cytometry analyses were performed using a FACS Calibur (BD Biosciences) with CELLQuest PRO3.3 software, with excitation at 488 nm to visualize YO-PRO 1 or annexin V-FITC fluorescence (530/30 bandpass filter) and PI (585/42 bandpass filter).

2.3. GSH/GSSG Measurements

The procedure for cell preparation was adapted from [28]. Cells from 10 mL overnight cultures in CM were collected, the pellet was resuspended in 1 mL sterile distilled H_2O and transferred to Eppendorf tubes. After 1 min centrifugation, cells were resuspended in 200 µL of 100 mM potassium phosphate buffer with 2 mM EDTA pH 7.4, and 200 µL of 2 M $HClO_4$

was added. Cells were vortexed with glass beads for 30 s - this was repeated a further 5 times - and then centrifuged at 7000 rpm for 5 min at 4ºC. The supernatant was transferred to a new Eppendorf and neutralized by the addition of 2 M KOH, 0.3 M MOPS, until the mixture reached pH 6-7. After centrifugation, the supernatant was collected, divided in two new tubes and frozen at -70ºC for at least 1h.

The sum of GSH and GSSG concentrations, here called "total glutathione", was determined using the kinetic assay with GLR as described in [28]. The reaction was followed spectrophotometrically at 405 nm in a Shimadzu UV-160A spectrophotometer.

To measure the GSSG concentration, after the supernatant was thawed and centrifuged, 2 µL of vinylpyridine for each 100 µL of supernatant was added. After 60 min incubation at 4ºC with agitation, GSSG was determined as described for total glutathione.

2.4. Life Span

The chronological life spans of *K. lactis* wild type and Δ*Klglr1* mutant cells were determined by measuring colony forming units (CFU) following the procedure described in [29]. Cultures in 100 mL CM (2% glucose) were inoculated at OD_{600}=0.1 from fresh overnight cultures and an aliquot of the culture was plated on to YPD medium (2% glucose) each day to score viability. The colonies formed on the first day were considered to represent 100% survival. The assay was performed until less than 10% of the culture remained viable.

3. Results and Discussion

3.1. Growth of Wild Type and *ΔKlglr1* Strains in Liquid Medium

There are two principal ways of determining life span in yeast. The Replicative Life Span (RLS) is defined as the number of times that a mother cell is able to divide, or as the number of buds (daughter cells) that can be produced by the mother cell [30]. Yeast division is carried out by budding, a mechanism in which the mother cell retains a greater volume than the daughter cell [31]. The Chronological Life Span (CLS) is the time that a cell can remain viable after reaching a post-replicative state [32]. Which of these mechanisms better explain the situation for eukaryotic cells is still unclear, but they are related to each other, since cells in a post-replicative state have a reduced RLS once they re-enter the cell cycle [33].

To determine whether the survival of the cells could be compromised by the lack of a functional GLR in *K. lactis*, we first compared the growth curves of *rag2*::loxP and Δ*Klglr1* mutant strains by inoculating, 100 mL of standard CM at OD_{600nm}=0.1, and then incubating at 30ºC with agitation. Every two hours, an aliquot was taken to record the OD_{600nm}. As shown in Figure 1A, there were no apparent differences between *rag2*::loxP and the congenic Δ*Klglr1* strains, suggesting that the Δ*Klglr1* mutation does not affect growth in glucose.

Previously we have shown a similar result for the wild type strain NRRL-Y1140 and its congenic Δ*Klglr1* strain [12].

When we tested the effect of glucose concentration on growth by culturing the different yeast strains on agar plates containing 0.1% or 2% glucose, we observed that the growth of

Δ*Klglr1* mutants on low glucose media was improved, and this improvement was even more evident for the double mutant *rag2*::loxPΔ*Klglr1*.

The *rag2*::loxP is a strain in which all glucose is metabolized by the pentose phosphate pathway (PPP).

We suggest that the improved growth of Δ*Klglr1* strains on glucose is due to a diversion of cytosolic NADPH from the PPP to reoxidation by alternative external mitochondrial dehydrogenases [12, 24]. NRRL-Y1140 is able to divert glucose through glycolysis, meaning that the production of cytosolic NADPH should not be as high as in the *rag2*::loxP strain.

We also performed life span studies based on the CLS methodology, as described in the Materials and Methods section. NRRL-Y1140 and NRRL-Y1140Δ*Klglr1* have previously been found to show no difference in survival [12, 34]; in this study we found that there was no difference in survival for the *rag2*::loxP and double mutant *rag2*::loxPΔ*Klglr1* strains either (Figure 1B).

From all of the data obtained, we conclude that the presence of a functional GLR is not essential for cell growth in *K. lactis*.

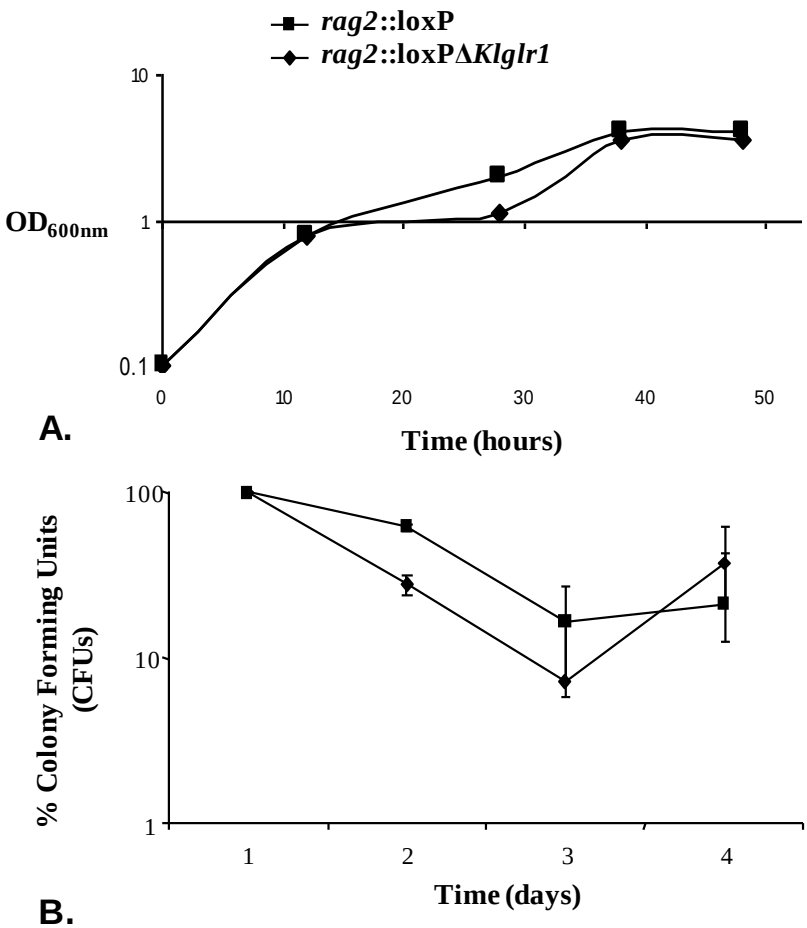

Figure 1. Growth curves (A) and life span (B) of *K. lactis rag2*::loxP and *rag2*::loxP*ΔKlglr1* in CM 2% glucose (n=2 for B).

3.2. Determination of GSH and GSSG Concentration in *K. lactis* Wild Type and Δ*Klglr1* Strains

We have previously shown that when cultures of *K. lactis* NRRL-Y1140 and NRRL-Y1140Δ*Klglr1* were in the exponential phase of growth, there were no differences in GSH and GSSG contents between the strains [12]. We suggested that, in the absence of a functional GLR, other proteins, most likely of the thioredoxin/thioredoxin reductase system, were responsible for maintaining GSH levels [34].

It is possible that cells in different phases of growth show different physiologies [35] and in consequence could differ in GSH metabolism, so we decided to investigate whether the GSH/GSSG concentrations were affected by the Δ*Klglr1*mutation in older cells (in stationary phase). As demonstrated in Figure 2, stationary phase Δ*Klglr1* cells showed an increase in GSSG levels, which is consistent with the results found in the literature for other yeasts [36, 37]. GSH levels also increase, although the GSH/GSSG ratio decreases more than 7-fold in comparison to wild type cells.

Taken together with the results obtained previously for exponential phase cells [12], these data suggest that glutathione metabolism in *K. lactis* is influenced by the culture phase. When cells enter the stationary phase, but not when they are in the exponential phase, GLR depletion causes the accumulation of GSSG.

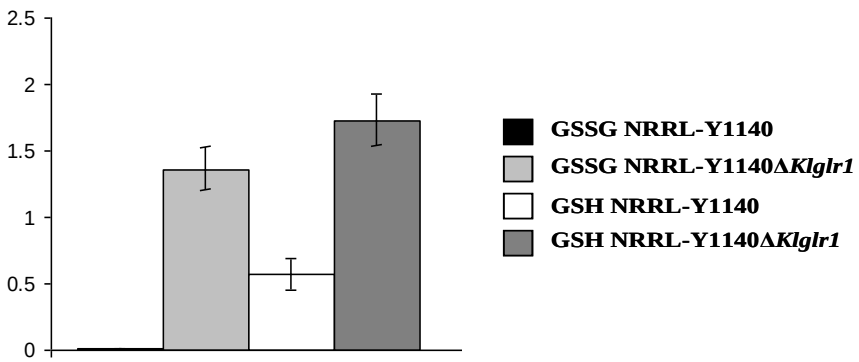

Figure 2. Total amounts of GSH and GSSG in *K. lactis* wild type strain NRRL-Y1140 and the congenic knock-out mutant Δ*Klglr1*. Data are expressed as μmol GSH or GSSG/mg protein (n=5-9).

3.3. Resistance of *K. lactis* to PCD Induced by Staurosporine and Phytosphingosine

Our data indicate that CLS in NRRL-Y1140 is not affected by the Δ*Klglr1* deletion [12] and that there is an accumulation of GSSG in stationary phase mutant cells.

These studies were performed in the absence of an external stimulus that could trigger the expression of defense mechanisms, as GSSG accumulation could compromise the defense mechanisms of the cell when it reaches stationary phase. For this reason, we decided to investigate whether GSH and GSSG levels were altered following the addition of STS or PHS to the media. Since cells lacking GLR in the exponential phase do not accumulate GSSG, these trials were carried out on cells that had already reached the stationary phase. Initially, to

determine the effect of these drugs on cell growth and survival, several dilutions of *K. lactis* cells were spotted on to CM plates supplemented with two different concentrations of the drugs (Figure 3). STS did not affect the growth of the *K. lactis* strains, with the exception of the *rag2*::loxP mutant, whose growth was slightly reduced by concentrations of 15 and 30 μM STS. In the case of PHS, growth of the wild type NRRL-Y1140 and *rag2*::loxP mutant strains was impaired by concentrations of 20 and 30 μg/mL of the drug, while both congenic strains that lack GLR were highly resistant to the drug.

STS is a non-specific inhibitor of cellular protein kinases [21] and it has been used for induction of apoptosis in diverse cellular models. ROS production, dissipation of the mitochondrial membrane potential and release of cytochrome c from mitochondria into the cytosol are the principal events responsible for the activation of the caspase cascade that leads to apoptosis [38, 39]. In certain human cells STS-induced PCD is preceded by an extracellular increase in GSH and a decrease in the intracellular GSH concentration, although without altering the GSH/GSSG ratio [21]. GSH efflux has not been described in yeast. It is known that *S. cerevisiae* can take up GSH from the medium using two membrane transporters, named GSH-P1 (high affinity) and GSH-P2 (low affinity) [40]. A third transporter of high affinity, HGT1, exists in *S. cerevisiae* [41] and has two homologues in *K. lactis*, KLLA0E00419g and KLLA0E00397g. KLLA0E00419g shows little sequence identity (34%) with HGT1, but KLLA0E00397 has 82% sequence identity with HGT1. Therefore, *K. lactis* is likely to be able to take up GSH from the medium, although GSH export proteins have not yet been reported for either of these two yeasts.

We investigated the effect of STS on GSH and GSSG concentrations in *K. lactis* cells, and found out that a 1h treatment of NRRL-Y1140 and knock-out mutant NRRL-Y1140Δ*Klglr1* with 15 μM STS did not significantly affect GSSG, but increased the GSH concentration in both strains (Figure 4). Thus, the GSH/GSSG ratio, commonly used as an indicator of the redox status of the cell, increased in both strains following STS treatment. The increase in the Δ*Klglr1* strain is less than in the wild type because these cells accumulate GSSG (Figures 2 and 4).

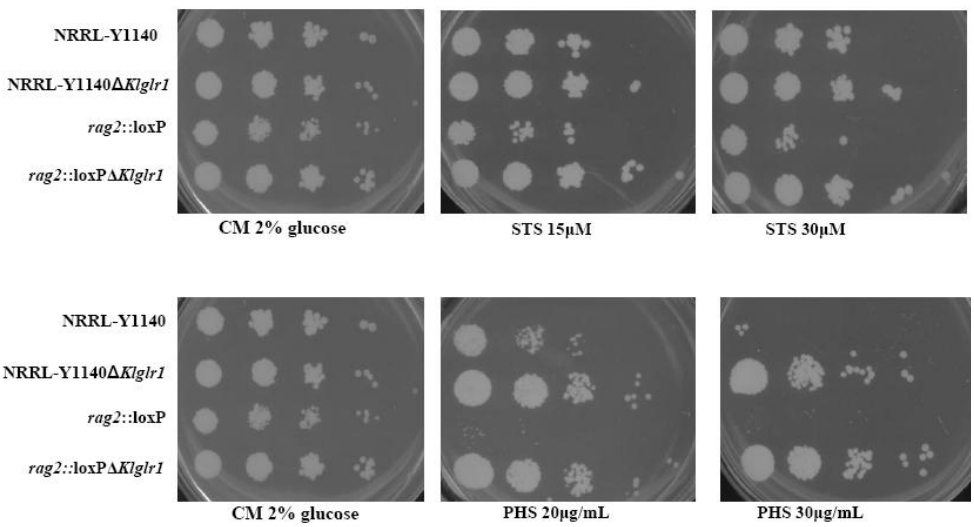

Figure 3. Effects of STS and PHS on the growth of *K. lactis* strains.

Further research will be necessary to confirm that there is no GSH efflux in *K. lactis*. We propose that increased levels of intracellular GSH make this yeast more resistant to the drug, since it does not lose GSH during STS-induced PCD, in contrast to human cells.

A striking result from these experiments was the unexpected sensitivity of the mutant *rag2*::loxP strain to STS (Figure 3). Previous studies carried out in our laboratory showed that this strain has increased catalase and GLR activities, a phenotype that has been related to a higher tolerance to oxidative stress (OS); while the *rag2* mutation also leads to increased levels of ROS [24]. As shown in Figure 5, the *rag2*::loxP strain contains more GSH than the wild type strain. This finding was as expected, since *rag2* strains have higher activities of GLR [24]. Levels of GSSG are also higher in *rag2*::loxP cells. We found there was an increase in the GSH/GSSG ratio in this strain following STS treatment (about 1.5-fold); but this increase was lower than for the strains depicted in Figure 4 (about 4.3-fold for NRRL-Y1140).

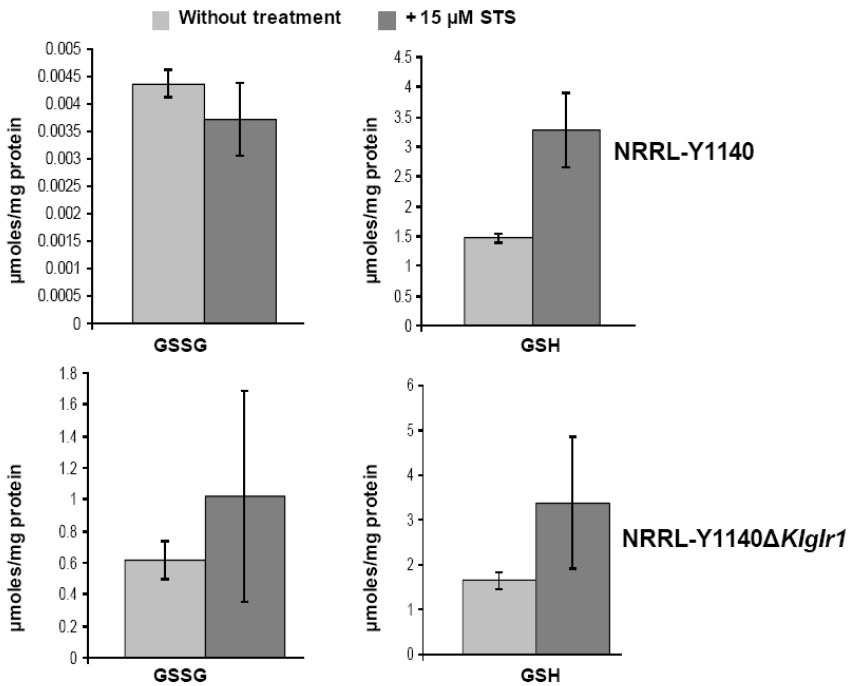

Figure 4. GSH and GSSG levels in wild type NRRL-Y1140 and NRRL-Y1140Δ*Klglr1* strains before and after culture for 1h with 15 µM STS. GSH and GSSG are expressed as µmol/mg protein (n=4).

To explain this we suggest that STS damage can be restored by increasing GSH levels, as a simple buffering mechanism. Since the increase of GSH levels in response to STS in *rag2*::loxP is quantitatively smaller than in NRRL-Y1140 and its congenic Δ*Klglr1*, it is possible that this is the cause of its slightly increased sensitivity to this drug.

Phytosphingosine (PHS) is a sphingolipid with antifungal properties, and it induces caspase-independent PCD in *Aspergillus nidulans*. Chromatin condensation, large-scale DNA fragmentation and externalization of phosphatidylserine are consequences of the treatment of the cells with this drug [42]. In *N. crassa* PHS causes apoptosis-like death: significantly

reduced viability, impairment of asexual spore germination, DNA fragmentation and production of ROS. Complex I mutants of this filamentous fungus are more resistant to PHS-induced PCD than the wild type strain. This resistance has been attributed to decreased ROS production in the mutants [22]. *K. lactis* also undergoes PCD upon PHS stimulus (Figure 6).

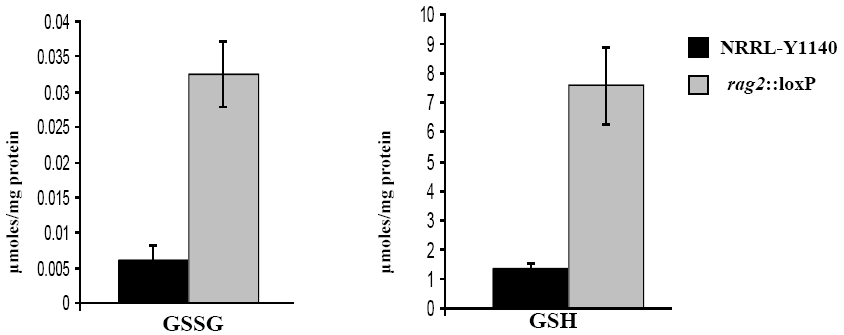

Figure 5. Comparison of GSSG and GSH levels between wild type NRRL-Y1140 and the congenic mutant *rag2*::loxP. GSH and GSSG are expressed as µmol GSSG or GSH/mg protein (n=4 for NRRL-Y1140; n=3 for *rag2*::loxP).

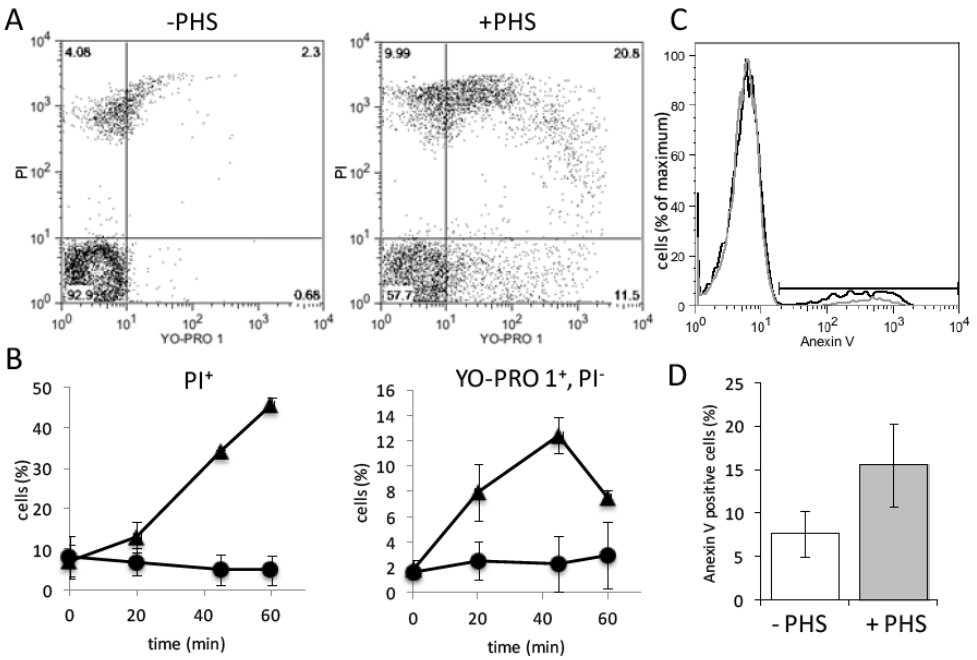

Figure 6. *K. lactis* undergoes programmed cell death induced by PHS. A) Flow cytometry measurement of propidium iodide (PI, y axis) and YO-PRO 1 (x axis) incorporation by *K. lactis* cultured for 4 hours followed by a further 45 min culture in the absence (left) or presence (right) of 30 µg/ml PHS. Numbers in each quadrant indicate cell percentages. B) Percentages of PI+ (necrotic, left) and YO-PRO+, PI- cells (apoptotic, right) measured as in A) in the absence (circles) or presence (triangles) of PHS. Average and standard deviation of two independent experiments are presented. C) Flow cytometry histograms of annexin V binding to *K. lactis* cells that were incubated in the absence (grey) or presence (black) of PHS. The gate indicates annexin V – positive cells. D) Average and standard deviation of two independent experiments measured as in C).

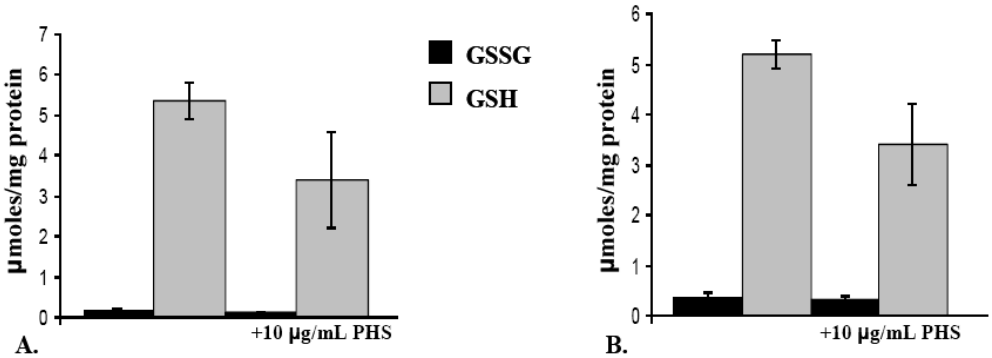

Figure 7. GSSG and GSH levels in A) NRRL-Y1140 (n=3) and B) NRRL-Y1140ΔKlglr1 (n=4) strains with and without 1 hour treatment with 10 µg/mL of PHS. GSH and GSSG are expressed as µmoles of GSSG or GSH/mg protein.

Early apoptotic cells are permeable to the YO-PRO 1 dye [27], but not to PI, which stains necrotic cells. Upon PHS treatment, both early apoptotic and necrotic cells increased in number (Figure 6A,B), with the apoptotic cells reaching a maximum at 45 min of incubation with 30 µg/ml PHS. Annexin V binding to phosphatidylserine-presenting cells, which is another early apoptotic hallmark, was also observed to increase after PHS treatment (Figure 6C,D). As shown in Figure 3, wild type *K. lactis* NRRL-Y1140 and mutant *rag2*::loxP cells were extremely sensitive to PHS and NRRL-Y1140Δ*Klglr1* and *rag2*::loxP*ΔKlglr1* were surprisingly tolerant to this drug. In order to understand whether GSH and GSSG levels were implicated in this phenotype, we measured GSSG and GSH concentrations in NRRL-Y1140 and its congenic Δ*Klglr1* after 1h of PHS treatment. We found that, while GSSG levels were unaffected by addition of the drug, GSH amounts were decreased in both strains (Figure 7).

This consequently caused a decrease in the GSH/GSSG ratio, of approximately the same magnitude for both wild type and Δ*Klglr1* mutant strains. The reason why NRRL-Y1140Δ*Klglr1* cells are more resistant than the wild type cells is still unknown, but since the *rag2*::loxP*ΔKlglr1* strain is more resistant than *rag2*::loxP cells, we cannot rule out involvement of GLR in PHS-induced PCD. We compared the effect of the addition of GSSG or GSH on the growth of *K. lactis* strains on solid media supplemented with PHS.

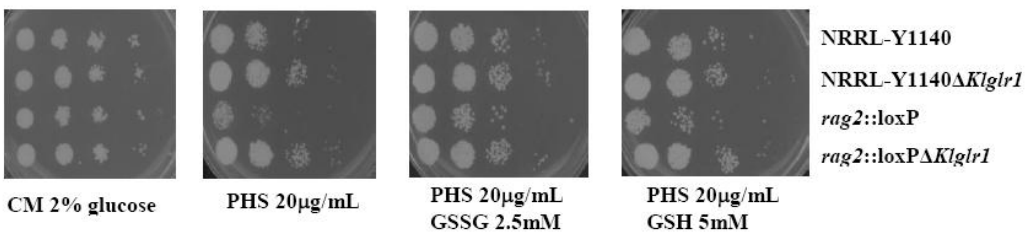

Figure 8. *K. lactis* cells grown on solid CM supplemented with PHS, PHS with GSSG and PHS with GSH.

As can be seen in Figure 8, cells from NRRL-Y1140 and *rag2*::loxP strains grown on solid CM supplemented with PHS and 2.5 mM GSSG or PHS with 5 mM GSH showed some growth improvement, compared with those grown on PHS alone.

This suggests that glutathione is involved in PCD, and that GLR plays an important role in triggering cell death, since cells lacking GLR seem to be less affected by PHS.

Conclusion

In this study we have demonstrated that, although GLR is an important enzyme in the defense against OS, it appears to be dispensable for the survival of *K. lactis*. Mutants lacking a functional GLR showed no differences in growth in liquid cultures with 2% glucose and their life span was also unaffected. We have also shown that STS does not trigger PCD in *K. lactis*. We propose that this phenotype could be related to the increased levels of GSH found in cells treated with STS. In human cells the response that leads to PCD is accompanied by a GSH efflux, an event that has not been observed in *K. lactis*. We also propose that GLR is involved in the PCD triggered by PHS, since cells lacking this protein were more resistant to the drug than the corresponding congenic cells, the wild type strain NRRL-Y1140 and the mutant *rag2*::loxP. The addition of GSSG or GSH to the media partially restored growth of NRRL-Y1140 and *rag2*::loxP strains in PHS, pointing to a role for this antioxidant in avoiding PHS-induced cell death.

Acknowledgments

We would like to thank Dr. Catarina Leitão from the IBMC's Advanced Flow Cytometry Unit for her help with flow cytometry experiments and analyses. This work was supported by a ''Programa Ciência'' financed by POPH-QREN (typology 4.2), funding from ESF and MCTES for ASF, and by grant BFU2009-08854 from MICINN (Spain) and FEDER. General support for the Spanish laboratory during 2008-11 was provided by Xunta de Galicia (Consolidación C.E.O.U.2008/008), and by FEDER. AGL acknowledges the grant received from "Axudas á mobilidade internacional UDC-Banco Santander" which made a 6-month internship at the IBMC possible.

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In: Glutathione: Biochemistry, Mechanisms of Action … ISBN: 978-1-62417-460-5
Editors: N. Labrou and E. Flemetakis © 2013 Nova Science Publishers, Inc.

Chapter XVIII

Biotechnological Production of Glutathione in Yeast

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Abstract

Glutathione (GSH), the most abundant non-protein thiol compound in living cells, is widely used in pharmaceutical, cosmetic and food industries. Compared with the chemical method, biotechnological production of GSH is an alternative approach because of the low cost and easy separation. Recent advances related to enzymatic method, fermentative method, as well as the recovery of GSH in yeast are highlighted in this review. The current bottlenecks of GSH production are presented based on our understanding of the regulation of GSH biosynthesis, and some possible strategies for overcoming these limitations are provided for further improving GSH production to expand application range of GSH.

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Keywords: Glutathione; *Saccharomyces cerevisiae*; Fermentative method; Metabolic Regulation

1. Introduction

GSH, γ-glutamyl-L-cysteinylglycine, is the most abundant bioactive tripeptide widely presented in almost all eukaryotic cells, except a few parasitic protozoans [1]. GSH is also distributed in aerobic Gram-negative bacteria such as cyanobacteria and proteobacteria, but absent in the strict anaerobes and Gram-positive bacteria (e.g., archaebacteria) [2]. Over 90% of GSH is normally reduced form in cells. Other forms of GSH e.g. GSH disulfide GSSG (oxidized GSH), GS-S-CoA, GS-S-Cys, and GS-S-protein can be turned to GSH by GSH reductase (GR) at the expense of NADPH [2, 3]. Although GSH is involved in many physiological processes, the key functions of GSH can be summarized into three ways: (1) an antioxidant for maintaining the redox homoeostasis in cells to protect DNA, proteins, and other biomolecules against oxidative damage; (2) an immunity booster via white blood cell production; and (3) a detoxifier in higher eukaryotic organisms through conjugation with exogenous electrophiles and diverse xenobiotics by glutathione-S-transferase [4, 5]. These characteristics make GSH important in the treatment of many diseases, such as liver cirrhosis, diabetes, neurodegenerative diseases, and aging [6]. Thus, GSH is considered to be one of the most important self-generated defense tripeptides [7].

2. GSH Biosynthesis and Regulation

GSH is sequential ATP-dependent synthesis through non-ribosomal peptide synthesis by two soluble enzymes in the cytosol, γ-glutamylcysteine synthetase (also known as glutamate-cysteine ligase, EC 6.3.2.2, Gsh1p) and GSH synthetase (EC 6.3.2.3, Gsh2p) [1]. First, the dipeptide γ-glutamylcysteine (γ-GCS) is formed from L-glutamic acid and L-cysteine by Gsh1p. Next, the C-terminal site of γ-GCS is conjugated with glycine to form GSH by Gsh2p (Figure 1). GSH biosynthesis thus forms part of the γ-glutamyl cycle in cells. The first reaction of GSH synthesis is generally considered to be the rate-limiting step because Gsh1p controlled by GSH feedback inhibition at both the transcriptional and post-translational levels to avoid GSH over-accumulation [8-12]. In addition to the activities of Gsh1p and Gsh2p directly dedicated for GSH biosynthesis, the levels of the substrates (cysteine, glutamate and glycine) and ATP in the cell are also affecting GSH level.

Although yeast cells can import/export GSH from/to the extracellular environment, the critical factor determining GSH production is GSH biosynthesis under tight regulation at 3 different levels: 1) *GSH1* regulation at the transcriptional level; 2) GSH1 regulation at the posttranslational level; 3) cysteine regulation at the substrate level [13]. *GSH1* expression is the rate-limiting step of GSH biosynthesis under tight transcriptional regulation. It is induced by the transcriptional regulator Yap1, which is activated by exposure to peroxides [14, 15]. *GSH1* expression is also mediated by another transcriptional activator, Met4, which is responsible for sulfur assimilatory pathways [12, 16]. Post-translational regulation is a non-allosteric feedback inhibition of Gsh1p activity [17]. The structural basis for feedback

inhibition of Gsh1p indicates that GSH binds to the glutamate and cysteine binding pockets, rather than to a separate site on the enzyme [18]. In addition to these two regulatory levels, cysteine is the major limiting precursor for GSH accumulation [13, 19].

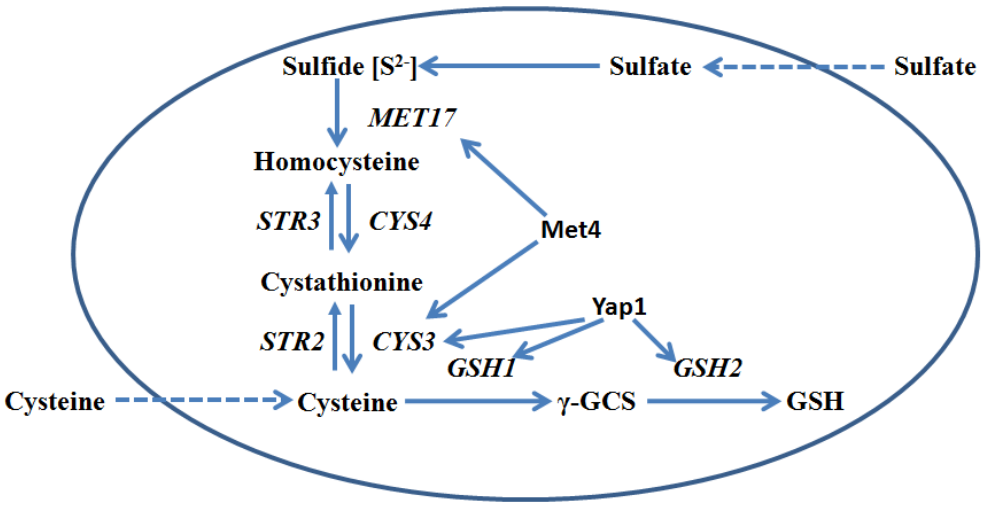

Figure 1. Biosynthetic pathways involved in the metabolism of cysteine and GSH. *MET17* homocysteine synthase, *CYS4* cystathionine γ-synthase, *CYS3* cystathionine β-lyase, *STR3* cystathionine β-lyase, *STR2* cystathionine γ-synthase, *GSH1* γ-glutamylcysteine synthetase, *GSH2* GSH synthetase, *Met4* leucine zipper transcriptional activator, *Yap1* redox sensitive transcriptional activator.

Cysteine addition could significantly increase the specific GSH production rate in fed-batch cultivation. Alfafara et al. [13] found that single-shot addition of cysteine is a better strategy to improve GSH yield in contrast to a continuous supplement maintained at a constant concentration.

3. Biotechnological Production of GSH: An Overview

GSH is now widely used as a medicine [20]. It also has great potential as a food additive and in the cosmetic industry [21]. The commercial demand for GSH will be expanded if the production cost can be further decreased [20]. Improved production method reduces production costs and ultimately decreases the price of GSH [22]. Since GSH discovery from ethanol extract of baker's yeast in the 1880s [23], its production has developed four methods: (1) extraction method; (2) chemical synthesis method; (3) enzymatic method; and (4) direct fermentative method. Interestingly, yeast remains the best choice for commercial GSH production. In the early days of its discovery, solvent extraction, as a preparative method, from natural sources of yeast and other living organisms provided GSH for laboratory studies and commercial demands. However, the limited availability of raw materials coupled with the relatively low intracellular content of GSH hampered its practical application and limited to the expanding commercial demands. Deciphering the chemical structure of GSH in the1920s paved the way for its chemical synthesis. In 1935, Harington and Mead demonstrated that

GSH could be synthesized using a modified version of benzylcarbonato method [24]. The critical step in synthetic GSH was the introduction and removal of sulfhydrl protective agents on the cysteine residue [23]. Despite this process being commercialized in the 1950s, the end product was an optically inactive mixture of the D-and L-isomers. Hence, it made the entire process more expensive, complicated and time-consuming to separate the physiologically active L-form of GSH.

With the understanding of GSH biosynthesis at the enzyme level, enzymatic method based on the use of microbial whole cells (e.g. *Escherichia coli* and *Saccharomyces cerevisiae*) or enzymes, the three precursor amino acids, ATP, necessary cofactors (Mg^{2+}), and a suitable pH was developed for the efficient production of GSH [25, 26]. Despite this method gives very high yields of GSH (up to 9 g/l), the consumption of ATP makes this process high cost and difficult to scale up in the industry [23]. Thus, a hot spot research is to construct a high efficient ATP regeneration system such as self-coupling system [25-28] and heterology-coupling system [29] (Figure 2).

Yoshida et al. developed a novel self-coupling ATP regeneration system through engineered glycolytic pathway to improve GSH productivity by up to 1.7-fold higher compared with the parental strain [27]. However, self-coupling ATP regeneration system is still inefficient to simultaneously improve GSH biosynthesis and ATP regeneration in one host. Despite the efficiency problem of ADP/ATP transport across the respective cell membranes, to date, heterology-coupling ATP regeneration system seems only feasible to use on an industrial scale.

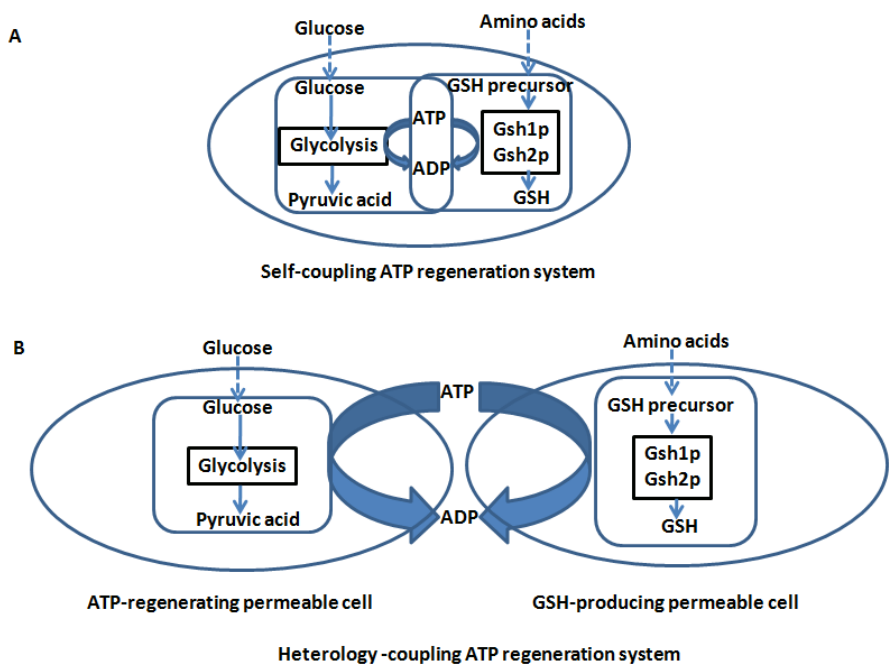

Figure 2. High efficient ATP regeneration system. (A) self-coupling system (B) heterolgoy-coupling system.

The currently commercial method is fermentative production using sugar substrate. *S. cerevisiae* and *Candida utilis* are the preferred microorganisms used in an industrial scale due

to higher intracellular GSH contents compared with other microorganisms. The ultimate goal of GSH production is to achieve high total GSH yield by increasing cell density and intracellular GSH content [22, 23]. To get maximum GSH production, high yield strain and high cell density (HCD) fermentation have been intensively explored in the recent 3 decades.

3.1. GSH High-Yield Strain

The traditional mutagenesis strategies including UV, X-radiation, NTG treatment, and GSH analogue (e.g. methionine and ethionine) resistance were employed to screen GSH over-producer.

Although these classical methods have power to improve GSH yield, it is laborious and time-consuming. In most cases, the molecular mechanism of higher yield and the mutation sites in the chromosome are not clear. With the rapid development of molecular biology, engineered yeast strains are available to achieve higher GSH yield. To solve the rate-limiting step of GSH biosynthesis, Ohtake et al. increased Gsh1p activity and GSH content more than 1000 folds and 3 folds, respectively, in a *S. cerevisiae* transformant by fusion *GSH1* gene from *E. coli* B with the promoter *P8* from *S. cerevisiae* [30]. Fan et al. constructed a recombinant plasmid pGMF containing *GSH1* from *S. cerevisiae* and a copper-resistance gene as a selection marker. GSH content of *S. cerevisiae* YSF-31 harboring plasmid pGMF was 1.5 fold of that in the control [31]. In addition to *S. cerevisiae*, other engineered yeast strains such as *Pichia pastoris* and *Hansenula polymorpha* also achieved the same effect by Gsh1p and Gsh2p overexpression [32, 33]. Although the GSH biosynthetic enzymes Gsh1p and Gsh2p have been overexpressed in yeast, there was still limited increasing GSH content in yeast. It might be attributed to the tight regulation of GSH1 coupled with its feedback inhibition by GSH.

3.2. GSH High Cell Density Fermentation

While intracellular GSH was improved by genetic engineering/random mutagenesis, HCD could be achieved by fermentation optimization and control. The effects of media components, environmental factors, yeast growth rate and amino acid supplementation on GSH production have been explored to get maximum yields. Medium recipe and culture conditions are important to GSH production, hence statistic experiment designs have been intensively applied in the bioprocess optimization such as single factorial design [20], central composition rotatable design (CCRD), fractional factorial design (FFD), orthogonal design (OD), D-optimal design, uniform design (UD), and response surface methodology (RSM) [34]. Santos et al. [35] used FFD and CCRD to obtain the optimal conditions of temperature, agitation rate, initial pH, inoculum size, and glucose concentration for GSH production in shake-flask culture of *S. cerevisiae* ATCC 7754. The highest GSH yield under the optimal culture condition achieved 154.5 mg/l, which increased ~50% than that of the control. Zhang et al. [36] was carried out UD to optimize 9 factors of the medium composition (glucose, yeast extract, peptone, malt extract, molasses, $MgSO_4$, $ZnSO_4$, $(NH_4)_2HPO_4$, and thiamine) for GSH production in shake-flask culture by *S. cerevisiae* T65. GSH yield at the optimal point achieved 74.6 mg/l, which was 1.81-fold higher than the control.

Fed-batch cultivation is the most common culture mode for achieving HCD in the laboratory and industrial scale [37]. Control methods such as constant-rate feeding [33, 38], exponential feeding [38, 39], dissolved oxygen (DO)-stat [32], pH-stat, and ethanol feedback [40] have been deployed for improving GSH production in HCD fed-batch cultivation (Table 1). Unfortunately, these control methods inevitably result in decreased cellular GSH content during the HCD cultivation [41].

Alternatively, addition of precursor amino acids during HCD fed-batch fermentation can help to maintain the GSH content [13, 42]. We implemented a simplified RQ feedback control strategy to control glucose feeding rate during the fed-batch phase to achieve simultaneous increase in cell density, GSH yield and GSH content [22].

RQ controlled at 0.65 resulted in the highest GSH productivity (46.9 mg/l/h) and cell productivity (3.5 g/l/h) as well as improved GSH yield (2.1 g/l) and intracellular GSH content (1.67%). We concluded that advantages of RQ control over ethanol control in the GSH production include lower byproduct concentration, shorter cultivation period, higher GSH content, and higher GSH yield. In addition, controlling the specific growth rate (μ) during fed-batch HCD culture is important to avoid a decrease in the GSH content [23]. Shimizu et al. [45] proposed an ideal profile of μ for maximizing GSH production by controlling glucose-feeding rate with the extended Kalamn filter and a programmed-controller/feedback compensator system.

Table 1. Summary of GSH production with different control strategies during the fed-batch phase (without amino acid addition)

Strain	Control mode	Biomass (g/l)	GSH (g/l)	GSH content (%)	GSH productivity (mg/l/h)
P. pastoris D18 [33]	Constant-rate feeding	90	0.92	1	32.8
S. cerevisiae T65 [22]	RQ feedback	112	1.5	1.34	46.9
H. polymorpha DL-1 [32]	DO-stat	72	0.91	1.3	22.7
H. polymorpha MOXp-GSH2 [32]	DO-stat	72	2.2	3.1	11
S. cerevisiae GE-2 [40]	Ethanol feedback[a]	90	0.9	~1	21.4
S. cerevisiae GE-2 [40]	Ethanol feedback[b]	90	0.85	~1	37.5
E. coli WSH-KE1 [38]	Constant-rate feeding	77.5	0.78	1	18.8
E. coli WSH-KE1 [38]	Artificial feedback feeding	64	0.45	0.7	7
E. coli WSH-KE1 [38]	Exponential feeding	80	0.88	1.1	35.2
S. cerevisiae KY5711 [43]	Ethanol feedback[c]	1	~0.05	5	12.5
C. utilis WSH 02-08 [44]	Constant-rate feeding	102.1	0.981	0.96	8.8
S.Cerevisiae ZJUS1 [39]	Exponential feeding	105	0.81	0.77	13.5

[a] Keeping ethanol concentration at ~1%.
[b] Keeping the descending rate of ethanol concentration between -0.1% and 0.15%.
[c] Maintaining a constant ethanol concentration by fuzzy logic control.

As a result, GSH yield was 41% higher than that of the control. In our previous work, we developed on-line biomass monitoring with an in situ capacitance probe in GSH fed-batch fermentation of *S. cerevisiae* T65 grown on an industrial complex medium [46]. Capacitance method has a good linear relationship with viable biomass and all off-line biomass assay such as dry cell weight (DCW), optical density at 600 nm wavelength (OD_{600}), packed mycelial volume (PMV) and number of colony forming units (CFU) (Figure 3). Furthermore, we applied capacitance method to real-time estimate μ. Compared with those determined by at-line indirect estimation methods such as oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER), the specific growth rates estimated by on-line capacitance measurement could be more reliable during GSH fermentation (Figure 4). Hence, capacitance method is a valuable tool for at-line controlling μ in order to achieve GSH fermentation optimization.

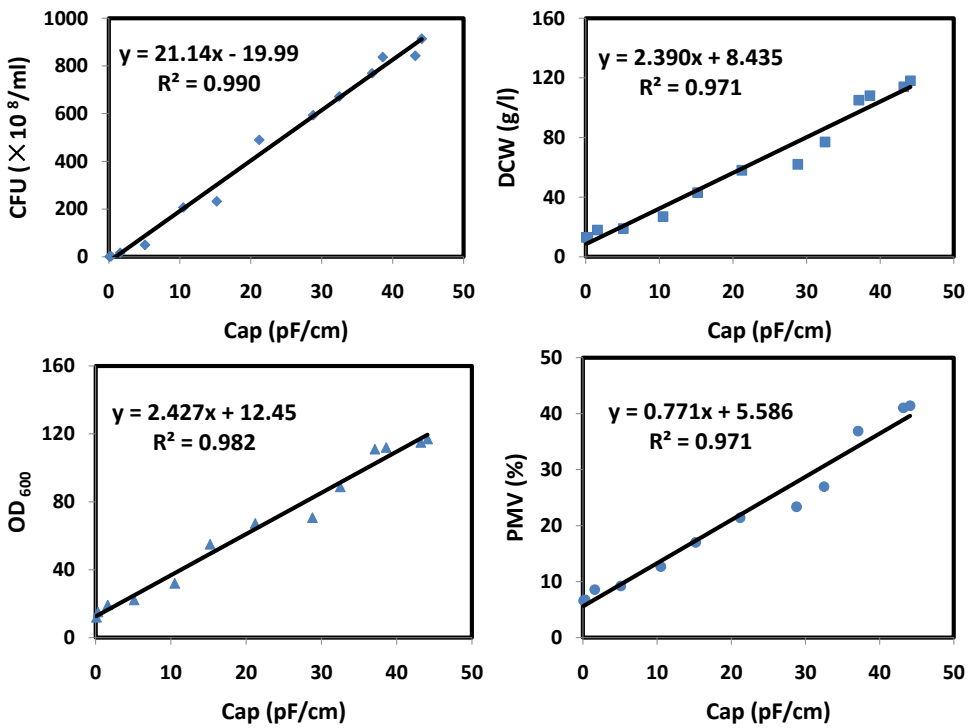

Figure 3. Linear relationship between capacitance (Cap) and biomass concentration estimated by CFU, DCW, OD_{600} or PMV method.

3.3. Downstream Processing and Purification of GSH

Although GSH production is a mature process, there is limited literature/patent on the downstream processing of GSH production. One reason might be that the outcome of research on the isolation and purification of GSH has not been published openly due to commercial significance [47]. Currently, GSH industry is used hot-water extraction method to isolate GSH from yeast cells. To seek more environmental friendly methods, decrease the solvent consumption, shorten the extraction time, increase the extraction yield, and enhance the

quality of extracts, some extraction techniques have been developed such as ultrasound-assisted extraction, supercritical fluid extraction, microwave-assisted extraction, hot-water extraction and solvent extraction [47]. Among these, ethanol extraction promises to be an inexpensive, simple and efficient technique which can be applied in the GSH industry [47]. In addition, ethanol extraction under appropriate conditions could maintain the cells with intact plasma membranes [48].

We found that GSH can be extracted efficiently from *S. cerevisiae* by treating with ethanol without disrupting yeast cells. Compared to hot water extraction, there was no significant advantage in GSH yield using ethanol extraction. But ethanol extraction has several advantages, such as lower energy consumption and lower protein concentration of extraction broth, which may reduce the complexity and cost of the purification process [47].

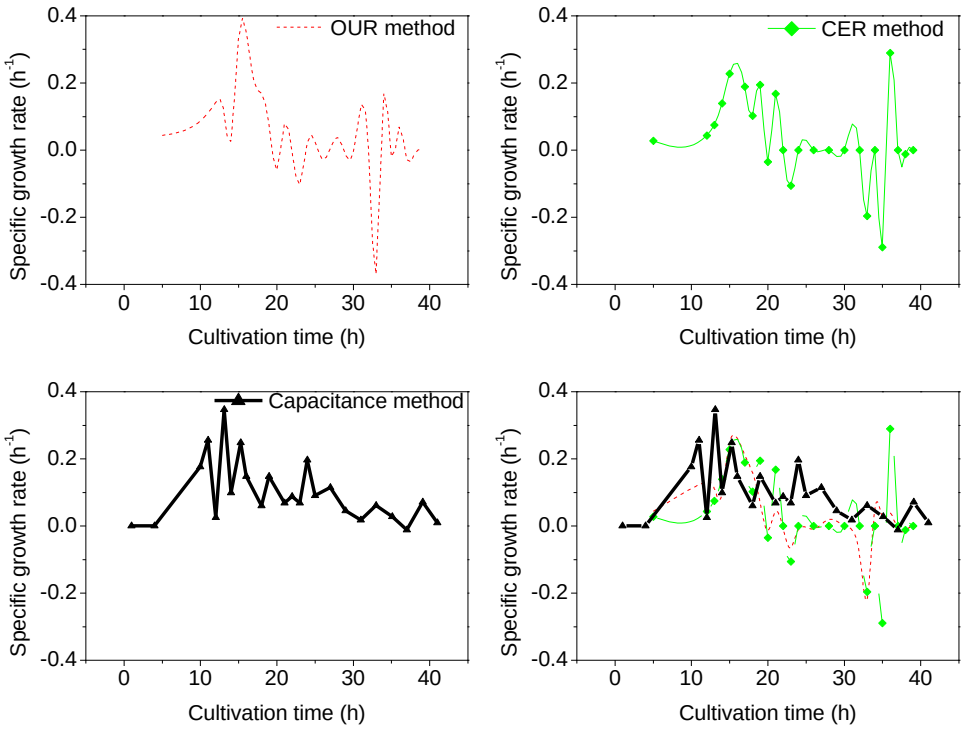

Figure 4. Comparison of specific growth rates estimated by at-line through direct estimation method (capacitance, solid line and closed triangles) and indirect estimation methods (CER, solid line and closed diamonds and OUR, dash line).

4. Current Bottlenecks of GSH Production in Yeast

GSH production in yeast has been widely investigated over a century. Although GSH yield significantly increased by HCD fed-batch fermentation, precursor addition, and Gsh1p overexpression in the genetic engineered yeast [13, 19, 31, 40, 44, 46], a better understanding of the current limitations and bottlenecks in GSH industry is necessary to develop new methods and strategies for further improvement. Based on our current understanding of GSH

metabolic pathway in yeast, two major bottlenecks need to be addressed: 1) GSH Feedback regulation of Gsh1p; 2) precursor cysteine conversion efficiency.

4.1. GSH Feedback Regulation

Several mutant strategies based on resistance to the mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NTG), methylglyoxal, sodium azide etc have been employed to isolate the feedback resistant mutants of Gsh1p [1]. While these mutants have led to higher GSH yields, information and mechanism about feedback resistant of the enzyme itself have not confirmed and understood. No additional information directly guided us to rational design for GSH-resistant enzyme.

Gsh1p evolves across different organisms in three distinct lineages: (1) the lineage including yeasts, mammals, fly, worm, fungi and parasites; (2) the bacterial lineage; and (3) the plant lineages [1]. We found that Gsh1p from *S. cerevisiae* is closed to *Zygosaccharomyces rouxii*, not *P. pastoris/C. utilis* by phylogenetic analysis (Figure 5). Gsh1p of *S. cerevisiae* has been crystallized in presence of $MgCl_2$ and glutamate, in complex with GSH, and in complex with $MgCl_2$, ADP and glutamate [9, 18]. The structure reveals an unusual binding pocket for an ATP-independent Mg^{2+} coordination site and the α-carboxylate group of the glutamate substrate. The catalytic subunit model suggests that post-translational modifications of cysteine residues may regulate Gsh1p activity [9]. The structure of Gsh1p also indicates that GSH occupies both the glutamate- and cysteine-binding sites and disrupts Mg^{2+} coordination in the ATP-binding site. These structures advance our understanding of the catalytic mechanism of Gsh1p and the molecular mechanism of feedback regulation by GSH [18]. Based on the above-mentioned knowledge of Gsh1p structure, we can engineer the glutamate-, cysteine- and ATP-binding sites of the catalytic subunit to increase substrates binding efficiency and decrease GSH bind efficiency to solve feedback inhibition problem. Thus, construction of a Gsh1p pool by site-directed mutagenesis and rational design is a way to isolate the GSH over-resistant enzyme.

An alternative way to solve the feedback regulation problem is to increase GSH excretion to prevent GSH over-accumulation in cells. GSH in the yeast secretes at low levels under normal growth conditions, which is taken up back into the cell by GSH transporter. A recent genome-wide screening identified ~270 genes (belong to 10 different functional classes) affecting GSH homeostasis [51]. These genes disruption have led to overexcrete GSH (2–37 fold) into the medium [51], e.g. GSH uptake transporter (*HGT1*) deletion mutant leads to threefold higher levels of GSH in the extracellular medium [51]. Although the GSH excretion mechanism is still not understood, many mutants exhibited altered plasma membrane permeability. For example, the vacuolar biogenesis *vps2* mutant that was defective in endosomal protein sorting was found to the highest GSH secretion [51]. In addition, there may have an efflux pump similar to *E. coli* CydDC which could export GSH redundant to the periplasm in yeast [52].

A second way to over-secretion is modulating the culture conditions. Low pH strategy can stimulate GSH secretion [53]. *C. utilis* have a significant GSH secretion into the medium at pH 1.5 for 3 h without any loss in cell viability [53]. Cell growth of *S. cerevisiae* at pH 3.5 leads to a significant GSH leakage while it is little or no GSH excretion at pH 6.

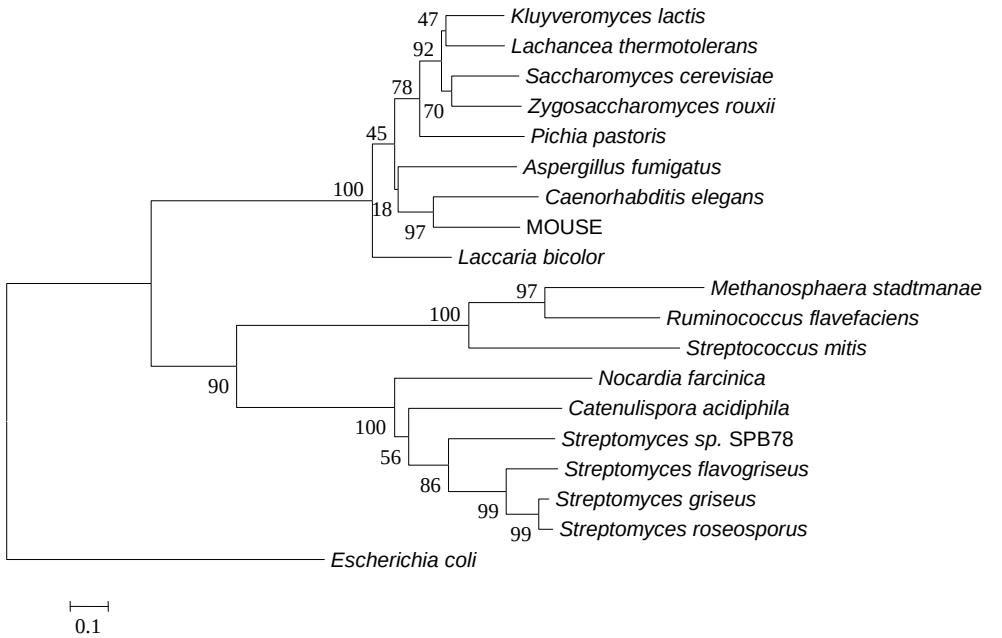

Figure 5. Neighbor-joining phylogenetic tree for Gsh1p based on the amino acid sequences with *E. coli* as the outgroup using MEGA 4.0 (Center for evolutionary medicine and informatics, Biodesign Institute, USA) [49]. Bootstrap values are based on 1,000 replicates. Scale bar represents 10 base substitutions per 100 bases. CLUSTAL X 2.0 (Conway Institute, USA) was used to generate alignment of Gsh1p from *S. cerevisiae* and other organisms [50].

Some genes responsible for the membrane compositions such as *ino1* and *ino4* may be related to inhibit GSH secretion at higher pH [51]. Moreover, surfactant addition during cultivation also affects GSH efflux into the medium [54, 55]. Low concentration of surfactants (e.g. 0.001-0.01 g/l SDS and CTAB) during cell growth achieves higher levels of extracellular GSH without significant affecting the growth and viability of the cells [54].

4.2. Precursor Cysteine Conversion Efficiency

Precursor fluxes through different metabolic cycles in the cell have a significant influence on the GSH biosynthesis. Although the addition of glutamate, glycine, serine, methionine, and arginine increases intracellular GSH content [19, 56], cysteine is the rate-limiting nutrient in GSH biosynthesis [13].

Manipulation of cellular cysteine level by extracelluar addition can lead to significantly increase GSH content [57, 58], but the conversion efficiency of extracelluar cysteine to GSH is limited to ~40% of the theoretical yield due to its requirement as a sulfur for protein synthesis whereby the sulfate assimilation is down-regulation [42]. In addition, shot-wise supplementation of cysteine substantially increases the production cost. Hence, engineered cysteine biosynthetic pathway becomes a practicable alternative to improve GSH yield and decrease the process cost. Despite the sulphur assimilation pathway and cysteine biosynthesis are under a tight transcriptional regulation by the intracellular cysteine levels [59], overexpression of some key enzymes (e.g. *CYS4*, cystathionine β-synthase and *MET4*, leucine

zipper transcriptional activator) in the cysteine biosynthetic pathway can enhance cysteine and GSH biosynthesis [32, 60].

Conclusion and Perspectives

Yeast continues to play a key role in GSH production and research being taken on understanding of GSH biosynthesis and its cellular redox homeostasis, although there are a lot of studies using other organisms/systems such as *E. coli* [61], Lactic acid bacteria [62-64] and *Actinobacteria* [65]. Despite the discovery of GSH over 120 years and the development of GSH fermentative method over 30 years, GSH yield still maintains at 2-5 g/l on an industrial scale.

Engineered industrial strains are still limited in 2-3 genes relevant to GSH biosynthesis, lacking a globe modification level to optimize GSH production. The transcription factors of both the sulphur assimilation and GSH biosynthesis have disclosed in the past few years, a better understanding of global sulphur metabolism and γ-glutamyl cycle in cells provides us a chance to further utilize them. For example, we can reprogram gene transcription (Yap1 and Met4) to elicit GSH overproducer phenotype through global transcription machinery engineering (gTME) [66].

We can also program cells to fine-tune all related genes and pathways of GSH biosynthesis by multiplex automated genome engineering (MAGE) [67]. Moreover, genetic engineering methods such as DNA shuffling and genome shuffling [68] are still efficient to strain engineering/improvement. Ribosome engineering [69] also allows us to discover a higher yield strain of GSH by selection of drug-resistant mutants carrying mutations in RNA polymerase and/or the ribosomal protein S12.

Lastly, the studies at the protein level have lagged behind to understand GSH transcriptional regulation. Site-directed evolution and mutagenesis of key proteins (Gsh1p, Gsh2p, Yap1p and Met4p) have a great potential to revise the substrate-binding sites and/or the catalytic regions for GSH over-accumulation along with the elucidation of these proteins structure.

In conclusion, GSH is successfully produced by fermentative method on an industrial scale and widely used in the pharmaceutical, food additive, and cosmetic industries. With various new techniques/tools emergence and application in the GSH industry, the price of GSH will further decrease by reducing the production costs, which will further expand its application and utilization.

Acknowledgments

This chapter was supported by the National Natural Science Foundation of China (grant no. 31100073), the Open Funding Project of the State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, and the Fundamental Research Funds for the Central Universities.

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Index

#

1,2-dimethylhydrazine, 203
2-oxoaldehydes, 304
3-Nitrotyrosine, 112, 122
4-hydroxynonenal, 115, 208, 215, 241
4-nitroquinoline 1-oxide, 202
6-hydroxy dopamine, 96

A

α,β-unsaturated carbonyls, 167
α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate, 36, 45
α-ketoglutarate dehydrogenese, 235
Actinobacteria, 351
acute myeloid leukemia, 213
acute respiratory distress syndrome, 4, 20, 127
adrenergic receptors, 127
adrenocorticotropic hormone, 36, 37
Agrobacterium, 242
AIDS, 65, 81, 111, 116, 120, 122, 123, 136, 137, 159, 160, 161
allelic variants, 167, 168, 180, 181, 194
allicin, 329
aminopropylcadaverine, 312
amoebiasis, 298, 317, 318
antiapoptotic activity, 195
antimalarial drugs, 298, 300, 302
Antimycin A, 238
antimycobacterial action, 150
antioxidant-responsive element (ARE), vi, 4, 6, 7, 8, 9, 10, 11, 13, 14, 15, 16, 17, 18, 20, 23, 24, 35, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 49, 50, 55, 56, 57, 59, 60, 62, 63, 65, 67, 68, 69, 70, 80, 81, 82, 83, 84, 85, 86, 87, 88, 95, 96, 97, 98, 99, 100, 102, 103, 104, 105, 106, 111, 113, 114, 115, 116, 117, 118, 119, 120, 122, 123, 124, 125, 127, 128, 129, 131, 132, 143, 144, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 165, 166, 167, 168, 170, 171, 172, 173, 174, 176, 177, 178, 179, 181, 187, 188, 189, 190, 191, 192, 194, 195, 199, 200, 201, 203, 204, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 223, 224, 227, 233, 234, 235, 236, 237, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 250, 251, 252, 253, 254, 255, 256, 264, 265, 266, 267, 268, 271, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 295, 297, 298, 299, 300, 301, 302, 304, 305, 306, 307, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 328, 329, 331, 333, 334, 335, 336, 337, 341, 342, 344, 345, 350, 351
antioxidants, 14, 15, 17, 23, 25, 26, 28, 60, 71, 80, 81, 84, 85, 86, 87, 88, 89, 91, 113, 119, 122, 123, 125, 128, 129, 130, 132, 144, 158, 211, 235, 242, 243, 244, 245, 247, 248, 249, 250, 251, 252, 254, 256, 258, 259
apoptosis, xi, 7, 13, 23, 26, 84, 115, 121, 123, 128, 129, 151, 154, 160, 165, 172, 194, 195, 202, 215, 235, 254, 255, 265, 303, 328, 329, 334, 335
Arbuscular Mycorrhiza (AM), 281
arene oxides, 167
ascorbic acid, 81, 113, 234, 264
ascorbic acid oxidase, 264
astrocyte, 95, 97, 98, 99, 103, 131
atheromatosis, 181
atherosclerosis, 15, 18, 27, 30, 33, 143, 150, 156, 157, 158, 159, 160, 161, 162, 174, 179, 185, 186, 253
autoimmune disease, 180

B

β-mercaptoethylamine, 128

benznidazole, 309
brain microvascular endothelial cells, 120
Breast cancer, 10, 25, 28, 29, 31, 166, 173, 174, 175, 213, 214, 255
bronchial hyperactivity, 127
buthionine sulphonine, 144, 145

C

cadaverine, 312
cadmium, 66, 233, 234, 236, 242, 271, 286, 329
cardiovascular disease, 15, 20, 33, 84, 156, 157, 158, 159
caspase, 115, 121, 129, 130, 150, 329, 334, 335
catalase, 4, 14, 66, 81, 84, 86, 87, 112, 113, 114, 158, 234, 243, 256, 264, 269, 271, 278, 283, 284, 297, 300, 310, 317, 335
cataracts, 65, 84, 118, 131, 132, 235, 253
CCAAT/enhancer binding protein, 208, 211, 212
central nervous system, 20, 22, 33, 36, 37, 96, 119
cerebral ischemia, 119
Chagas disease, 253, 295, 298
chemotherapy, 10, 20, 65, 86, 138, 178, 194, 195, 201
chlorambucil, melphalan, 214
chloromethane, 216
cholecalciferol, 63, 64
chronic gastritis, 255
Chronic oxidative stress, 105, 180
cisplatin, 65, 195, 208, 214
c-jun N-terminal kinase, 188
claudin, 120, 121
coenzyme Q, 238, 253
colorectal cancer, 23, 31, 178, 179, 194, 213, 255, 256
complementary RNA, 208
copy number variations, 208, 213
coronary artery disease, 144, 156, 157, 158, 179
corticotropin-releasing factor, 36, 37
coulombic surface analysis, 192
curcumin, vii, 95, 96, 102, 103, 104, 105
cyclooxygenases, 113
cysteinil-glycin-dipeptidase, 82
cysteinyl-glycine, xi, 35, 36, 42, 43, 64, 111, 265, 297
cystic fibrosis, 19, 21, 111, 116, 117
cytochrome b, 238
cytochrome c, 115, 123, 334
cytochrome oxidase, 238
cytochrome P_{450}, 4, 16, 24, 62, 126, 180, 228, 238, 239
cytochrome P450 family enzymes, 4
cytomegalovirus, 122
cytoprotection, 208

D

D-cysteine, 36, 48
dehydroascorbate, 60, 84, 171, 172, 233, 234, 243, 247, 264, 271, 283
dehydroascorbic acid, 167, 234, 303
dementia, 120, 122
diabetes, 15, 18, 26, 66, 74, 111, 116, 117, 158, 165, 179, 204, 221, 225, 235, 253, 260, 342
diabetic nephropathy, 4, 18, 29
diallyldisulfide, 328, 329
dichloromethane, 208, 210
Diesel engine exhaust, 125
dihydrolipoamide dehydrogenase, 304, 307
dihydrolipoic acid (DHLA), 235
dithiolic glutaredoxin, 312, 316
DNA internucleossomic fragmentation, 84
DNA shuffling, 210, 351
DnaK suppressor protein, 146
dopamine, 100, 112, 124
dopaminergic system, 123
downstream processing, 347
drought stress, 243, 269, 287

E

eicosanoid metabolism, 165
electron Paramagnetic Resonance, 240
endothelial cell barrier, 122
energy expenditure, 35, 36, 42
Entamoeba histolytica, 68, 296, 316
epidemiological studies, 224, 227
epidermal growth factor, 208
essential amino acids, 36, 45
ethoxyquin, 167

F

feedback regulation, 349
Fenton reaction, 158, 236, 244, 252, 299
Fermentative method, 341, 342, 343, 351
fetus abortion, 86
flavin-adenine-dinucleotide, 4

G

Gastric cancer, 29, 176, 177, 213
gastric metaplasia, 255
gene polymorphisms, 29, 31, 180, 201, 223, 224, 229

genome shuffling, 351
genotoxic electrophiles, 207
genotype frequencies, 223, 224, 225
Giardia lambia, 318
Glaucoma, 180, 253
glucagon-like peptide-1, 36, 41, 50
glucocorticoid-responsive element, 208, 210
glucose oxidase, 158
glutamate cysteine ligase, 19, 59, 145
glutamate toxicity, 119, 130
glutamate-cysteine ligase, 319, 342
glutamatergic receptors, 130, 131
glutamylcysteine ethyl ester, 66
glutamylcysteine synthase, 97, 98
glutaredoxins, 172, 295, 297, 300, 302, 312
glutathione disulfide, 5, 60, 64, 66, 79, 80, 82, 113, 114, 118, 300, 304
glutathione disulfide *S*-monoxide, 304
glutathione oxidase, 3, 4, 9
glutathione peroxidase, 3, 5, 9, 10, 14, 39, 59, 62, 64, 79, 80, 81, 83, 84, 91, 113, 119, 124, 132, 234, 252, 268, 269, 296, 297, 300, 305, 307, 308, 310, 314, 315, 316
glutathione reductase, 3, 5, 9, 20, 23, 33, 35, 38, 40, 41, 59, 60, 80, 81, 83, 84, 113, 234, 243, 247, 271, 278, 295, 296, 297, 300, 302, 304, 307, 308, 310, 317, 327, 328, 329
glutathione sulfenic acid, 304
glutathione synthetase, 60, 62, 82, 245, 284, 301, 311
glutathione thiyl radical, 304
glutathione transferase, 3, 5, 8, 24, 25, 31, 80, 81, 83, 88, 187, 188, 195, 228, 234, 263, 264, 267, 268, 271, 272
glutathione/ascorbate cycle, 233, 235, 247
glutathione-dependent peroxidases, 265, 300
glutathione-S-transferase, 24, 29, 30, 32, 114, 117, 305, 342
glutathionylspermidine synthetase, 296, 312, 315
glycine, 3, 4, 7, 8, 35, 36, 38, 43, 59, 64, 79, 81, 82, 97, 98, 99, 102, 113, 116, 117, 145, 166, 240, 248, 281, 284, 296, 297, 301, 308, 311, 315, 342, 350
glycolytic pathway, 344
glyoxalase system, 181, 304, 305
glyoxalases, 295, 300, 304
granulosa cell tumor cell line, 214
G-site, 188, 192

H

halogenonitrobenzenes, 167
Hansenula polymorpha, 345
head and neck squamous cell carcinoma, 5, 18, 30, 31, 32, 178
heavy metal tolerance, 264, 286, 287
hematopoietic diseases, 203
hemoglobin, 118, 299, 301, 306
hepatic nuclear factor 1, 208, 211, 212
hepatocarcinogenesis, 200, 201, 203, 204
hepatocellular carcinoma, 31, 177, 200, 203
herbicide detoxification, 264
high density lipoproteins, 144, 157
HIV, 5, 10, 23, 65, 68, 111, 112, 113, 116, 117, 120, 121, 122, 123, 136, 143, 151, 152, 153, 154, 155, 156, 159, 160, 161
homoglutathione, 282, 284
homoglutathione sythetase, 284
homotrypanothione, 312
H-site, 188, 192
human immuno-deficiency virus, 143
human monocyte derived macrophages, 144, 149
humoral cytokines, 153
hydrogen peroxide, 5, 9, 10, 14, 15, 22, 36, 39, 57, 60, 80, 81, 83, 114, 128, 146, 157, 203, 234, 235, 236, 279, 297, 299, 316, 317
hydroperoxides, 57, 83, 114, 123, 179, 188, 191, 265, 313, 314, 315, 316
hydroxyl radical, 15, 57, 81, 114, 128, 158, 234, 235, 236, 237, 240, 241, 243, 249, 297, 299, 317
hypochlorous acid, 57, 158, 317
hypothalamic-pituitary-adrenal axis, 36

I

idiopathic pulmonary fibrosis, 127
immature cortical neurons, 119
Indinavir, 112, 122
inducible nitric oxide synthase, 96, 98, 149
inflammation, 21, 33, 116, 127, 156, 166, 179, 235, 317
interleukin-6, 96
intracellular symbiotic interactions, 281, 282
ionizing radiation, 10, 13, 15, 113, 128, 129, 166
ionotropic glutamate receptors, 36
ischemic stroke, 180
Isothiocyanates, 189

L

L-2-oxothiazolidine-4-carboxylate, 63
Lactic acid bacteria, 351
L-buthionine-[S-R]-sulfoximine (BSO), 96, 97, 100, 132, 285

L-cysteine, 7, 9, 35, 36, 38, 43, 48, 49, 59, 62, 67, 69, 102, 113, 117, 311, 317, 318, 342
leishmaniasis, 309
Leucotrienes, 62
L-glutamate, 35, 36, 38, 43, 113, 145, 311
linoleate, 237
lipid hydroperoxides, 87, 191, 236, 241
lipoamide dehydrogenase, 248
lipoic acid, LA, 235
liposomal GSH, 144, 156, 157
lipoxygenases, 113
low density lipoprotein, 144, 157
L-serine, 36, 48, 318
lung cancer, 18, 31, 32, 67, 168, 173, 176, 194, 201, 213, 214

M

macrophages, 125, 126, 128, 143, 148, 149, 150, 152, 153, 154, 155, 156, 157, 158, 159, 171, 313, 317
major depressive disorder, 65
malaria, 215, 295, 298, 299, 300, 301, 303, 304, 305, 306
maleylacetoacetate isomerase, 166, 172
malondialdehyde (MDA), 70, 115
malondialdyde, 144, 145
medulloblastoma, 62
Mehler reaction, 239, 243
melanin, 62, 63
melatonin, 64
membrane-associated proteins in eicosanoid and glutathione metabolism, 5, 166, 167, 210
metabotropic glutamate receptors, 36
metal chelator, 245, 297
methamphetamine, 112, 113, 118, 121, 122, 123, 138
methionine glyase, 318
methylglyoxal, 62, 300, 304, 305, 349
Methylobacterium, 216
metronidazole, 318
Michael adducts, 122
microsomal epoxide hydrolase enzymes, 5
microtubule associated protein 2, 96
mitogen-activated protein kinase, 208, 212, 276
mitogen-activated protein kinase kinase 1, 208, 212
mitogen-activated protein kinase-activated protein kinase 2, 208
Monobromobimane, 70
monodehydroascorbate radical (MDA), 247
monodehydroascorbate reductase (MDHAR), 234, 247, 283
mouse embryonic fibroblasts, 208, 212
multidrug resistance-associated proteins, 178
multiple drug resistances, 5, 19
multiple myeloma, 224, 226, 227, 228, 229
Mycobacterium tuberculosis, 143, 144, 159
myelodysplasia, 215, 228

N

N-acetylated L-cysteine, 144, 145
N-acetylcysteine, 64, 111, 117, 118, 130
N-acetylcysteine amide, 111, 117
N-acetyl-L-cystine, 96
N-acetyl-*p*-benzoquinone imine, 62
N-acetyl-*p*-benzoquinone imine (NAPQI), 62
NADH dehydrogenase, 238
NADPH-oxidase, 283
natural killer cells, 143, 150
neoplastic transformation, 233, 234, 235, 242, 244
neuroblastoma cells, 119, 124
neurocognitive disorders, 120, 122, 123
neurons, 39, 40, 43, 47, 48, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 130
neurotoxicity, 102, 106, 118, 119, 120, 122, 124, 129, 130, 131, 138
nifurtimox, 309
nitric oxide, 33, 58, 60, 98, 102, 113, 144, 145, 148, 149, 158, 159, 276, 279, 299, 317
nitric oxide synthase, 113, 144, 145
nitrosoglutathione, 144, 148, 296, 304, 307, 308
N-methyl-*D*-aspartate, 36, 38
N-methyl-N'-nitro-N-nitrosoguanidine, 349
normal granulosa cells (NGC), 214

O

oligodendroglia, 119
ovothiol A, 311
oxidative stress, xi, 3, 6, 7, 9, 11, 14, 15, 17, 18, 19, 20, 21, 23, 24, 25, 26, 27, 29, 33, 38, 39, 55, 56, 58, 59, 60, 65, 66, 67, 69, 79, 81, 85, 86, 87, 90, 95, 100, 101, 102, 103, 105, 111, 113, 114, 116, 118, 119, 120, 121, 122, 123, 124, 125, 127, 128, 130, 131, 132, 133, 143, 144, 145, 146, 147, 150, 154, 157, 158, 167, 179, 188, 191, 195, 201, 203, 207, 209, 211, 212, 213, 216, 234, 235, 236, 240, 242, 244, 248, 250, 253, 254, 255, 256, 258, 263, 264, 265, 277, 280, 284, 287, 297, 299, 303, 304, 305, 312, 317, 318, 319, 328, 329, 335
oxidized-LDL, 144, 150

P

peripheral blood mononuclear cell, 144, 150

peroxidase, 8, 9, 23, 91, 97, 112, 114, 144, 150, 157, 233, 234, 243, 247, 252, 266, 269, 271, 283, 287, 296, 304, 305, 307, 308, 314, 317
peroxynitrite, 58, 98, 122, 299, 317
phosphatidylcholine, 87
phosphatidylethanolamine, 87
phosphatidylinositol 3-kinase, 208
phospholipase, 45, 130, 131, 278
phospholipid hydroperoxide, 252
phospholipid hydroperoxide GSH peroxidases, 60
phosphotidylinositol 3-kinase (PI3K), 212
phytochelatins, 245
phytosphingosine, 327, 328, 329, 333, 335
Pichia pastoris, 345
planar aromatic compounds, 167
Plasmodium falciparum, 298, 299, 300, 308, 319
plasmoredoxin, 296, 303, 304, 308
polycyclic aromatic hydrocarbons, 12, 125, 166, 171
Polyunsaturated fatty acids, 80, 85, 114
precancerous lesions, 255
probucol, 119
Prostate cancer, 30, 31, 32, 175, 195, 213, 214
protein kinase C, 14, 27, 303
pyruvate ferredoxin oxidoreductase, 317, 318

R

radioprotectors, 128
radiotherapy, 86, 128
rapid-eye-movement sleep, 36, 39
reactive nitrogen intermediates, 144, 157
reactive nitrogen species (RNS), 15, 26, 58, 80, 87, 166, 317
reactive oxygen intermediates, 143, 144
reactive oxygen species (ROS), 5, 6, 13, 14, 15, 20, 23, 26, 27, 38, 39, 57, 58, 59, 60, 65, 80, 81, 83, 84, 85, 86, 87, 88, 89, 104, 113, 114, 115, 116, 117, 119, 121, 123, 124, 126, 127, 128, 129, 130, 148, 154, 157, 166, 179, 191, 207, 209, 211, 264, 271, 275, 276, 279, 280, 282, 283, 284, 287, 295, 297, 299, 300, 306, 314, 317, 328, 329, 334, 335, 336
redox sensitive yellow fluorescent protein, 70
redox-sensitive green fluorescent protein, 70
respiratory quotient, 36, 42
rheumatoid arthritis, 20, 33, 174
rhizobia, 281, 282, 283, 284
ribonucleotide reductase, 297, 303, 306, 313, 314, 315
ribosome engineering, 351
RNA interference, 208, 269, 311
root nodules, 281, 283

S

S. cerevisiae, 329, 334, 344, 345, 346, 347, 348, 349, 350
S-2-hydroxyacylglutathione, 304
S-adenosylmethionine, 64, 308
salinity, 250, 264, 268
Schizophrenia, 37, 65, 98, 102
Sedative and Hypnotic Effects, 39, 49
S-glutathionylation, 194, 280, 303, 304
S-hexyl-GSH, 191, 193
sickle cell anemia, 111, 116
signal-regulating kinase 1, 188, 194
single nucleotide polymorphisms, 5, 17, 173, 181
skin papillomas, 213
sleeping sickness, 295, 298, 309
sleep-promoting substance, 36, 39, 40
S-methylglutathione, 36, 39
S-nitrosation, 304
sodium azide, 349
sperm cryopreservation, 88, 89
Spermatogenesis, 85
spermidine, 295, 309, 310, 312, 313, 317
sphingomyelin, 87
staurosporine, 327, 328, 329, 333
staurosporine (STS), 327, 328, 329
stress signalling mechanism, 188, 195
structure, viii, 8, 11, 24, 28, 45, 46, 81, 89, 90, 101, 131, 167, 171, 187, 189, 190, 192, 193, 209, 218, 237, 240, 249, 292, 302, 305, 311, 319, 343, 349, 351
sulfotransferase enzyme, 5
sulphonamidase activity, 191
superoxide anion, 15, 57, 81, 98, 114, 158, 297, 306, 317
superoxide dismutase, 5, 14, 59, 80, 81, 84, 86, 87, 98, 113, 158, 234, 238, 243, 264, 269, 271, 283, 296, 297, 300, 306, 308, 317
symbiosome membrane, 281
symbiosomes, 281
systemic lupus erythematosus, 20, 166, 180

T

temperature stress, 264
teratocarcinoma cells, 96
Thiobarbituric acid reactive substances, 70
thioltransferases, 302, 314
thioredoxin, 23, 192, 209, 249, 256, 277, 285, 295, 296, 299, 302, 303, 304, 306, 307, 308, 310, 314, 318, 333

thioredoxin reductase, 296, 299, 302, 304, 306, 307, 308, 310, 318, 333
tocopherol, 26, 27, 81, 84, 119, 233, 235, 241, 243, 245, 246, 247, 249, 250, 251, 252, 256, 297
toll-like receptors, 144, 149
toxoplasmosis, 122
trace elements, 68
transgenic plants, 263, 264, 265, 266, 267, 268, 269, 270, 271
trans-stilbene oxide, 25, 167
trypanosomatids, 295, 309, 310, 311, 312, 313, 314, 315, 316, 317, 319
trypanothione, 295, 296, 297, 302, 307, 309, 310, 311, 312, 313, 314, 315, 317, 319
trypanothione synthetase (TS), 296, 312
trypanothione-dependent enzymes, 296
tryparedoxin, 296
tryparedoxin peroxidase, 297, 314, 315
trypomastigotes, 309
tuberculosis, 143, 144, 148, 155, 159
tumor necrosis factor receptor-associated factor 2, 188, 194
tumorigenesis, 174, 208, 209, 213, 214, 216, 217
tyrosinase, 62, 63

U

ubiquinone, 238
UDP-glucuronosyltransferases, 6
ultraviolet radiation, 299
unsaturated fatty acid, 237
uridine 5'-diphospho-glucuronosyltransferase, 6

V

vitamin D, 63, 64
vitamin D_3, 63, 64
vitamin E, 14, 26, 81, 84, 113, 233, 240, 245, 246, 252, 254, 255

X

xanthine oxidase, 113, 158
xenobiotic detoxification, 272, 299
xenobiotic-responsive element (XRE), 209, 210

Z

Zeta (ζ) class GST, 268

Γ

γ-glutamiltransferase (GGT), 82
γ-glutamyl transpeptidase, 7, 20, 99
γ-glutamyl-cysteine, 7, 19, 20, 36, 42, 59, 96, 97, 102, 277, 279, 284, 342, 343