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Antioxidants in Biology and Medicine

Essentials, Advances, and Clinical Applications

Yunbo Li

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ANTIOXIDANTS IN BIOLOGY AND MEDICINE: ESSENTIALS, ADVANCES, AND CLINICAL APPLICATIONS

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Preface

Molecular oxygen is essential for aerobic life, yet incomplete reduction or excitation of molecular oxygen during aerobic metabolisms results in the inevitable formation of reactive oxygen species (ROS). ROS are capable of causing damage to cellular constituents, including proteins, lipids, and nucleic acids. As such, aerobic organisms, including mammals have evolved a series of complementary antioxidant enzymes and non-enzymatic antioxidant molecules to control ROS and thereby protect critical biomolecules from oxidative damage. In addition, many diet-derived compounds have antioxidant activities and thus also contribute to the mammalian antioxidant defenses. Under physiological conditions, ROS and antioxidant defenses are generally in balance. However, increased formation of ROS or compromised antioxidant defenses may disrupt this balance, leading to oxidative stress that may eventually contribute to the development of many diseases.

The last two to three decades have witnessed the remarkable advances in our knowledge about ROS and antioxidants in biology and medicine. It has been increasingly recognized that ROS play an important role in diverse disease processes, including cardiovascular disorders, diabetes, neurodegeneration, and cancer. In line with this notion, accumulating evidence also demonstrates the effectiveness of antioxidant-based strategies for the intervention of an increasing number of disease processes that involve an oxidative stress mechanism. In this context, many synthetic antioxidant compounds have been developed, which can also be used to augment antioxidant defenses to protect against oxidative stress-associated disease pathophysiology. New knowledge in antioxidant biology and medicine will certainly further enhance our ability to develop more effective mechanistically-based strategies to combat human diseases that involve an oxidative stress component.

This book covers both the fundamentals and the most recent advances in antioxidant research in biology and medicine. The aim of the book is to provide a critical evaluation of the various endogenous and exogenous antioxidants with an emphasis on their role in health and disease. To this end, the book focuses on discussing solid scientific evidence obtained from basic research in mammalian models of human diseases as well as studies conducted directly in human subjects. The book is divided into four sections, as listed below.

- Section I: Introduction to Oxidative Stress and Antioxidants
- Section II: Antioxidants Synthesized by Cells
- Section III: Antioxidants Derived from the Diet
- Section IV: Synthetic Antioxidants

To set a stage for the subsequent discussion on how individual antioxidants function to protect against oxidative stress and disease processes, Section I introduces the basic knowledge on ROS and oxidative stress. It also provides an overview of the different kinds of antioxidants, the molecular regulation of antioxidant genes, and the antioxidant-based strategies for disease intervention. Section I has two chapters.

Section II consists of seventeen chapters devoted to the discussion of each of the major endogenous antioxidants synthesized by cells with respect to their basic biology and involvement in health and disease. Each chapter covers knowledge in three major areas: (1) basic biochemistry and molecular regulation, (2) animal studies on the role in disease processes, and (3) human studies and clinical applications.

Section III contains four chapters that cover the major antioxidants derived from the diet: vitamin C, vitamin E, carotenoids, and phenolic compounds. The biochemical properties and role in health and disease (including both experimental and clinical evidence) for each of these diet-derived antioxidants are discussed.

Section IV consists of four chapters on synthetic antioxidants, including antioxidant enzyme mimetics, glutathione precursors, spin traps, and nanomaterials. These chapters describe the chemical and biochemical properties of each of the above four classes of synthetic antioxidants with an emphasis on their protective effects in disease processes in both experimental animals and human subjects.

It is hoped that this book by integrating knowledge on antioxidants from essentials to advances, and from basic research to clinical applications will provide the reader a unique approach to understanding the rapidly evolving field of antioxidants in biology and medicine.

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January, 2011

SECTION I
INTRODUCTION TO OXIDATIVE STRESS AND ANTIOXIDANTS

Utilization of molecular oxygen by aerobic organisms, including mammals leads to the inevitable formation of reactive oxygen and nitrogen species (ROS/RNS) from various metabolic processes. ROS/RNS have the potential to damage cellular constituents. As such, aerobic organisms have evolved a number of antioxidants to protect critical biomolecules from ROS/RNS-mediated damage. Under physiological conditions, formation of ROS/RNS and functioning of antioxidants are generally in balance. However, increased ROS/RNS production or compromised antioxidant defenses may disrupt this balance, leading to potentially detrimental conditions, such as oxidative stress injury. Understanding of the basic biology of ROS/RNS and oxidative stress is crucial for developing antioxidant-based strategies for the intervention of diseases involving an ROS/RNS-dependent mechanism. Section I introduces the basics related to ROS/RNS and oxidative stress, and provides an overview of antioxidants and their gene regulation. This sets a stage for the subsequent chapters devoted to the discussion of individual antioxidants in biology and medicine. Section I consists of two chapters.

Chapter 1

Introduction to Oxidative Stress

Abstract

Reactive oxygen and nitrogen species (ROS/RNS) are collective terms that refer to a number of oxygen- and nitrogen-containing reactive species. ROS/RNS are produced from various cellular sources. They also exist as environmental pollutants. Although ROS/RNS fulfill important physiological functions, uncontrolled formation of ROS/RNS leads to detrimental conditions, such as oxidative stress injury. This chapter introduces the common types of ROS/RNS and summarizes the role of oxidative stress in disease development.

1. Historical Overview

Molecular oxygen is essential for aerobic life, yet incomplete reduction or excitation of molecular oxygen during aerobic metabolisms generates oxygen-containing reactive species that pose a serious threat to aerobic organisms. Although more than two centuries ago J. Priestley stated that "oxygen might not be so proper for use in the usual healthy state of the body…", insight into the mechanisms of oxygen toxicity was provided in an article published in 1954 by R. Gerschman and coworkers, hypothesizing that oxygen poisoning and radiation injury may have at least one common basis of action, possibly through the formation of oxidizing free radicals [1]. Another important event in the investigations of oxygen toxicity was the finding by J.M. McCord and I. Fridovich in 1969 of an enzymatic function of a protein containing both copper and zinc, which then was known alternatively as erythrocuprein [2]. The function of this enzyme (Cu,Zn superoxide dismutase) is catalysis of dismutation of superoxide (a free radical) to produce hydrogen peroxide and molecular oxygen. The discovery of Cu,Zn superoxide dismutase has triggered extensive investigations into the free radical mechanisms of oxygen toxicity. A large body of evidence over the last several decades demonstrates a critical role for oxygen-derived free radicals and related reactive species in the pathophysiology of a wide variety of diseases. While free radicals and related reactive species cause deleterious effects in biological systems, it is important to note that these reactive species may also play important physiological roles, such as antimicrobial activity and participation in cell signal transduction [3, 4].

2. Free Radicals and Reactive Oxygen and Nitrogen Species (ROS/RNS)

2.1. Definitions

2.1.1. Free Radicals

Free radical is a commonly encountered term in biology and medicine. It refers to any chemical species capable of independent existence that contains one or more unpaired electrons. An unpaired electron refers to the one that occupies an atomic or molecular orbital by itself. For example, the superoxide ($O_2^{\cdot -}$) noted above is a free radical. The superscript dot indicates the unpaired electron whereas the superscript minus sign indicates that the free radical is negatively charged. Both anionic and cationic free radicals exist in biological systems. In general, free radicals are reactive species. However, as described below, reactive species are not necessarily free radicals.

2.1.2. ROS/RNS

Reactive oxygen species (ROS) is a term also frequently used in biology and medicine. This term can be simply defined as oxygen-containing reactive species. It is a collective term to include superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), singlet oxygen ($^1O_2$), peroxyl radical ($LOO^{\cdot}$), alkoxyl radical ($LO^{\cdot}$), lipid hydroperoxide (LOOH), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), and ozone (O_3). Similarly, the term reactive nitrogen species (RNS) has been coined to include nitric oxide ($^{\cdot}NO$), peroxynitrite, nitrogen dioxide ($^{\cdot}NO_2$), and other oxides of nitrogen or nitrogen-containing reactive species [5]. It is of note that peroxynitrite is classified as both an ROS and an RNS in the literature.

Among the ROS listed above, some contain unpaired electrons, and thus belong to free radicals. They are also called oxygen free radicals. Examples of oxygen free radicals include superoxide, hydroxyl radical, peroxyl radical, and alkoxyl radical. Some ROS do not contain any unpaired electrons, and thus they are not free radicals. Examples of the non-radical ROS include hydrogen peroxide, peroxynitrite, hypochlorous acid, and ozone. Singlet oxygen can exist in two states: the delta state ($^1\Delta_g$) and sigma state ($^1\Sigma_g^+$). ($^1\Sigma_g^+$)$^1O_2$ is a free radical because it contains two unpaired electrons. On the other hand, ($^1\Delta_g$)$^1O_2$ is a non-radical. Due to its highly unstable nature, ($^1\Sigma_g^+$)$^1O_2$ is generally regarded to lack biological significance. As such, in biology and medicine, singlet oxygen usually refers to the $^1\Delta_g$ state and is considered a non-radical species.

2.2. Sources of ROS/RNS

Utilization of molecular oxygen by aerobic organisms leads to the formation of ROS/RNS. ROS/RNS are generated from various endogenous cellular sources, such as NAD(P)H oxidases and mitochondria. ROS/RNS are also produced from a variety of exogenous sources, including physical agents, xenobiotics, and biologic agents (Table 1-1).

2.2.1. Endogenous Sources

The various endogenous sources of ROS/RNS in mammals are described below.

Table 1-1. Sources of reactive oxygen and nitrogen species in biology and medicine

Source	Description
Endogenous Sources	NAD(P)H oxidases Mitochondria (electron transport chain and oxidases) Xanthine oxidoreductase Cytochrome P450 enzymes Uncoupled nitric oxide synthases Peroxisomes
Exogenous Sources	Physical agents and particulate matter Xenobiotics Biologic agents (bacteria and viruses)

2.2.1.1. NAD(P)H OXIDASES

The NAD(P)H oxidase in phagocytic cells produces large amounts of superoxide during respiratory burst. Phagocytic NAD(P)H oxidase is perhaps the best characterized cellular source of ROS. This multicomponent enzyme consists of the catalytic subunit $gp91^{phox}$ (also referred to as NOX2), together with the regulatory subunits $p22^{phox}$, $p47^{phox}$, $p40^{phox}$, $p67^{phox}$, and the small GTPase Rac. For many years, this enzyme was thought to exist as a single isoform only in phagocytes and its function restricted to antimicrobial action. This notion changed with the discovery of multiple NOX forms that represent an enzyme family containing at least five members, namely, NOX1, NOX2, NOX3, NOX4, and NOX5. NOX stands for NAD(P)H oxidase. The NOX enzymes are expressed in various types of cells, and represent an important source of ROS under a wide variety of physiological and pathophysiological conditions [6].

2.2.1.2. MITOCHONDRIA

Another important cellular source of ROS is mitochondria. In mammalian cells, mitochondrial respiration usually accounts for 80-90% of the oxygen consumed by the cells. Under physiological conditions, both complexes I and III of the mitochondrial electron transport chain are involved in the univalent reduction of molecular oxygen to superoxide [7]. It is estimated that about 0.1% of the oxygen utilized by mitochondria is converted to superoxide. The superoxide formed in mitochondria undergoes dismutation to yield hydrogen peroxide either spontaneously or catalyzed by manganese superoxide dismutase in mitochondrial matrix. The formation of ROS by mitochondrial electron transport chain is increased under various pathophysiological conditions, including tissue ischemia-reperfusion [8]. Certain xenobiotics, such as quinone compounds undergo redox cycling catalyzed by mitochondrial electron transport chain complexes, leading to increased ROS formation. ROS formed through this mechanism are believed to contribute to the cardiotoxicity of doxorubicin, a quinone-containing anticancer drug [9]. In addition to mitochondrial electron transport chain, NOX4 is recently identified to be present in mitochondria, and may contribute to ROS formation in this organelle [10]. Monoamine oxidase associated with mitochondria is also known to produce hydrogen peroxide. Hence, ROS formed in mitochondria may result from two general pathways: mitochondrial electron transport chain complexes and oxidases present in mitochondria. Besides ROS, nitric oxide is also generated in mitochondria via the action of

mitochondrial nitric oxide synthase. Uncontrolled production of mitochondria-derived ROS/RNS has been implicated in diverse pathophysiological processes, such as tissue ischemia-reperfusion injury and neurodegeneration [11].

2.2.1.3. Other Endogenous Sources

Other cellular sources of ROS include xanthine oxidoreductase, cytochrome P450 enzymes, uncoupled nitric oxide synthases, and peroxisomes. Xanthine oxidoreductase has two interconvertible forms: xanthine dehydrogenase and xanthine oxidase. Both forms catalyze the conversion of hypoxanthine to xanthine, and xanthine to uric acid. Both forms also catalyze one- and two-electron reduction of molecular oxygen to form superoxide and hydrogen peroxide, respectively, with xanthine oxidase being more active in generating the ROS. Xanthine oxidoreductase-derived ROS are implicated in pathophysiological processes, such as tissue ischemia-reperfusion injury. Cytochrome P450 enzymes, particularly P4502E1 can generate significant amounts of ROS when being induced by xenobiotics [12]. The ROS formed from P4502E1 contribute to alcoholic liver injury. Nitric oxide synthases normally catalyze the formation of nitric oxide. Under certain conditions, such as substrate deficiency or oxidative modifications, these enzymes can catalyze one-electron reduction of molecular oxygen to form superoxide. The organelle peroxisome contains enzymes, especially the flavoprotein dehydrogenases that produce significant amounts of hydrogen peroxide. Peroxisome also contains large quantities of catalase, which decomposes hydrogen peroxide (see Chapter 4 for discussion of catalase).

2.2.2. Exogenous Sources

2.2.2.1. Physical Agents and Particulate Matter

Ionizing radiation causes homolysis of water to form hydroxyl radical, which contributes to radiation-induced tissue injury. Hydroxyl radical is also formed from ultraviolet light-induced homolysis of hydrogen peroxide (Section 2.3.3). Exposure of biological systems to ionizing radiation and ultraviolet light may result in the formation of other ROS/RNS as well. In addition to ionizing radiation and ultraviolet light, exposure to asbestos fibers or silica dusts leads to the formation of ROS/RNS in biological systems. The metal ions associated with asbestos fibers participate in Fenton- or Fenton-type reaction to yield ROS (see Section 2.3.1 for description of Fenton reaction). Similarly, the surface redox chemical properties of silica dusts play a role in ROS formation. On the other hand, both asbestos fibers and silica dusts can provoke inflammatory responses, resulting in production of ROS/RNS. Various types of nanomaterials, including silicon, gold, and copper nanoparticles as well as CdTe quantum dots have been shown to induce ROS/RNS formation in biological systems though the detailed mechanisms remain to be elucidated [13]. In contrast, cerium oxide nanoparticles and fullerene-derived nanomaterials exert antioxidant effects via scavenging ROS in biological systems (see Chapter 27 for discussion of nanomaterials).

2.2.2.2. Xenobiotics

As described in Section 2.2.1.2 above, certain xenobiotics, including drugs are able to cause increased ROS/RNS levels in cells and tissues, leading to oxidative injury. There exist four general mechanisms for xenobiotic-augmented ROS/RNS levels in biological systems, as listed below.

- Redox cycling of xenobiotics, such as quinone compounds
- Interference with mitochondrial electron transport chain, causing increased electron leakage and thereby the augmented formation of superoxide
- Induction of ROS/RNS-producing enzymes, such as P4502E1
- Inhibition of cellular antioxidants

In addition, some ROS/RNS, such as ozone and nitrogen oxides exist as air pollutants. Exposure to these ROS/RNS air pollutants via inhalation may cause pulmonary inflammation and oxidative injury.

2.2.2.3. Biologic Agents

Biologic agents, including bacteria and viruses may cause increased levels of ROS/RNS in target cells and tissues. As outlined below, there are four potential mechanisms for the increased ROS/RNS levels caused by biologic agents.

- Biologic agents induce activation of inflammatory cells, such as phagocytic cells, resulting in ROS formation from NAD(P)H oxidase.
- Biologic agents can cause activation of other ROS/RNS-producing enzymes, such as inducible nitric oxide synthase to generate large amounts of nitric oxide. As described below, nitric oxide reacts with superoxide, generating the highly reactive species peroxynitrite (Section 2.3.4).
- Certain bacteria contain ROS-producing enzymes that may release significant amounts of ROS.
- Some bacteria-derived toxins may downregulate tissue antioxidants. Viral infections may also decrease the expression of certain antioxidants in host cells via modulating cell signal transduction [14, 15].

2.3. Chemical Reactivity of Major Types of ROS/RNS

As noted above, ROS/RNS are generated from various endogenous and exogenous sources. The effects of ROS/RNS in biological systems are determined by multiple factors, among which the chemical reactivity of these species plays an important part in determining their biological effects. This section summarizes the major chemical reactivity for each of the ROS/RNS commonly encountered in biology and medicine.

2.3.1. Superoxide ($O_2^{\cdot -}$)

Superoxide is formed from one-electron reduction of molecular oxygen with NAD(P)H oxidases, mitochondria, and xanthine oxidoreductase as major cellular sources (see Section 2.2 for discussion of sources of ROS/RNS). Due to its lower reduction potential, superoxide often acts as a reducing agent rather than an oxidizing species. As such, the ability of superoxide to directly oxidize biomolecules is much limited. Nevertheless, superoxide is an important ROS that can result in cell and tissue injury [16]. The biological damaging potential of superoxide is related to several chemical properties of this free radical species, which are summarized below.

- Although it generally acts as a reducing agent, superoxide does oxidize the iron-sulfur clusters in several enzymes, such as aconitase. Oxidation of the iron-sulfur clusters by superoxide in these enzymes leads to the release of iron from the enzymes and the subsequent enzyme inactivation.
- Superoxide reacts with nitric oxide ($^{\cdot}NO$) at an almost diffusion-limited rate to form the highly damaging species peroxynitrite ($ONOO^-$; Section 2.3.4) (Reaction 1).

$$O_2^{\cdot-} + {}^{\cdot}NO \longrightarrow ONOO^- \quad (1)$$

- In the presence of transition metal ions (e.g., iron) superoxide reacts with hydrogen peroxide, producing hydroxyl radical, an extremely reactive species (Section 2.3.3). The reaction of superoxide and hydrogen peroxide in the presence of iron to produce hydroxyl radical is referred to as iron-catalyzed Haber-Weiss reaction (Reaction 2).

$$H_2O_2 + O_2^{\cdot-} \xrightarrow{Fe} {}^{\cdot}OH + OH^- + O_2 \quad (2)$$

In the absence of iron this reaction proceeds slowly. The presence of iron dramatically accelerates the reaction to produce hydroxyl radical. The iron-catalyzed Haber-Weiss reaction can be written in two sub-reactions (Reactions 3 and 4). Reaction 4 is also referred to as Fenton reaction.

$$O_2^{\cdot-} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \quad (3)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\cdot}OH + OH^- \quad (4)$$

Superoxide undergoes spontaneous dismutation to form hydrogen peroxide and molecular oxygen, and this dismutation reaction is catalyzed by superoxide dismutase (SOD) with a reaction rate constant being over three orders of magnitude greater than that of spontaneous dismutation (Reaction 5) (see Chapter 3 for discussion of SOD).

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (5)$$

2.3.2. Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide is formed through either spontaneous or SOD-catalyzed dismutation of superoxide. As such, the cellular sources for superoxide formation are also the ones for hydrogen peroxide. Similar to superoxide, hydrogen peroxide is not a potent oxidant, and the direct reactions between hydrogen peroxide and most biomolecules are much limited. However, some enzymes, such as protein phosphatases can be directly inhibited by hydrogen peroxide through oxidation of the sulfhydryl (SH) groups of the enzymes [17]. Although it is not a potent oxidant, hydrogen peroxide at high levels can lead to cell and tissue injury. As summarized below, several mechanisms may account for the damaging effects of hydrogen peroxide in biological systems.

- Hydrogen peroxide reacts with the sulfhydryl groups of certain enzymes, leading to enzyme inactivation.

- Reaction of hydrogen peroxide with iron produces hydroxyl radical, an extremely potent ROS. The Fenton reaction is believed to be an important mechanism by which hydrogen peroxide mediates oxidative damage. Other metal ions, such as cuprous ion ($Cu^{1+)}$ also catalyze the formation of hydroxyl radical from hydrogen peroxide via a similar reaction called Fenton-type reaction (Reaction 6).

$$Cu^{1+} + H_2O_2 \longrightarrow Cu^{2+} + {}^{\cdot}OH + OH^- \quad (6)$$

- Reaction of hydrogen peroxide with chloride (Cl^-) generates hypochlorous acid (HOCl), another potent oxidant (Section 2.3.8). This reaction is catalyzed by myeloperoxidase (MPO) found in phagocytic cells (Reaction 7). Hypochlorous acid is involved in the killing of invading microorganisms as well as tissue injury associated with phagocytic cell activation [18].

$$H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + OH^- \quad (7)$$

Hydrogen peroxide is decomposed to water by three major types of enzymes in mammals, which are listed below.

- Catalase (Chapter 4)
- Glutathione peroxidase (Chapter 6)
- Peroxiredoxin (Chapter 11)

2.3.3. Hydroxyl Radical (·OH)

One-electron reduction of hydrogen peroxide forms hydroxyl radical. One source of hydroxyl radical formation in biological systems is the reaction of hydrogen peroxide with transition metal ions through Fenton or Fenton-type reactions (Reactions 4 and 6). As outlined below, there are several other potential sources of hydroxyl radical formation, which may also have biological relevance.

- Ionizing radiation induces homolytic cleavage of water to form hydroxyl radical and hydrogen atom ($H^{\cdot}$) (Reaction 8). Ultraviolet (UV) light-induced homolytic cleavage of hydrogen peroxide also yields hydroxyl radicals (Reaction 9). The hydroxyl radicals formed mediate ionizing radiation- and UV light-induced injury.

$$H_2O \xrightarrow{\text{Ionizing Radiation}} H^{\cdot} + {}^{\cdot}OH \quad (8)$$

$$H_2O_2 \xrightarrow{\text{UV Light}} 2{}^{\cdot}OH \quad (9)$$

- Reaction of hypochlorous acid (HOCl) with superoxide produces hydroxyl radical (Reaction 10). This reaction may be particularly relevant to sustained activation of inflammatory cells (e.g., phagocytic cells) during which both hypochlorous acid and superoxide are formed in large quantities.

$$HOCl + O_2^{\cdot-} \longrightarrow O_2 + Cl^- + {}^{\cdot}OH \quad (10)$$

- Peroxynitrite ($ONOO^-$) becomes protonated to form peroxynitrous acid (ONOOH) (Reaction 11). Decomposition of peroxynitrous acid yields hydroxyl radical and nitrogen dioxide radical ($^{\cdot}NO_2$) (Reaction 12) [19].

$$ONOO^- + H^+ \longrightarrow ONOOH \quad (11)$$

$$ONOOH \longrightarrow {}^{\cdot}OH + {}^{\cdot}NO_2 \quad (12)$$

- Reaction of electronically excited nitric dioxide ($^{\cdot}NO_2$*) with water is reported to form hydroxyl radical (Reaction 13).

$$^{\cdot}NO_2^* + H_2O \longrightarrow {}^{\cdot}OH + HONO \quad (13)$$

HONO is nitrous acid. The above reaction is considered a chemical mechanism of atmospheric hydroxyl radical production [20].

Hydroxyl radical is known as the most reactive ROS. Due to its extremely high oxidizing capacity, hydroxyl radical reacts with the first biomolecule that it bumps into at a reaction rate constant very near the diffusion limit. Thus, hydroxyl radical reacts non-selectively with both less vital biomolecules (e.g., cellular antioxidants) and vital biomolecules (e.g., DNA).

2.3.4. Peroxynitrite ($ONOO^-$)

Peroxynitrite is formed from the reaction of superoxide and nitric oxide (Reaction 1). This reaction occurs at an almost diffusion-limited rate. Thus, peroxynitrite forms if a biological system simultaneously generates both superoxide and nitric oxide. As indicated above, peroxynitrite is negatively charged and undergoes protonation to form peroxynitrous acid (ONOOH) (Reaction 11).

Peroxynitrite is a potent oxidant capable of causing biological damage. Although it is not a free radical, peroxynitrite is much more reactive than its parent molecules, superoxide and nitric oxide. In this regard, as described below, formation of peroxynitrite is believed to mediate the detrimental effects of high levels of nitric oxide formed during inflammation (Section 2.3.5). Peroxynitrite reacts directly or indirectly with most critical biomolecules, including lipids, proteins, and nucleic acids. Several chemical mechanisms contribute to the biological damaging effects of peroxynitrite, which are outlined below [19].

- Peroxynitrite and its protonated form (peroxynitrous acid) can directly cause oxidation of biomolecules.
- Decomposition of peroxynitrous acid leads to the formation of hydroxyl radical and nitrogen dioxide (Reaction 12), both of which are highly reactive species capable of damaging biomolecules.
- Peroxynitrite reacts with carbon dioxide (CO_2), resulting in the formation of carbonate radical ($CO_3^{\cdot-}$) and nitrogen dioxide via an unstable intermediate nitrosoperoxycarbonate ($ONOOCO_2^-$) (Reaction 14).

$$ONOO^- + CO_2 \longrightarrow [ONOOCO_2^-] \longrightarrow CO_3^{\cdot-} + {}^{\cdot}NO_2 \quad (14)$$

Carbonate radical is a potent oxidant capable of oxidizing biomolecules. Due to the presence of high levels of carbon dioxide in cells and tissues, the above reaction pathway is believed to play a major role in peroxynitrite-induced biological damage.

Glutathione (Chapter 5) is perhaps the most important biomolecule in the detoxification of peroxynitrite in mammalian systems. Both glutathione peroxidase (Chapter 6) and peroxiredoxin (Chapter 11) are able to catalyze the conversion of peroxynitrite to nitrite in vitro. However, the role of these two enzymes in protecting against peroxynitrite toxicity in vivo remains to be established.

2.3.5. Nitric Oxide (·NO) and Related Nitrogen Oxides

Nitric oxide, also referred to as nitric monoxide, has one unpaired electron, and is thus a free radical. The dot in ·NO stands for the unpaired electron. However, nitric oxide is often written as NO with the dot omitted. Nitric oxide is the first gaseous species identified as an endogenously generated signaling molecule. Nitric oxide plays important roles in many physiological processes, such as vascular homeostasis, neurotransmission, and host defense [21]. Nitric oxide is formed in biological systems by two general pathways: nitric oxide synthase-dependent pathway and nitrate-nitrite-nitric oxide pathway [22].

Nitric oxide possesses important physiological functions; however, under certain conditions, such as inflammation it also contributes to pathophysiological processes. Although nitric oxide is reported to exert various adverse effects, most of them are more likely mediated by its oxidation products rather than nitric oxide itself [19]. Indeed, the direct reactions of nitric oxide with most biomolecules, including proteins (except for heme-containing proteins), lipids, and nucleic acids are much limited. Because of its free radical nature, however, nitric oxide reacts readily with other free radical species. As noted earlier, nitric oxide reacts with superoxide at an almost diffusion-limited rate to form the highly damaging species peroxynitrite (Reaction 1). In addition, nitric oxide-mediated redox reactions may lead to the formation of secondary nitrogen oxides, such as nitrogen dioxide. Nitrogen dioxide is a free radical species of high reactivity to various biomolecules. It also exists as an important air pollutant.

2.3.6. Peroxyl Radical (LOO·) and Alkoxyl Radical (LO·)

Alkoxyl and peroxyl radicals are formed from lipid peroxidation. Hydroxyl radical abstracts a hydrogen atom from a lipid molecule (LH), leading to the formation of a carbon-centered radical (L·). In the presence of molecular oxygen, the carbon-centered radical is converted to a peroxyl radical (LOO·) (Reactions 15 and 16).

$$\cdot OH + LH \longrightarrow L\cdot + H_2O \quad (15)$$

$$L\cdot + O_2 \longrightarrow LOO\cdot \quad (16)$$

Both alkoxyl and peroxyl radicals can abstract hydrogen atoms from adjacent lipid molecules, resulting in the propagation of lipid peroxidation. After abstracting a hydrogen atom (H) from an adjacent lipid molecule, peroxyl radical is reduced to lipid hydroperoxide (LOOH) (Reaction 17). Decomposition of lipid hydroperoxide in the presence of metal ions, such as iron leads to the formation of alkoxyl radical or peroxyl radical depending on the form of the iron ions (Reactions 18 and 19).

$$LOO^{\cdot} + H \longrightarrow LOOH \quad (17)$$

$$LOOH + Fe^{2+} \longrightarrow LO^{\cdot} + Fe^{3+} + OH^{-} \quad (18)$$

$$LOOH + Fe^{3+} \longrightarrow LOO^{\cdot} + Fe^{2+} + H^{+} \quad (19)$$

Alkoxyl and peroxyl radicals are strong oxidants. In addition to propagating lipid peroxidation in biomembranes or lipoproteins, alkoxyl and peroxyl radicals are also able to oxidize proteins, leading to protein dysfunction and enzyme inactivation. Oxidation of DNA by these free radicals results in oxidative base modifications [23]. Notably, peroxyl radicals react with each other, yielding singlet oxygen [24], a potent oxidant capable of damaging biomolecules (Section 2.3.7).

Both alkoxyl and peroxyl radicals react readily with cellular non-protein antioxidants, including vitamin E, vitamin C, glutathione, and bilirubin (see later chapters for discussion of these antioxidants). These antioxidants act to protect more vital cellular constituents from damage by alkoxyl and peroxyl radicals. At present, no specific enzymes have been found to catalytically metabolize alkoxyl and peroxyl radicals in mammals.

2.3.7. Singlet Oxygen ($^1O_2$)

Singlet oxygen is an electronically excited form of molecular oxygen. As noted earlier in Section 2.1.2, the term singlet oxygen in biology and medicine usually refers to the $^1\Delta_g$ state, which is not a free radical. As described below, singlet oxygen is formed by different pathways or chemical mechanisms in biological systems.

- Photosensitivity reaction: In a photosensitivity reaction, an endogenous photosensitizer molecule (PS) (e.g., a porphyrin compound) is converted to an excited state (PS*) upon illumination by light. The excitation energy is then transferred to molecular oxygen, converting it to singlet oxygen, while the photosensitizer molecule returns to the ground state (Reactions 20 and 21).

$$PS + light \longrightarrow PS^{*} \quad (20)$$

$$PS^{*} + O_2 \longrightarrow PS + {}^1O_2 \quad (21)$$

- Phagocytic cell respiratory burst: Phagocytic cell activation during inflammation leads to the formation of hypochlorous acid (HOCl), which reacts with hydrogen peroxide, forming singlet oxygen (Reaction 22) [25].

$$HOCl + H_2O_2 \longrightarrow {}^1O_2 + H_2O + HCl \quad (22)$$

- Lipid peroxidation: As stated above in Section 2.3.6, peroxyl radicals ($LOO^{\cdot}$) generated during lipid peroxidation react with each other to yield singlet oxygen along with the formation of alcohol (LOH) and ketone (LO). This reaction proceeds via a linear tetraoxide intermediate (LOOOOL) (Reaction 23).

$$LOO^{\cdot} + LOO^{\cdot} \longrightarrow [LOOOOL] \longrightarrow LOH + LO + {}^1O_2 \quad (23)$$

Singlet oxygen is a strong oxidant that displays considerable reactivity towards biomolecules, including lipids, proteins, and nucleic acids [26, 27]. The interactions between singlet oxygen and biomolecules may occur via two modes: direct chemical reaction and physical quenching. Physical quenching results in energy transfer and de-excitation of the singlet state without chemical changes in the energy acceptor. Direct chemical reaction is the major mode of reactivity of singlet oxygen in biological systems. Attack of lipid molecules in biomembranes or lipoproteins by singlet oxygen triggers lipid peroxidation. Reaction of singlet oxygen with proteins can result in multiple effects, including oxidation of side-chains, backbone fragmentation, aggregation, unfolding, enzyme inactivation, and enhanced degradation by proteasome pathway. Reaction of singlet oxygen with DNA leads to base modifications and DNA strand breaks.

A number of non-protein antioxidants react with singlet oxygen and thereby protect vital biomolecules from oxidative damage. Notably, dietary carotenoids, such as β-carotene and lycopene (Section 2 of Chapter 22) have been shown to potently quench singlet oxygen. To date, no enzymes have been shown to selectively detoxify singlet oxygen through a catalytic mechanism.

2.3.8. Hypochlorous Acid (HOCl)

Hypochlorous acid is formed from the reaction of hydrogen peroxide and chloride catalyzed by phagocytic myeloperoxidase during inflammation (Reaction 7). Myeloperoxidase also utilizes hydrogen peroxide to oxidize bromide (Br^-) and thiocyanate (SCN^-) to generate two other potent oxidants hypobromous acid (HOBr) and hypothiocyanous acid (HOSCN), respectively. Both HOBr and HOSCN are minor products as compared with hypochlorous acid [28].

Hypochlorous acid is a potent oxidizing species that reacts with proteins, lipids, nucleic acids, and carbohydrates. Formation of hypochlorous acid by phagocytic cells during respiratory burst plays an integral part in innate immune defense system. Hypochlorous acid along with other oxidizing species derived from hypochlorous acid, including hydroxyl radical and singlet oxygen contributes to the killing of microorganisms. However, under certain conditions, such as chronic inflammation, uncontrolled formation of hypochlorous acid may attack normal cellular biomolecules, leading to cell and tissue injury [29]. In this context, hypochlorous acid has been implicated in the pathogenesis of various human diseases, including atherosclerosis [30].

Cellular glutathione has been suggested to play a major role in the detoxification of hypochlorous acid. Hypochlorous acid also reacts with the sulfonate amino acid taurine to form an unreactive chloroamine product. Taurine is present at high concentrations in neutrophils, and may act to detoxify hypochlorous acid.

2.3.9. Ozone (O_3)

Ozone is a gas in the stratosphere that protects animals on earth from solar irradiation. Ozone is also an air pollutant near the earth's surface, where it is formed by photochemical reactions. Ozone pollution still represents a significant environmental issue in many urban areas. Recently, it is reported that ozone may be produced by antibodies in biological systems [31]. However, this finding has been questioned by other investigators. Ozone is not a free radical, but has potent oxidizing capacity. It reacts with biomolecules, including lipids, pro-

teins and nucleic acids, resulting in cell and tissue damage. Oxidative injury is a significant mechanism of ozone-induced pulmonary disorders.

2.4. Molecular Targets of ROS/RNS and Cellular Consequences

As discussed above, ROS/RNS are reactive species capable of damaging cellular constituents, including proteins, lipids, and nucleic acids, leading to cell and tissue injury. Reactions of ROS/RNS with proteins can cause enzyme inactivation, structural alterations, and enhanced protein degradation. Attack of lipids by ROS/RNS causes lipid peroxidation, leading to membrane disruption as well as the formation of secondary reactive species, such as aldehydes. Lipid peroxidation-derived reactive aldehydes (e.g., malondialdehyde, 4-hydroxy-2-nonenal, acrolein) are cytotoxic and mutagenic. They also modulate cell signal transduction. Direct DNA damage by ROS/RNS can be manifested as DNA base modifications, strand breakage, and DND-DNA or DNA-protein cross-links. Damage of the above vital biomolecules by ROS/RNS may lead to cell dysfunction, senescence, death, or malignant transformation. Due to the detrimental nature of ROS/RNS, aerobic organisms, including mammals have evolved diverse antioxidant defenses to protect vital biomolecules from ROS/RNS-mediated damage. In addition, a number of compounds derived from dietary sources or synthesized in the laboratories also exhibit antioxidant activities in biological systems. An introduction to the antioxidant system is provided in Chapter 2. The delicate balance between ROS/RNS and antioxidant system is an integral part of physiological homeostasis in mammals. Disruption of this balance may lead to disease pathophysiology.

3. Oxidative Stress and Diseases

3.1. Definitions

3.1.1. Oxidative Stress

The term oxidative stress refers to a condition where the levels of ROS significantly overwhelm the capacity of antioxidant defenses in a biological system. Oxidative stress condition can be caused by either increased ROS formation or decreased activity of antioxidants or both in a biological system. Oxidative stress condition is associated with oxidative damage to biomolecules, including proteins, lipids, and nucleic acids. Moderate oxidative stress may cause cell dysfunction, whereas overt oxidative stress usually causes cell death. It should be noted that increases in ROS levels in a biological system are not always associated with injury. Under certain circumstances, small transient increases in ROS levels can be employed as a signaling mechanism, leading to physiological cellular responses [4, 17]. In this context, the disease conditions in which oxidative stress plays a role usually involve sustained formation of relatively large amounts of ROS via various mechanisms, including activation of inflammatory cells.

Oxidative stress and inflammation are intimately related processes. On the one hand, inflammation leads to the production of ROS/RNS from cellular sources, including NAD(P)H oxidase and inducible nitric oxide synthase. Such formed ROS/RNS mediate the detrimental

effects associated with dysregulated inflammation. On the other hand, ROS/RNS cause increased expression of proinflammatory cytokines and adhesion molecules, thus augmenting or propagating the dysregulated inflammatory process. Such a dysregulated inflammatory state is sometimes referred to as inflammatory stress. As oxidative stress and inflammation are closely intertwined, the compound term oxidative/inflammatory stress has been used in the literature to describe settings where both oxidative stress and dysregulated inflammation contribute to disease pathophysiology.

3.1.2. Other Related Terms

Analogous terms have been coined to describe the conditions associated with increased levels of RNS. For example, the term nitrative stress is defined as a condition under which the levels of RNS significantly overwhelm the capacity of RNS-detoxification mechanisms in a biological system. Nitrative stress condition is associated with nitration of biomolecules, leading to cell and tissue injury. Nitration refers to the addition of a nitro (-NO_2) group to a compound. The RNS peroxynitrite readily causes nitrative stress in biological systems. Another term nitrosative stress is defined as a condition induced by nitric oxide or related species, leading to nitrosylation of critical protein cysteine thiols (*S*-nitrosylation) and metallocofactors of proteins. Nitrosylation refers to the addition of a nitroso (-NO) group to a thiol group or a redox active metal ion center of a protein. While dysregulation of nitrosylation is associated with pathophysiological conditions, well-controlled nitrosylation plays an important role in cell signal transduction and provides a mechanism of redox-based physiological regulation [32].

3.2. Oxidative Stress in Diseases

3.2.1. Different Roles of Oxidative Stress in Diseases

Increased formation of ROS/RNS and subsequent oxidative damage to biomolecules have been consistently observed under various disease conditions. However, these observations do not necessitate a causal relationship between oxidative stress and disease development. Possible relationships between oxidative stress and diseases, and the effects of controlling ROS/RNS on disease development are summarized below.

- ROS/RNS act as the only initial cause of the disease. Control of the ROS/RNS will prevent or stop the disease development.
- ROS/RNS act as one of the initial causes of the disease. Control of the ROS/RNS will mitigate the disease development.
- ROS/RNS act as a contributing factor in the disease progression. Control of the ROS/RNS will slow the disease progression.
- ROS/RNS are formed as a consequence of the disease and contribute to the progression of the disease pathophysiology. Control of the ROS/RNS will slow the disease progression.
- ROS/RNS are formed as a consequence of the disease and play no role in disease initiation or progression. Control of the ROS/RNS will have no effects on disease process.

3.2.2. Diseases Involving an Oxidative Stress Mechanism

Establishment of a causal or contributing role for ROS/RNS in a particular disease is the basis for developing strategies to prevent and treat that particular disease. Based on findings from extensive investigations in animal models of human diseases as well as studies in human subjects over the last several decades, it has been recognized that ROS/RNS play a causal and/or a contributing role in a wide variety of diseases [33]. Table 1-2 lists some of the disease processes and related conditions in which oxidative stress plays a role. Another line of evidence supporting an important role for elevated ROS/RNS in diseases is the protective effects of diverse antioxidants on disease pathophysiology, which are the main focus of this book.

Table 1-2. Disease processes and related conditions in which reactive oxygen and nitrogen species play a causal or contributing role

Disease	Description
Cardiovascular Diseases	Hypertension, Atherosclerosis Myocardial ischemia-reperfusion injury Heart failure, Cardiotoxicity
Diabetes and Metabolic Syndrome	Diabetes, Diabetic complications Obesity, Insulin resistance
Neurological Diseases	Alzheimer's disease, Parkinson's disease Stroke, Amyotrophic lateral sclerosis
Pulmonary Diseases	Chronic obstructive pulmonary disease Asthma, Hyperoxia-induced lung injury Pulmonary toxicity
Hepatic and Gastrointestinal Diseases	Alcoholic fatty liver disease Nonalcoholic fatty liver disease Hepatotoxicity, Inflammatory bowel disease Ischemia-reperfusion injury
Renal Diseases	Diabetic nephropathy Ischemia-reperfusion injury, Nephrotoxicity
Eye Diseases	Cataract Age-related macular degeneration
Skin Diseases	Ultraviolet light-induced skin injury Scleroderma, Contact dermatitis, Psoriasis
Cancer	Chemical carcinogenesis Spontaneous cancer development Angiogenesis, Cancer metastasis
Aging	Lifespan Aging-related organ degeneration
Arthritic Diseases	Rheumatoid arthritis Other types of arthritis
Sepsis	Septic shock Multiple organ dysfunction
Infections	Viral infections Bacterial infections

4. Conclusion

ROS/RNS are able to cause damage to a variety of cellular constituents, including proteins, lipids, and nucleic acids, leading to tissue injury and disease processes. Under certain circumstances, well-controlled production of ROS/RNS also fulfils important physiological roles, including antimicrobial activity and participation in cell signaling. The biological effects of ROS/RNS, including both beneficial and detrimental effects are dependent on multiple factors. These include the chemical reactivity of the ROS/RNS, the concentrations and duration of exposure, and the types of tissues/cells that the ROS/RNS are produced from or act on. The causal or contributing role of ROS/RNS in various diseases makes it imperative to develop antioxidant-based strategies to mitigate the tissue injury and therefore protect against the disease development. The subsequent Chapter 2 is intended to provide an overview on antioxidants in biology and medicine.

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Chapter 2

Introduction to Antioxidants

Abstract

A wide variety of antioxidants have been evolved to combat reactive oxygen and nitrogen species-mediated damage in mammals. The endogenous antioxidants include proteins and non-protein compounds synthesized by cells. Mammalian cells and tissues also contain antioxidant compounds derived from the diet. In addition, a number of synthesized antioxidant compounds have become available. This chapter defines the term antioxidant and provides an overview of the various classes of antioxidants in biology and medicine. The molecular regulation of endogenous antioxidants and antioxidant-based strategies for disease intervention are also introduced in this chapter.

1. Definition and Mode of Antioxidant Action

The term antioxidant is frequently used in biology and medicine, and has been defined in various ways in the literature. One common way to define this term is that antioxidant is any substance that can prevent, reduce, or repair the reactive oxygen and nitrogen species (ROS/RNS)-induced damage to a target biomolecule [1]. There are three potential modes of action by which antioxidants protect biomolecules from ROS/RNS-induced damage. They are described as follows.

- Scavenging of ROS/RNS: Many antioxidants are able to directly scavenge ROS/RNS. Scavenging of ROS/RNS may occur through enzyme-catalyzed reactions. For example, superoxide dismutase catalyzes the dismutation of superoxide to form hydrogen peroxide and molecular oxygen (Chapter 3). Non-enzymatic antioxidants may scavenge ROS/RNS through direct chemical reactions.
- Inhibition of ROS/RNS formation: ROS/RNS are formed from various cellular sources, such as mitochondria and NAD(P)H oxidases (Section 2.2 of Chapter 1). Antioxidants may act on these cellular sources, inhibiting or preventing the formation of ROS/RNS. For example, mono-*O*-methylated flavanols inhibit NAD(P)H oxidases in vascular cells, leading to decreased formation of superoxide [2] (Section 2.1.2 of Chapter 23).

- Removal or repair of the ROS/RNS-induced Damage: ROS/RNS can cause damage or modifications to biomolecules. Mammalian cells are equipped with various mechanisms to remove or repair the ROS/RNS-modified biomolecules. For example, ROS/RNS oxidize methionine residues in proteins, forming methionine sulfoxide. Such formed methionine sulfoxide can be reduced back to methionine by methionine sulfoxide reductase [3] (Section 2.1 of Chapter 14). Methionine sulfoxide reductase is an important repairing system for oxidative protein damage in mammals. Mammalian cells are also equipped with enzymes for repairing oxidative damage of lipids and nucleic acids.

2. Classification of Antioxidants

Antioxidants are generally classified into two major categories based on their sources, as listed below.

- Endogenous antioxidants
- Exogenous antioxidants

Endogenous antioxidants refer to those made by cells. Endogenous antioxidants can be further classified into two subgroups: (1) protein antioxidants and (2) non-protein antioxidants. Endogenous protein antioxidants include enzymes, such as superoxide dismutase and catalase, and non-enzymes, such as ferritin and metallothionein. Examples of endogenous non-protein antioxidants include the reduced form of glutathione and bilirubin.

Exogenous antioxidants refer to those that are not made by cells. Exogenous antioxidants can be either derived from dietary sources or synthesized in laboratories. Typical examples of exogenous antioxidants derived from the diet include vitamin C, vitamin E, and polyphenols. Antioxidant enzyme mimetics, spin traps, and cerium oxide nanoparticles are examples of synthetic antioxidants. Table 2-1 summarizes the various classes of antioxidants covered in this book.

Table 2-1. Classification of antioxidants in biology and medicine (continued on next page)

Source	Name of Antioxidant	Chapter
Endogenous Protein Antioxidants Made by Cells	Superoxide Dismutase	3
	Catalase	4
	Glutathione-synthesizing Enzymes	5
	Glutathione Peroxidase	6
	Glutathione Reductase	7
	Glutathione S-Transferase	8
	Glutaredoxin	9
	Thioredoxin	10
	Peroxiredoxin	11
	Thioredoxin Reductase	12
	Sulfiredoxin	13

Source	Name of Antioxidant	Chapter
	Methionine Sulfoxide Reductase	14
	Heme Oxygenase	15
	NAD(P)H:Quinone Oxidoreductase	16
	Paraoxonase	17
	Ferritin	18
	Metallothionein	18
Endogenous Non-protein Antioxidants Made by Cells	Glutathione	5
	Bilirubin	19
	Coenzyme Q	19
	Estrogens	19
	α-Lipoic Acid	19
	Melatonin	19
	Pyruvate	19
	Uric Acid	19
Exogenous Antioxidants Derived from the Diet	Vitamin C	20
	Vitamin E	21
	Carotenoids	22
	Phenolic Compounds	23
Exogenous Antioxidants Synthesized in Laboratories	Antioxidant Enzyme Mimetics	24
	Glutathione Precursors	25
	Spin Traps	26
	Nanomaterials	27

3. Antioxidant Gene Regulation

Regulation of antioxidant gene expression is an integral part of cellular defenses against ROS/RNS-induced damage in aerobic organisms, including mammals. Multiple mechanisms are involved in the regulation of mammalian antioxidant gene expression. At the transcriptional level, nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) plays a central role in the regulation of a series of antioxidant genes in mammals. Under certain conditions, other transcription factors may also be involved in antioxidant gene regulation. These include Nrf1, Nrf3, nuclear factor kappa-B (NF-κB), and AP-1 [4-6]. This section focuses on discussing Nrf2 as a central regulator of mammalian antioxidant gene expression and its role in protection against ROS/RNS-mediated biological damage.

3.1. Regulation of Antioxidant Gene Expression by Nrf2 Signaling

3.1.1. Historical Overview

Early studies during the 1980's showed that administration of phenolic chemicals to experimental animals resulted in simultaneous induction of multiple antioxidants, including glutathione, glutathione S-transferase, and NAD(P)H:quinone oxidoreductase 1, suggesting a possible existence of a central regulator for the above cellular defenses. Studies in the early 1990's demonstrated a crucial role for a cis-acting element, named antioxidant response ele-

ment (ARE) in the regulation of antioxidant gene expression [7]. In 1997, M. Yamomoto and coworkers identified Nrf2 as a critical transcription factor that interacts with the ARE, leading to increased expression of various antioxidant and cytoprotective genes [8]. Subsequent studies from multiple research groups have established that Nrf2 plays a central role in regulating both constitutive and inducible expression of a wide variety of antioxidant genes in mammalian systems [9].

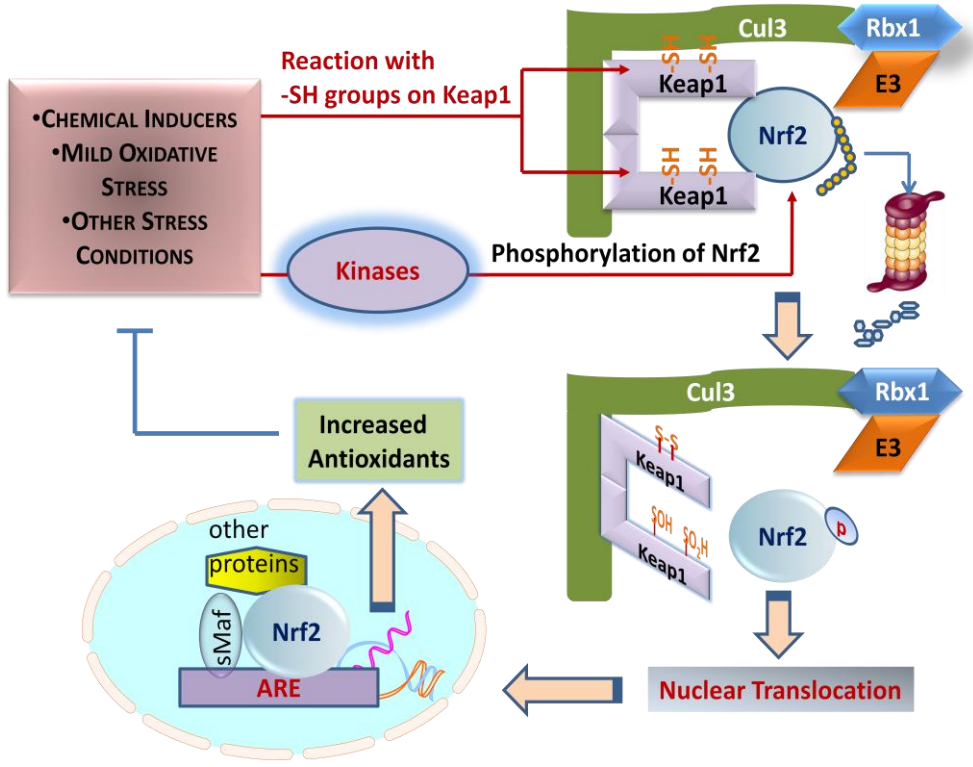

Figure 2-1. Schematic illustration of Keap1-Nrf2-ARE signaling. See text (Section 3.1.2) for detailed description.

3.1.2. Keap1-Nrf2-ARE Signaling

Nrf2 normally resides in the cytosolic compartment through association with a cytosolic actin-binding protein, Keap1 (Kelch-like ECH-associated protein 1). Keap1 plays a central role in controlling Nrf2 activity [10]. Keap1 exists as dimers inside the cells and functions as a substrate linker protein for interaction of Cul3/Rbx1-based E3-ubiquitin ligase complex with Nrf2. This protein-protein interaction leads to continuous ubiquitination of Nrf2 and its proteasomal degradation (Figure 2-1). Hence, the continuous degradation of Nrf2 under basal conditions keeps the Nrf2 level low and thereby the low basal levels of Nrf2-regulated antioxidants. When the cells are exposed to moderate amounts of ROS/RNS or chemical inducers, Nrf2 dissociates from Keap1, becomes stabilized, and translocates into the nuclei. Inside the nuclei, Nrf2 interacts with other protein factors, including small Maf (sMaf), and binds to

ARE, resulting in increased transcription of antioxidant genes. The increased production of antioxidants helps detoxify the ROS/RNS and may even render cells more resistant to higher levels of ROS/RNS. Thus, Nrf2-dependent upregulation of antioxidants by moderate levels of ROS/RNS can be viewed as an adaptive response to oxidative stress. Two potential mechanisms have been proposed to explain the dissociation of Nrf2 from Keap1 under stress conditions, which are summarized below [10, 11].

- Oxidation of cysteine residues of Keap1: Keap1 is a cysteine-rich protein, and the cysteine residues may serve as redox sensors for ROS/RNS or chemical inducers. Modifications of these cysteine sulfhydryl groups on Keap1 protein is thought to cause dissociation of Nrf2 from Keap1.
- Phosphorylation of Nrf2: Several protein kinases, including protein kinase C, mitogen-activated protein kinase, and phosphatidylinositol 3-kinase have been suggested to phosphorylate Nrf2, leading to dissociation of Nrf2 from Keap1.

3.1.3. Nrf2-regulated Antioxidants

Numerous in vitro and in vivo studies demonstrate that Nrf2 plays an indispensable role in the regulation of a wide variety of antioxidant gene expression. The antioxidant enzymes known to be regulated by Nrf2 through an ARE-driven mechanism in mammalian cells include ferritin heavy subunit, both catalytic and modifier subunits of γ-glutamylcysteine ligase, glutathione reductase, heme oxygenase-1, peroxiredoxin-1 and -6, multiple isozymes of glutathione S-transferase, NAD(P)H:quinone oxidoreductase 1, NRH:quinone oxidoreductase 2, sulfiredoxin, Cu,Zn superoxide dismutase, thioredoxin, and thioredoxin reductase-1. In addition to the antioxidant genes, Nrf2 signaling is also found to affect the expression of numerous other genes whose products are involved in various cellular processes, such as generation of NADPH and metabolism of xenobiotics [10, 11].

3.2. Nrf2-dependent Induction of Antioxidants and Protection against Oxidative Stress

It has been established that Nrf2 plays a crucial role in mediating the induction of cellular antioxidants by many chemical inducers. Several classes of chemicals are commonly used for induction of cellular antioxidants to protect against disease conditions associated with oxidative stress. These compounds, also known as chemoprotective agents include the following four chemical classes.

- Phenolic compounds
- Dithiolethiones
- Isothiocyanates
- Triterpenoids

The above chemical agents have been shown to play a protective role in a variety of animal models of disease pathophysiology, including oxidative tissue injury, inflammatory disorders, and chemical carcinogenesis [11]. Studies using Nrf2-knockout mouse model

demonstrate that the protective effects of the above chemoprotective agents occur primarily via activation of Nrf2 and the subsequent induction of antioxidants as well as other cytoprotective proteins. As coordinated actions of various antioxidants are essential for efficient detoxification of ROS/RNS, the simultaneous upregulation of Nrf2-dependent antioxidants by chemoprotective agents may represent an effective strategy for the intervention of disease conditions involving an oxidative stress component.

4. Antioxidant-based Disease Intervention

The critical involvement of ROS/RNS in a number of disease conditions makes it imperative to develop strategies to control these reactive species-mediated pathophysiological processes. Intervention of ROS/RNS-associated disease conditions could be potentially achieved through various approaches, among which is the antioxidant-based intervention.

4.1. Overall Strategies of Antioxidant-based Disease Intervention

Antioxidant-based intervention of ROS/RNS-associated disease conditions may include two overall strategies, as described below.

- Administration of exogenous antioxidant compounds to scavenge ROS/RNS associated with disease conditions: For disease conditions caused by increased levels of ROS/RNS, scavenging of these reactive species by exogenously administered antioxidant compounds may prevent or diminish the tissue injury caused by the ROS/RNS. In this context, both diet-derived antioxidant compounds (e.g., vitamin C, vitamin E, polyphenols) and synthetic antioxidant chemicals (e.g., antioxidant enzyme mimetics) have been investigated extensively for the intervention of various disease processes in animal models and human subjects (Chapters 20-26).
- Administration of antioxidant enzyme inducers (e.g., chemoprotective agents) to augment endogenous cellular antioxidant defenses: As noted above, the chemical inducibility of endogenous antioxidants makes it possible for protecting against ROS/RNS-mediated injury by administration of antioxidant-inducing agents. Induction of cellular antioxidants and other cytoprotective enzymes by chemoprotective agents has been demonstrated to be a valid approach to the intervention of diseases involving an oxidative stress mechanism in animal models. This approach has also been applied for cancer chemoprotection in humans [12]. An alternative approach to increasing cellular antioxidant defenses is through transgenic overexpression of the individual antioxidant proteins. This is usually done in experimental animals.

4.2. Animal Studies on Antioxidant-based Disease Intervention

Animal studies provide important information on the safety and efficacy as well as mechanisms of actions for drugs that are used for the prevention and treatment of human diseas-

es. This is also true for the development of antioxidant-based strategies for the intervention of disease conditions involving an ROS/RNS-mediated pathophysiological component. Three approaches as outlined below are commonly used to study antioxidant intervention of diseases in experimental animals.

- Administration of exogenous antioxidant compounds
- Transgenic overexpression of antioxidant proteins
- Induction of endogenous antioxidants by chemoprotective agents

Transgenic approach provides the most convincing information regarding the effects of overexpression of a particular antioxidant protein on a disease condition. This also provides the strongest evidence for the involvement of a particular ROS/RNS in disease pathophysiology [13]. Using exogenous antioxidant compounds with selective ROS/RNS-scavenging properties may also yield important insights into the potential role of ROS/RNS in disease pathophysiology. In contrast, the effectiveness of chemical inducers of antioxidants in protecting against a disease condition may result from the simultaneous induction of multiple endogenous antioxidants. Indeed, chemical inducers usually upregulate a series of different antioxidant enzymes via activating Nrf2 signaling [14]. As noted earlier, Nrf2 acts as a central regulator of a wide variety of mammalian antioxidants and other cytoprotective factors. Figure 2-2 summarizes the major approaches to augmenting tissue antioxidant defenses for the intervention of disease conditions with ROS/RNS as etiological or contributing factors.

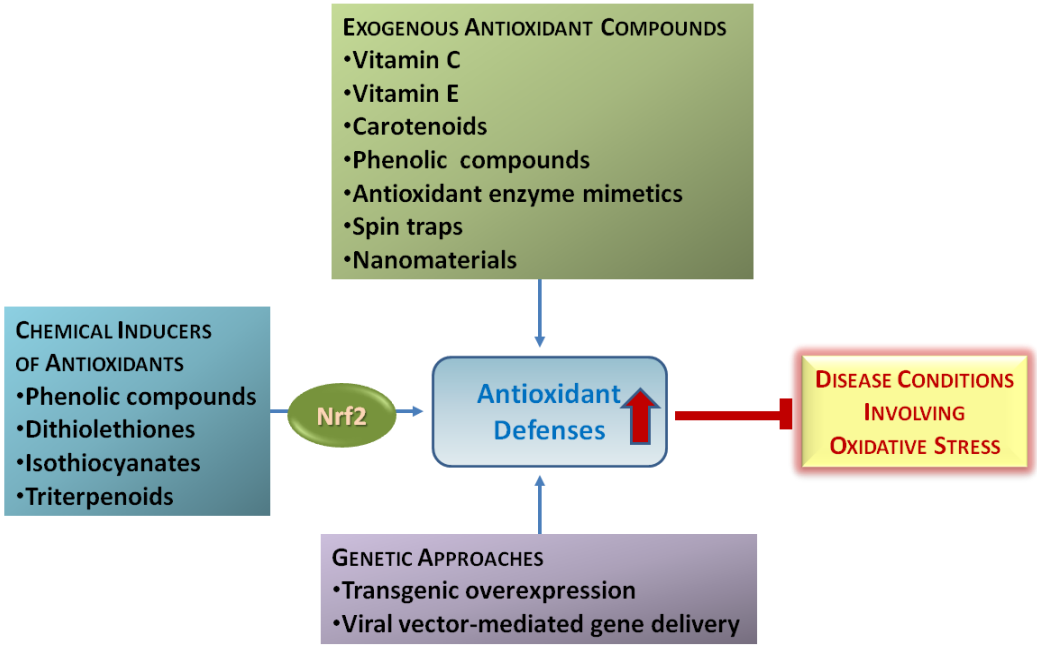

Figure 2-2. Major experimental approaches to augmenting tissue antioxidant defenses for the intervention of disease conditions that involve an oxidative stress mechanism. See text (Section 4.2) for detailed description.

4.3. Human Studies on Antioxidant-based Disease Intervention

The three approaches used in experimental animal studies, as discussed in Section 4.2 could also be applied to human studies though at present the use of genetic approach (gene therapy) is much limited in humans. In this regard, a more practical antioxidant-based approach in human studies involves the use of exogenous antioxidant compounds or chemoprotective agents capable of inducing endogenous antioxidants. In this context, randomized double-blind, placebo-controlled clinical trials are usually conducted to determine the efficacy of antioxidant-based strategies in the intervention of human diseases that are believed to have an oxidative stress component. The interventional clinical trials can be either preventive or therapeutic.

5. Caveats on Antioxidants and Antioxidant-based Disease Intervention

5.1. Multiple Biological Activities of Antioxidants

Many antioxidants have well-established ability to scavenge ROS/RNS, inhibit their formation, or repair the damage caused by ROS/RNS. However, it is important to note that in addition to their effects on ROS/RNS antioxidants, including both protein and non-protein antioxidants may also exert other biological effects irrelevant to their antioxidant activities. For example, the antioxidant enzyme catalase decomposes hydrogen peroxide to form water and molecular oxygen, a well-known antioxidant activity of catalase. This antioxidant enzyme also exhibits peroxidase and oxidase activities toward a number of substrates, including alcohols [15] (Section 2.1 of Chapter 4). Another example is α-tocopherol, which is known to inhibit lipid peroxidation by scavenging lipid peroxyl radical. α-Tocopherol has been shown to also exert non-antioxidant effects, such as inhibition of protein kinases [16] (Section 2.3 of Chapter 21). Thus, the response of a biological system to a particular antioxidant may result from: (1) the antioxidative activity of the compound only, (2) the non-antioxidative activity of the compound only, or (3) the combined antioxidative and non-antioxidative activities. In antioxidant biology and medicine, another important consideration is that many antioxidants, such as glutathione and glutathione S-transferase are also important factors in the detoxification of electrophilic species, including reactive aldehydes [17]. As described in Section 2.4 of Chapter 1, reactive aldehydes are produced from lipid peroxidation elicited by ROS/RNS, and they can cause damage to biomolecules.

5.2. Genetic Manipulations of Antioxidants

Transgenic overexpression or knockout of particular antioxidant genes is frequently used to study the biological activities of endogenous antioxidant proteins in experimental animals. While these genetic animal models provide the most convincing evidence regarding the biological functions of particular antioxidant proteins, interpretation of the data should be done with caution. This is because deletion of one antioxidant gene may lead to compensatory

upregulation of others. Likewise, overexpression of one antioxidant gene may also lead to downregulation of others. Thus, changes in the phenotypes of these genetically manipulated animals likely result from the combined actions of all the antioxidants whose expression is altered due to the genetic manipulation of a single antioxidant gene. Another notion regarding transgenic overexpression of antioxidant genes is that the levels of the overexpressed antioxidant proteins are usually several or even over ten-fold higher than the basal physiological levels. The physiological significance of such dramatically overexpressed antioxidant proteins thus needs to be evaluated with caution. Regardless of the caveats, genetic manipulation of antioxidant genes in experimental animals continues to serve as an important approach to understanding the biology of endogenous antioxidants.

6. Conclusion

Due to the detrimental effects of ROS/RNS, aerobic organisms, including mammals have evolved a number of endogenous antioxidants to counteract ROS/RNS-mediated biological damage. In addition, various exogenous antioxidant compounds have been found in dietary sources or synthesized in laboratories. The complementary effects of diverse antioxidants make it possible to effectively detoxify ROS/RNS and protect against disease conditions involving an oxidative stress mechanism. In this context, coordinated regulation of gene expression of a series of antioxidants primarily through Nrf2 signaling is an integral part of mammalian antioxidant defense mechanisms against oxidative stress. As accumulating evidence suggests a critical role for ROS/RNS in the pathophysiological processes of a wide variety of diseases, it is imperative to develop more effective antioxidant-based strategies for disease intervention. Development of such strategies relies on a profound understanding of the basic biology of antioxidants and their role in health and disease. The subsequent chapters are devoted to the discussion of the various kinds of antioxidants in biology and medicine.

7. References

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SECTION II
ANTIOXIDANTS SYNTHESIZED BY CELLS

Aerobic organisms, including mammals have evolved a wide variety of antioxidants to combat oxidative stress. These endogenous antioxidants synthesized by cells include both proteins and non-proteins. The protein antioxidants further comprise enzymes and non-enzymes. Superoxide dismutase and catalase, as mentioned in previous chapters are typical examples of antioxidant enzymes synthesized by cells. Examples of non-enzyme protein antioxidants include ferritin and metallothionein. A number of non-protein antioxidants are also synthesized by mammalian cells, which include glutathione, bilirubin, and coenzyme Q. These various endogenous antioxidants, either proteins or non-proteins, have unique antioxidative properties and often act coordinately to detoxify reactive oxygen and nitrogen species. In addition, they also possess other novel biological activities. The seventeen chapters in Section II are devoted to the discussion of each of the major endogenous antioxidants with respect to their basic biology and implications in health and disease.

Chapter 3

Superoxide Dismutase

Abstract

Since its discovery in 1969, superoxide dismutase (SOD) has been extensively studied in biology and medicine. There are three isozymes of SOD in mammals, namely, copper, zinc SOD, manganese SOD, and extracellular SOD. All three isozymes catalyze dismutation of superoxide to hydrogen peroxide and molecular oxygen with a similar reaction rate constant. SOD isozymes play an important role in protecting against various disease processes in animal models, including cardiovascular diseases, diabetes, neurodegeneration, and cancer. This notion from another angle demonstrates a crucial involvement of superoxide in disease pathophysiology. Studies in human subjects also provide evidence indicating protective effects of SODs in certain disease conditions.

1. Overview

1.1. Definition and History

The term superoxide dismutase (SOD) refers to a family of enzymes that catalyze the dismutation of superoxide to hydrogen peroxide and molecular oxygen. There are three isozymes of SOD in mammals, as listed below.

- Copper, zinc superoxide dismutase (Cu,ZnSOD or SOD1)
- Manganese superoxide dismutase (MnSOD or SOD2)
- Extracellular superoxide dismutase (ECSOD or SOD3)

Here the term isozyme (also known as isoenzyme or isoform) refers to enzymes that differ in amino acid sequence but catalyze essentially the same biochemical reaction.

As introduced in Chapter 1, the enzymatic activity of Cu,ZnSOD was first identified by J.M. McCord and I. Fridovich in 1969 [1]. But the protein was discovered before. T. Mann and D. Keilin had purified the protein 30 years earlier from bovine blood and liver as copper-binding protein of unknown function. The protein was then named erythrocuprein, hepatocuprein, or cytocuprein. Superoxide, the substrate of SOD was discovered by L. Pauling in

the 1930's, and it was not known then if this free radical could be produced biologically. In 1969, P.F. Knowles and coworkers showed that the enzyme xanthine oxidase could produce superoxide. J.M. McCord and I. Fridovich subsequently demonstrated that the copper protein purified by T. Mann and D. Keilin could catalytically eliminate the superoxide initially discovered by L. Pauling. This copper-containing protein is known as Cu,ZnSOD today. In 1970 and 1973, I. Fridovich and coworkers further discovered MnSOD and FeSOD (FeSOD is not present in mammals), respectively. The third SOD in mammals, namely, ECSOD, was discovered by S.L. Marklund and coworkers in 1982 [2].

1.2. Basic Characteristics

In mammals, Cu,ZnSOD is a homodimer with a molecular mass of 32 kDa. Both MnSOD and ECSOD are homotetramers with a molecular mass of 86-88 and 135 kDa, respectively. ECSOD also contains copper and zinc. Cu,ZnSOD is present in cytosol. It is also found in nuclei and mitochondrial intermembrane space. MnSOD exists in mitochondrial matrix. ECSOD is associated with plasma membrane or present in extracellular space. The human genes for Cu,ZnSOD, MnSOD, and ECSOD are localized on chromosomes 21q22, 6q25, and 4q21, respectively. Table 3-1 summarizes the basic characteristics of the three SOD isozymes in mammals.

Table 3-1. Basic Characteristics of the three isozymes of mammalian superoxide dismutase

Isozyme	Molecular Mass	Assembly of Subunits	Metal Ions	Cellular Location	Chromosomal Localization
Cu,ZnSOD	32 kDa	Homodimer	Cu and Zn	Cytosol, nuclei, mitochondrial intermembrane space	21q22
MnSOD	86-88 kDa	Homotetramer	Mn	Mitochondrial matrix	6q25
ECSOD	135 kDa	Homotetramer	Cu and Zn	Plasma membrane, extracellular space	4q21

2. Biochemistry and Molecular Regulation

2.1. Biochemistry

All three isozymes of SOD catalyze dismutation of superoxide ($O_2^{\cdot-}$) to form hydrogen peroxide (H_2O_2) and molecular oxygen with a similar reaction rate constant of ~1.6 x 10^9 $M^{-1}s^{-1}$ (Reaction 1).

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (1)$$

The reaction rate constant of SOD-catalyzed dismutation of superoxide is over three orders of magnitude greater than that of spontaneous dismutation, which is ~5 x 10^5 $M^{-1}s^{-1}$ at pH 7.0. Here the term dismutation refers to a chemical reaction in which the same reactant is both oxidized and reduced. In the dismutation reaction of superoxide, one superoxide is oxidized to molecular oxygen and another is reduced to hydrogen peroxide.

The overall mechanism by which SOD functions has been called "ping-pong" mechanism as it involves the sequential reduction and oxidation of the metal center, with the concomitant oxidation and reduction of superoxide (Reactions 2 and 3, where M stands for the redox metal ion of the SOD).

$$M^{n+}\text{-SOD} + O_2^{\cdot-} \longrightarrow M^{(n-1)+}\text{-SOD} + O_2 \quad (2)$$

$$M^{(n-1)+}\text{-SOD} + O_2^{\cdot-} + 2H^+ \longrightarrow M^{n+}\text{-SOD} + H_2O_2 \quad (3)$$

Cu,ZnSOD also exhibits peroxidase activity. Interaction of hydrogen peroxide with Cu,ZnSOD in the presence of bicarbonate yields carbonate radical [3]. As stated in Section 2.3.4 of Chapter 1, carbonate radical is a potent oxidant capable of inducing damage to biomolecules in vitro. In addition, Cu,ZnSOD catalyzes tyrosine nitration by peroxynitrite [4]. However, the in vivo significance of these reactions remains to be elucidated.

2.2. Molecular Regulation

A number of transcription factors have been shown to play a role in regulating constitutive or inducible expression of SOD genes. These transcription factors include NF-κB, AP-1, AP-2, SP1, and CCAAT-enhancer-binding protein (C/EBP) [5]. Recently, it has been shown that Nrf2 regulates Cu,ZnSOD expression via an antioxidant response element-driven mechanism [6]. In addition to the regulation by transcription factors, SOD genes are also subject to epigenetic regulation, such as hypermethylation, leading to epigenetic silencing. The term epigenetic regulation refers to heritable changes at the level of gene expression not related to the underlying DNA sequence.

SOD expression is regulated not only at transcriptional level, but also at post-transcriptional level via changes in mRNA stability, mRNA translation, and post-translational modifications [7]. Alterations in the regulation of SOD expression at these various levels may change tissue SOD activity and thereby modulate the susceptibility to disease pathophysiology involving increased formation of superoxide.

3. Role in Health and Disease

3.1. Animal Studies

3.1.1. Experimental Approaches

Various approaches have been used to study the role of SODs in health and disease in experimental animals. These approaches include the following.

- Gene knockout
- Transgenic overexpression or viral vector-mediated gene delivery
- Administration of exogenous SODs or SOD derivatives
- Pharmacological inhibition or induction of endogenous SODs

Gene knockout and transgenic overexpression models provide the most convincing evidence on the biological activities of SODs. A gene knockout mouse is a mouse that has had one or more specific genes artificially deleted from its genome. For a target gene, if both copies of the gene are deleted (as indicated by the superscript $^{-/-}$), the animal is called homozygous. On the other hand, if only one copy of the gene is deleted (as indicated by the superscript $^{+/-}$), it is called heterozygous. In contrast to gene knockout, transgenic overexpression animal models are used to achieve selective overexpression of a particular antioxidant protein. Transgenic overexpression animals are created by introducing new DNA sequences into the germline.

Direct administration of antioxidant enzymes or proteins to animals is another way to study the biological activities of the antioxidants. However, limited bioavailability is a major drawback of such an approach. In this regard, antioxidant enzyme mimetics have been commonly used owing to their favorable bioavailability (see Chapter 24 for discussion of antioxidant enzyme mimetics). Here the term bioavailability in pharmacology refers to the fraction of an administered dose of unchanged drug that reaches the systemic circulation.

In addition to genetic approaches, pharmacological inhibition or induction of antioxidant enzymes is also used to study their biological activities. For example, diethyldithiocarbamate is often utilized as an inhibitor of Cu,ZnSOD to study its biological activities in both cell cultures and experimental animals. However, lack of specificity is a major limitation associated with the use of pharmacological inhibitors, including diethyldithiocarbamate. A number of chemical agents, such as polyphenolic compounds have been shown to induce SODs in cell cultures and experimental animals. Again, the limitation is the lack of selectivity as these chemical inducers usually cause upregulation of a battery of antioxidant genes. In addition, they may also exert non-antioxidant effects.

3.1.2. Role in Cardiovascular Diseases

Both gene knockout and transgenic overexpression animal studies demonstrate a critical protective role for all three isozymes of SOD in cardiovascular disorders, including hypertension, atherosclerosis, myocardial ischemia-reperfusion injury, cardiac hypertrophy, heart failure, and drug/xenobiotic-induced cardiovascular complications [8, 9]. With the above animal models of human cardiovascular diseases, knockout of SODs leads to exacerbation, whereas transgenic overexpression of SODs attenuates the pathophysiological processes. It is noteworthy that while homozygous knockout of either Cu,ZnSOD or ECSOD does not affect animal survival, deletion of MnSOD in mice is embryonically lethal or causes early neonatal death due primarily to dilated cardiomyopathy [10].

3.1.3. Role in Diabetes

SODs play an important role in protecting against various complications of diabetes in animal models. Overexpression of Cu,ZnSOD or MnSOD attenuates diabetic renal injury, neuropathy, cardiomyopathy, and retinopathy [11-14]. Conversely, diabetes-induced renal

injury and cataract are exacerbated in Cu,ZnSOD-deficient mice. Cu,ZnSOD deficiency also potentiates diabetic embryopathy in experimental animals, indicating an involvement of superoxide in hyperglycemia-induced adverse effects on fetus. In addition to protecting against diabetic complications, Cu,ZnSOD and MnSOD may also prevent the genesis of diabetes. Pancreatic β-cell-selective overexpression of Cu,ZnSOD in mice protects the β-cells from oxidative stress and enhances the resistance of the animals to the development of alloxan-induced diabetes. Alloxan is a compound capable of causing the pancreatic β-cell destruction and thereby the development of type 1 diabetes in experimental animals. Adenoviral vector-mediated overexpression of MnSOD in the pancreatic islets extends islet graft function in a mouse model of autoimmunity-induced diabetes. These experimental findings suggest a critical role for both Cu,ZnSOD and MnSOD in protecting against the pancreatic β-cell injury and the development of diabetes. In contrast, overexpression of ECSOD in the pancreatic islets has been reported to provide no effects on the development of diabetes in nonobese diabetic mice [15].

3.1.4. Role in Neurological Diseases

SOD overexpression via genetic approaches provides neuroprotection in various animal models of neurological disorders, including ischemic brain injury, Parkinson's disease, and Alzheimer's disease [16, 17]. Intraperitoneal injection of Tat-Cu,ZnSOD fusion protein also protects against ischemic brain injury in mice [18]. Similarly, various SOD mimetics are neuroprotective in animal models of neurological diseases (Chapter 24).

Gene knockout animal models have provided important insights into the protective role of SODs in neurodegeneration. Dilated cardiomyopathy, mitochondrial abnormalities, hepatic lipid accumulation, and early neonatal death have been reported to occur in homozygous MnSOD knockout ($MnSOD^{-/-}$) mice. Treatment with the SOD mimetic Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP; Chapter 24) rescues the $MnSOD^{-/-}$ mice from the above systemic pathological changes and dramatically prolongs their survival. These animals instead develop pronounced neurodegeneration, which is believed to result from excessive mitochondrial production of superoxide in neuronal cells due to the inability of MnTBAP to cross the blood-brain barrier [19]. These observations are consistent with the notion that mitochondria are a major source of cellular superoxide, and uncontrolled excessive formation of superoxide leads to cell and tissue injury.

In contrast to MnSOD, deletion of Cu,ZnSOD in animals causes milder phenotypes. Homozygous Cu,ZnSOD knockout ($Cu,ZnSOD^{-/-}$) mice develop normally and show no overt abnormal phenotypes when they reach adulthood except that females show reduced fertility. However, these mice exhibit increased vulnerability to motor neuron loss after axonal injury, suggesting that the neuroprotective effects of Cu,ZnSOD may become evident only when neurons are under stress or exposed to insults [20].

3.1.5. Role in Pulmonary Diseases

A beneficial role for all three isozymes of SOD in pulmonary hyperoxic injury has been demonstrated in experimental animals via both gene knockout and transgenic overexpression [21, 22]. SODs attenuate pulmonary inflammatory responses, oxidative stress, and tissue remodeling following hyperoxia. Overexpression of SODs also results in amelioration of pulmonary injury induced by drugs (e.g., bleomycin), radiation, and environmental chemicals or

dusts (e.g., tobacco smoke, asbestos, air pollutants). Administration of exogenous SOD protein via intratracheal instillation or inhalation protects against oxidative and inflammatory lung injury in experimental animals. Conversely, targeted disruption of SODs aggravates the pathophysiological processes of the pulmonary disorders.

3.1.6. Role in Hepatic and Gastrointestinal Diseases

Multiple animal studies demonstrate a protective role for SODs in hepatic disorders, including liver ischemia-reperfusion injury, graft dysfunction after liver transplantation, nonalcoholic fatty liver disease, and liver injury induced by alcohol and other drugs/xenobiotics. Viral vector-mediated delivery of ECSOD gene to liver tissue suppresses ischemia-reperfusion-induced hepatic injury in mice. Viral vector-mediated delivery of either Cu,ZnSOD or MnSOD, but not ECSOD increases animal survival and attenuates graft dysfunction after transplantation of fatty livers in rats. These protective effects may result from the SOD-mediated suppression of NF-κB activation and proinflammatory cytokine production [23]. It is also reported that Cu,ZnSOD deficiency causes ApoB degradation and increases hepatic lipid accumulation in mice [24]. Homozygous MnSOD-deficient mice die within the first 10 days of life with dilated cardiomyopathy and accumulation of lipid in liver [10]. These observations suggest a critical pathophysiological role for superoxide and oxidative stress in hepatic lipid dysregulation and the possible development of nonalcoholic fatty liver disease.

Overexpression of either Cu,ZnSOD or MnSOD in mice inhibits alcohol-induced liver injury, whereas knockout of Cu,ZnSOD aggravates the liver damage [25, 26]. Overexpression of Cu,ZnSOD also protects against carbon tertrachloride-elicited hepatotoxicity in mice. However, mice deficient in Cu,ZnSOD are resistance to acetaminophen-induced hepatotoxicity, suggesting a possible pro-oxidant role for the physiological level of Cu,ZnSOD activity in acetaminophen-mediated liver injury [27].

A protective role for SODs in gastrointestinal disorders has been demonstrated in several animal models. For example, transgenic overexpression of Cu,ZnSOD in mice protects tissue from neutrophil infiltration and lipid peroxidation during intestinal ischemia-reperfusion. Oral coadministration of Cu,ZnSOD and catalase is reported to attenuate stress-induced gastric mucosal lesions in rats. In an animal model of chemically-induced acute colitis, transgenic overexpression of Cu,ZnSOD results in amelioration of colonic inflammation and improves animal survival [28].

3.1.7. Role in Renal Diseases

Either viral vector-mediated or transgenic overexpression of SODs is reported to attenuate renal ischemia-reperfusion injury in animal models. Administration of lecithinized SOD reduces ischemia-induced chronic allograft dysfunction in rats. Targeting a synthetic cationic SOD to renal proximal tubular cells inhibits oxidative stress and nephrotoxicity of cisplatin and increases the survival of cancer-bearing mice [29]. Cisplatin is an anticancer drug that may cause severe renal injury. Viral delivery of Cu,ZnSOD gene reduces cyclosporine A-induced free radical formation and nephrotoxicity. Cyclosporine A is a commonly used immunosuppressive agent whose clinical use in organ transplantation is hampered by its nephrotoxicity. On the other hand, Cu,ZnSOD deficiency causes salt sensitivity and aggravates hypertension in hydronephrosis in mice [30]. Similarly, mice with heterozygous knockout of

MnSOD ($MnSOD^{+/-}$) develop salt-sensitive hypertension and accelerated renal senescence [31]. Together, these findings from both transgenic overexpression and gene knockout animal models indicate a critical involvement of superoxide in various kidney pathophysiological processes, and also point to the feasibility of using SOD-based modalities for the intervention of renal diseases.

3.1.8. Role in Skin Diseases

Oxidative stress is involved in a variety of skin disorders, including ultraviolet light-induced injury, skin carcinogenesis, and skin aging [32]. A number of animal studies demonstrate an important role for SODs in protecting against the above skin pathophysiological processes. It is also reported that treatment with Cu,ZnSOD ameliorates skin ischemic injury in experimental animals [33].

3.1.9. Role in Cancer

Oxidative stress is implicated in multistage carcinogenesis, including initiation, promotion, and progression. It is not surprising that SODs are also found to have a beneficial effect in experimental carcinogenesis. Many studies with gene knockout models show that long-term reduction of either Cu,ZnSOD or MnSOD results in increased DNA damage, mutations, and higher incidence of cancer development, including hepatocarcinogenesis [34, 35]. Recently, it has been reported that overexpression of ECSOD attenuates heparanase expression and inhibits breast carcinoma cell growth and invasion [36]. As mentioned above (Section 3.1.8), SOD overexpression also inhibits chemically-induced skin carcinogenesis in experimental animals.

The protective effects of SODs in cancer development have also been investigated via examining their involvement in cancer cell growth in vitro. However, the results from these in vitro studies are inconsistent, with some studies showing SODs as cancer cell survival factors, and others indicating the opposite [36, 37]. Regardless of these in vitro observations, studies using gene knockout and transgenic animal models have provided convincing evidence supporting a critical role for SODs in protecting against carcinogenesis.

3.1.10. Role in Other Diseases and Conditions

SODs may be important protectors in anemia. Deficiency of either Cu,ZnSOD or MnSOD in mice causes oxidative stress in red blood cells and hemolytic anemia [38, 39]. Studies also demonstrate a protective role for SODs in aging and aging-related cognitive decline and muscle atrophy [40]. Moreover, gene transfer of ECSOD to the penis reduces superoxide levels and improves erectile function in aged or diabetic rats [41, 42].

As discussed above, substantial experimental evidence demonstrates an important role for SODs in protecting against various pathophysiological conditions. However, it has also been reported that SODs may exert unfavorable effects under certain experimental conditions. One example is the aforementioned role for Cu,ZnSOD in potentiating acetaminophen-induced hepatotoxicity [27]. As another example, $Cu,ZnSOD^{-/-}$ mice are shown to produce less amounts of caspase-2-dependent cytokines and be less susceptible to lipopolysaccharide-induced endotoxic shock compared with wild-type littermates [43]. These observations point to the complicated involvement of superoxide and SODs in physiology and disease. Nevertheless, animal studies continue providing important insights into the biological activities of

antioxidant enzymes and lay a foundation for investigations on their potential health benefits in human subjects.

3.2. Human Studies and Clinical Perspectives

3.2.1. Study Approaches

Since the discovery of Cu,ZnSOD more than four decades ago, there have been extensive studies on the biological activities of SODs in human health and disease. As outlined below, two major approaches are employed to study the biological activities of SODs as well as other antioxidant enzymes in human subjects.

- Genetic variation studies: Gene mutations and polymorphisms of SODs have been studied in human populations in relation to disease conditions. Here the term polymorphism refers to a genetic variation in the DNA sequence with a measurable frequency of detection above 1%. Any polymorphism in the chromosomal DNA (coding and non-coding) can affect mRNA processing, maturation, and translation as a result of changes in conformation. A polymorphism in an antioxidant gene may result in a decrease, an increase, or no change in the activity/level of the protein encoded by the gene.
- Clinical interventional studies (trials): Recombinant SODs as well as synthetic SOD mimetics have been investigated for their potential use in the intervention of certain human disease conditions.

Another approach of clinical potential in humans is gene therapy. Gene therapy with the use of antioxidant genes is emerging as a promising approach for select disease conditions and is an especially lucrative option for patient groups not suitable for conventional therapies [8]. However, this approach is much limited in human studies due to safety concerns.

3.2.2. Genetic Variation Studies

The discovery in 1993 of the Cu,ZnSOD mutations in familial amyotrophic lateral sclerosis, a fatal, adult-onset neurodegenerative disease primarily affecting motor neurons, has attracted broad attention on the biological role of SODs in human disorders [44]. As illustrated in Figure 3-1, induction of this disease in humans is believed to be due, at least partially, to a toxic gain of function (e.g., an increased pro-oxidant activity) of the mutant Cu,ZnSOD protein, rather than a loss of SOD activity.

Many, though not all, human population studies have demonstrated that gene polymorphisms exist for all three SOD isozymes, and are associated with altered risk of developing various human disease conditions. These include cardiovascular diseases, diabetes, neurodegenerative disorders (e.g., Parkinson's disease, Alzheimer's disease), lung diseases, and certain types of cancer [45-50]. However, it should be borne in mind that the above observational studies do not prove a causal relationship between gene polymorphisms and disease development. In addition to the gene polymorphism studies, there are also reports in human subjects showing a correlation between changes in SOD levels and disease pathophysiology. Again, such studies do not establish a causal role for SODs in the genesis or progression of disease pathophysiology.

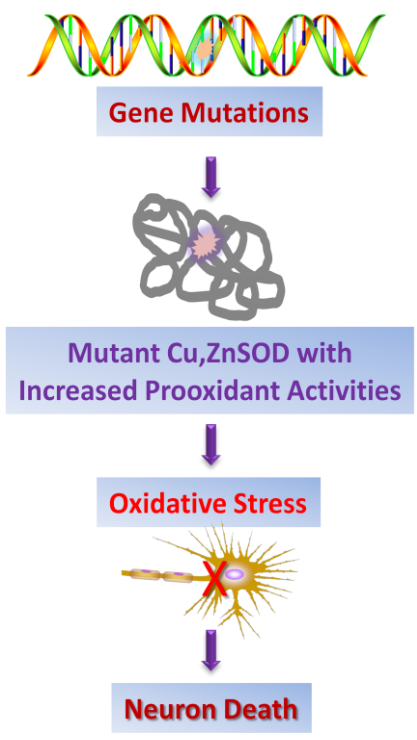

Figure 3-1. Schematic illustration of the potential involvement of Cu,ZnSOD-mediated oxidative stress in the pathogenesis of familial amyotrophic lateral sclerosis. It is believed that the mutations in Cu,ZnSOD gene result in production of mutant Cu,ZnSOD with increased pro-oxidant activities. This toxic gain of function causes oxidative stress, leading to motor neuron death. This notion is supported by studies in experimental models showing oxidative neuron degeneration induced by the mutant Cu,ZnSOD protein.

3.2.3. Interventional Clinical Trials

There have been studies in using SODs for the intervention of certain human disease conditions, but the results are inconsistent. An early clinical study reported that recombinant human SOD failed to improve recovery of ventricular function in patients undergoing coronary angioplasty for acute myocardial infarction [51]. A randomized double-blind, placebo-controlled trial showed a beneficial effect of recombinant human SOD on acute and chronic rejection in recipients of cadaveric renal transplants [52]. It has also been reported that in kidney transplantation use of a modified perfusate containing polyethylene glycol-SOD (PEG-SOD), prostaglandin E1, and nitroglycerin improves delayed graft function and survival [53]. In certain clinical studies, topical application of SODs was reported to improve skin or mucosa lesions associated with various skin disorders, including progressive systemic sclerosis, burns, and pruritis. Cu,ZnSOD has also been used in certain creams for cosmetic purposes with a hope to retard skin aging. The protective effects of SODs when used as exogenously administered agents in human disorders remain to be established through more clinical studies. One major drawback associated with using native SODs for disease intervention is the protein nature of the SODs, which limits their membrane permeability, bioavailability, and

half-life. In this context, small molecular mass SOD mimetics have been developed. These SOD mimetics have been studied for the intervention of ROS-mediated disease processes (Chapter 24).

4. Conclusion and Future Directions

Extensive animal studies demonstrate an important role for all three forms of SOD in protecting against a wide variety of disease conditions. The protective effects apparently result from SOD's ability to scavenge superoxide and attenuate oxidative and inflammatory stress. Consistent with the observations made in animal models, human population studies demonstrate that SOD gene polymorphisms are associated with altered risk of developing various disease conditions. It is established that mutations in Cu,ZnSOD gene causally contribute to the pathogenesis of familial amyotrophic lateral sclerosis. However, randomized controlled trials on using SODs for the intervention of human diseases have been much limited with inconsistent results. Future studies should focus on developing more effective superoxide scavengers, such as small molecular mass SOD mimetics, and testing their effectiveness in disease intervention in well-designed clinical trials. Such studies will further enhance our understanding of the role of superoxide and SODs in human health and disease. They will also contribute to the development of SOD-based modalities to combat human diseases involving a superoxide-mediated oxidative stress mechanism.

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Chapter 4

Catalase

Abstract

Mammalian catalase is a heme-containing enzyme that catalyzes the decomposition of hydrogen peroxide to water and molecular oxygen. It is widely distributed in various tissues and primarily localized in peroxisomes. A number of transcription factors, co-activators, and other signaling molecules are implicated in the regulation of mammalian catalase expression. Studies in animal models of catalase deficiency or overexpression demonstrate a protective role for catalase in disease conditions ranging from cardiovascular injury and diabetes to cancer and aging. Inheritable catalase deficiency and catalase gene polymorphisms have also been identified in humans. Studies on both hereditary catalase deficiency and gene polymorphisms suggest a potential beneficial role for catalase in human health and disease.

1. Overview

1.1. Definition and History

Mammalian catalase belongs to a family of iron-protoporphyrin IX-containing proteins that include a variety of cytochromes, globins and peroxidases. Catalase is one of the best characterized antioxidant enzymes in mammalian tissues. It plays an important role in regulating intracellular hydrogen peroxide levels in mammalian cells. Another major enzyme for detoxifying hydrogen peroxide is glutathione peroxidase (Chapter 6).

Catalase was identified and named by O. Loew in 1900. O. Loew named it catalase due to its catalytic action on hydrogen peroxide [1]. A congenital absence of erythrocyte catalase, named acatalasemia was first identified by S. Takahara about six decade ago among some Japanese patients who had oral gangrene [2]. The disease is also called Takahara's disease. In 1964, R.N. Feinstein and coworkers established catalase-deficient mouse strains, including acatalasemic and hypocatalasemic mice through a large-scale screening of the progeny of irradiated C3H mice [3]. The development of these catalase-deficient mice as well as gene knockout and transgenic overexpression animal models has greatly helped understand the biological activities of mammalian catalase.

1.2. Basic Characteristics

Mammalian catalase is a homotetramer with a molecular mass of ~240 kDa. It is widely distributed throughout the body with high levels in liver, kidney, and red blood cells. In cells, catalase is primarily localized in peroxisomes. It is also found in other intracellular compartments, including mitochondria, nuclei, and endoplasmic reticulum. Human catalase gene is localized on chromosome 11p13.

2. Biochemistry and Molecular Regulation

2.1. Biochemistry

As stated above, mammalian catalase is a homotetramer, and each subunit contains a heme (ferriprotoporphyrin IX) at the active site. The active site is internally located and approachable by a passageway that becomes narrow, thereby explaining the ability of the enzyme to use only substrates of small size [4].

Catalase is best known for its ability to catalyze the decomposition of hydrogen peroxide (H_2O_2) to form water and molecular oxygen (Reaction 1). This is a dismutation reaction because one molecule of hydrogen peroxide is reduced to water and another molecule is oxidized to molecular oxygen.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \qquad (1)$$

Catalase catalyzes decomposition of hydrogen peroxide via a two-stage process. In the first step, one hydrogen peroxide molecule oxidizes the heme iron of the resting enzyme to form an oxyferryl species with a π-cationic porphyrin radical, termed compound I (Reaction 2). The second hydrogen peroxide molecule is utilized as a reductant of compound I to regenerate the resting-stage enzyme along with the formation of water and molecular oxygen (Reaction 3, where Por denotes porphyrin).

$$\text{Catalase}(\text{Por-Fe}^{\text{III}}) + H_2O_2 \longrightarrow \text{Compound I}(\text{Por}^{+\cdot}\text{-Fe}^{\text{IV}}\text{=O}) + H_2O \qquad (2)$$

$$\text{Compound I}(\text{Por}^{+\cdot}\text{-Fe}^{\text{IV}}\text{=O}) + H_2O_2 \longrightarrow \text{Catalase}(\text{Por-Fe}^{\text{III}}) + H_2O + O_2 \qquad (3)$$

The sum of Reactions 2 and 3 is: $2H_2O_2 \longrightarrow 2H_2O + O_2$

Catalase also possesses peroxidase or oxidase activities toward a number of substrates, including low molecular mass alcohols, the tryptophan precursor indole, and the neurotransmitter precursor β-phenylethylamine [5]. In general, catalase exhibits the peroxidatic activity in the presence of low concentrations of hydrogen peroxide. Reaction 4 illustrates the peroxidatic activity of catalase in converting alcohol (AH_2) to aldehyde (A) in the presence of low levels of hydrogen peroxide.

$$H_2O_2 + AH_2 \xrightarrow{\text{Catalase}} A + 2H_2O \qquad (4)$$

2.2. Molecular Regulation

Catalase expression is regulated at multiple levels, including transcription, post-transcription, and post-translation. Regulation of mammalian catalase expression has only been recently studied more carefully, and much still remains unknown. It has been reported that the forkhead transcription factor Foxo3a plays an important role in the upregulation of catalase gene expression in mammalian cells [6]. Other regulatory factors involved in the regulation of catalase expression include the transcriptional co-activator PGC-1α (peroxisome proliferator-activated receptor γ co-activator 1α), the non-receptor tyrosine kinases c-Abl and Arg, Sirt1, Sirt3, telomerase, and AMP-activated protein kinase α1 [7-10]. Recently, Nrf2 is also shown to control catalase gene expression though it remains unclear if the promoter of catalase gene contains the antioxidant response element [11]. At the post-translational level, catalase is shown to undergo ubiquitination and proteasomal degradation by a mechanism dependent on c-Abl- and Arg-mediated phosphorylation [12].

3. Role in Health and Disease

3.1. Animal Studies

3.1.1. Experimental Approaches

The experimental approaches described in Chapter 3 are also frequently used to study the biological activities of catalase in animal models. Like superoxide dismutase, much of our current knowledge on the biological activities of mammalian catalase comes from studies in knockout, acatalasemic, and transgenic overexpression animal models, as well as the direct use of catalase or catalase derivatives. A number of catalase derivatives have been developed to selectively deliver the chemically-modified catalase to different tissues or various cell populations within the same tissue [13]. Viral vector-mediated gene delivery is also frequently employed to overexpress catalase in target tissues of experimental animals to study the effects on disease pathophysiology. The chemical inhibitor aminotriazole is a commonly used tool to study the biological activities of catalase in cellular systems.

3.1.2. Role in Cardiovascular Diseases

Studies using both gene knockout and overexpression animal models demonstrate a critical role for catalase in protecting against various cardiovascular disorders with oxidative stress as an important mechanism. These include atherosclerosis, hypertension, myocardial ischemia-reperfusion injury, cardiomyopathy, cardiac hypertrophy, and chronic heart failure [14-16]. Notably, overexpression of catalase targeted to mitochondria attenuates murine cardiac aging, suggesting a crucial role for mitochondria-derived hydrogen peroxide in aging-associated myocardial dysfunction and degeneration [17]. In addition, catalase also protects against cardiotoxicity induced by drugs and xenobiotics, including doxorubicin and ethanol in experimental animals [18].

3.1.3. Role in Diabetes

Overexpression of catalase attenuates oxidative stress, inflammation, and development of

both type 1 and type 2 diabetes in experimental animals. Catalase overexpression also ameliorates diabetic complications, including diabetic cardiomyopathy [19]. In vitro, mitochondrial overexpression of catalase protects the pancreatic β-cells from oxidative stress- and inflammatory cytokine-induced injury [20]. On the other hand, acatalasemic mice have been shown to be more susceptible to chemically-induced pancreatic β-cell injury and diabetes [21]. In contrast to the large body of literature showing a protective role of catalase in diabetes, one study reported that cytoplasmic catalase overexpression in nonobese mice accelerated diabetes development [22].

3.1.4. Role in Neurological Diseases

Homozygous catalase knockout (catalase$^{-/-}$) mice develop normally and show no gross abnormalities. As compared with wild-type littermates, catalase$^{-/-}$ mice are not more susceptible to hyperoxia-induced lung injury. However, brain mitochondria of the catalase$^{-/-}$ mice are more vulnerable to trauma-induced impairment in oxidative phosphorylation than those of wild-type mice [23]. This suggests a potential role for catalase in neuroprotection. Indeed, transgenic overexpression or viral vector-mediated gene delivery of catalase attenuates the disease severity in various animal models of neurodegenerative disorders, including multiple sclerosis, optic neuropathy, and Friedreich's ataxia [24]. Similarly, administration of the synthetic superoxide dismutase/catalase mimetics (Chapter 24) has also been reported to protect against neurodegeneration in experimental animals [25, 26].

3.1.5. Role in Pulmonary Diseases

As stated above in Section 3.1.4, catalase-deficient mice are not more susceptible to hyperoxic pulmonary injury than wild-type mice [23]. But, viral vector-mediated delivery of catalase gene to lung attenuates hyperoxia-induced lung injury and mortality in mice [27]. Immunotargeting of catalase to the pulmonary endothelium also alleviates oxidative stress and reduces acute lung transplantation injury in rats [28]. However, a study shows that overexpression of catalase in lung tissue of mice fails to attenuate allergic airway disease, in which oxidative stress and inflammation play an important role [29].

3.1.6. Role in Hepatic and Gastrointestinal Diseases

Several studies demonstrate that delivery of catalase gene to liver results in increased hepatic catalase activity and attenuation of liver necrosis and inflammation following ischemia-reperfusion in experimental animals. Administration of a catalase derivative, succinylated catalase to mice also increases catalase activity in liver tissue and prevents neutrophil-mediated hepatic ischemia-reperfusion injury [30]. Catalase and glutathione peroxidase-1 double-knockout mice are found to be more susceptible to ethanol-induced liver injury than wild-type mice [31], indicating a potential protective role for catalase in hepatotoxicity. Indeed, catalase derivatives are shown to protect against liver failure induced by a number of hepatotoxicants, including carbon tetrachloride and thioacetamide [13]. Delivery of catalase derivatives to liver nonparenchymal cells (e.g., Kupffer cells) is effective in inhibiting liver injury during septic shock in mice. This finding implicates that targeting catalase to Kupffer cells may suppress liver injury due to Kupffer cell activation (i.e., release of reactive oxygen species) during septic shock. Administration of catalase derivatives also attenuates experimental colitis in animal models, suggesting a causal involvement of hydrogen peroxide in the

development of colitis. It is also reported that acatalasemic mice exhibit increased susceptibility to oxidant-mediated peritoneal injury and fibrosis. Peritoneal fibrosis is a critical complication of peritoneal dialysis [32].

3.1.7. Role in Renal Diseases

Catalase deficiency in mice sensitizes these animals to renal oxidative stress and fibrosis due to nephronectomy or ureteral obstruction [33, 34]. On the other hand, transgenic overexpression of catalase attenuates interstitial fibrosis and tubular apoptosis in db/db diabetic mice, suggesting a critical involvement of hydrogen peroxide and oxidative stress in diabetic nephropathy [35]. Administration of a catalase derivative (cationized catalase) to mice leads to accumulation of the catalase derivative in kidney, and attenuation of cisplatin-induced nephrotoxicity without compromising its antitumor activity. The survival of the cisplatin-treated tumor-bearing animals is also improved by administration of the catalase derivative [36]. These observations support the oxidative stress mechanism of cisplatin-induced nephrotoxicity and point to the feasibility for using catalase-based modalities to enhance the therapeutic index of cisplatin. Cisplatin is an important anticancer drug whose clinical use is limited by its nephrotoxicity.

3.1.8. Role in Skin Diseases

Multiple studies suggest a protective effect of catalase in ultraviolet (UV) light-induced skin injury. It is known that UV light causes the formation of reactive oxygen species (ROS), including hydrogen peroxide in skin tissue. Viral vector-mediated overexpression of catalase protects normal human reconstructed epidermis from UV light-induced sunburn cell formation, hypertrophy, and oxidative DNA damage. Catalase overexpression also reduces UV light-induced apoptosis in a human xeroderma pigmentosum reconstituted epidermis [37]. It is further reported that catalase overexpression in cultured human keratinocytes inhibits UV light-induced apoptosis via preventing DNA damage caused by ROS [38].

Animal studies demonstrate that skin wound healing is subject to redox regulation. Viral vector-mediated overexpression of catalase in mouse skin is found to retard skin wound healing and tissue remodeling, suggesting a critical role for hydrogen peroxide in promoting skin wound healing in experimental animals [39]. In this regard, hydrogen peroxide at moderate concentrations has been suggested as a potential modality to facilitate the process of skin wound healing. This represents another example that ROS may play a beneficial role in health and disease.

3.1.9. Role in Cancer

Unlike superoxide dismutase (Section 3.1.9 of Chapter 3), the involvement of catalase in experimental carcinogenesis has not been investigated in gene knockout or transgenic overexpression models. Several studies using catalase derivatives have found a protective effect of catalase in tumor metastasis in animal models, including hepatic and pulmonary metastasis as well as peritoneal dissemination of tumor cells [13]. These findings provide evidence for a causal involvement of hydrogen peroxide in tumor metastasis. In vitro, catalase overexpression suppresses tumor cell proliferation and invasiveness. Together, these studies suggest that targeted delivery of catalase to sites of tumor cell metastasis may be a promising approach to inhibiting metastatic tumor growth.

3.1.10. Role in Other Diseases and Conditions

Catalase may also play a beneficial role in other diseases and conditions, including arthritis, eye disorders, and aging. Overexpression of catalase targeted to mitochondria results in extension of murine lifespan and delayed development of cardiac pathology and cataract. Tissue oxidative damage, mitochondrial enzyme inactivation, and mitochondrial deletion are also attenuated in the catalase-overexpressing animals [40]. A recent study also demonstrates that ectopic catalase expression in mitochondria by viral vector-mediated delivery leads to enhanced exercise performance in mice, possibly by increased removing of excessive, pathogenic hydrogen peroxide in muscle [41].

3.2. Human Studies and Clinical Perspectives

The significance of catalase in human health and disease has been investigated largely via studying the phenotypic changes in patients with hereditary catalase deficiency and catalase gene polymorphisms in general populations.

3.2.1. Hereditary Catalase Deficiency

As stated above in Section 1.1, acatalasemia in humans was identified about six decades ago in some Japanese patients. This rare genetic disorder was originally thought to predispose people to oral and facial infections. However, no such predisposition was found in other Japanese people who were later shown to have the same genetic disorder. Hereditary catalase deficiency has been subsequently also observed in people from Switzerland and other countries. Although catalase makes much of the proteins in peroxisomes, patients with hereditary catalase deficiency have no obvious features of peroxisomal diseases. However, these patients may be more susceptible to oxidative stress injury and have an increased risk of developing diabetes [42, 43].

3.2.2. Gene Polymorphisms

There exist various polymorphisms in the catalase gene in human populations. Extensive studies demonstrate an association of gene polymorphisms with a number of disease conditions, including hypertension, diabetes, neurodegeneration, asthma, inflammatory disorders, vitiligo, osteonecrosis, and certain types of cancer [44-52]. Although population gene polymorphism studies do not establish a causal relationship between genetic variations of catalase gene and development of disease conditions, they do provide important clues to the biological significance of this antioxidant enzyme in human health and disease. Such knowledge may serve as a basis for developing strategies to minimize the potential risk associated with genetic variations of catalase gene in human subjects.

4. Conclusion and Future Directions

Studies in various animal models provide a large body of data supporting a critical role for catalase in protecting against a wide range of disease conditions. These studies also provide evidence for a causal involvement of hydrogen peroxide in disease pathophysiology.

Although studies in human subjects have not been as convincing as those in experimental animals, there is evidence that catalase may exert beneficial effects in certain human disease conditions, including hypertension, diabetes, and cancer. Future studies should emphasize translational research and focus on developing novel catalase derivatives or mimetics with improved bioavailability and safety profile and testing their efficacy in disease intervention in clinical trials. As superoxide dismutase and catalase act coordinately in the sequential metabolism of superoxide to hydrogen peroxide, and hydrogen peroxide to water (Figure 4-1), more attention needs to be paid to the combined effects of these two antioxidant enzymes in health and disease. Future efforts should also be directed to the continued characterization of novel gene polymorphisms and their impacts on tissue catalase expression as well as individual's susceptibility to disease development.

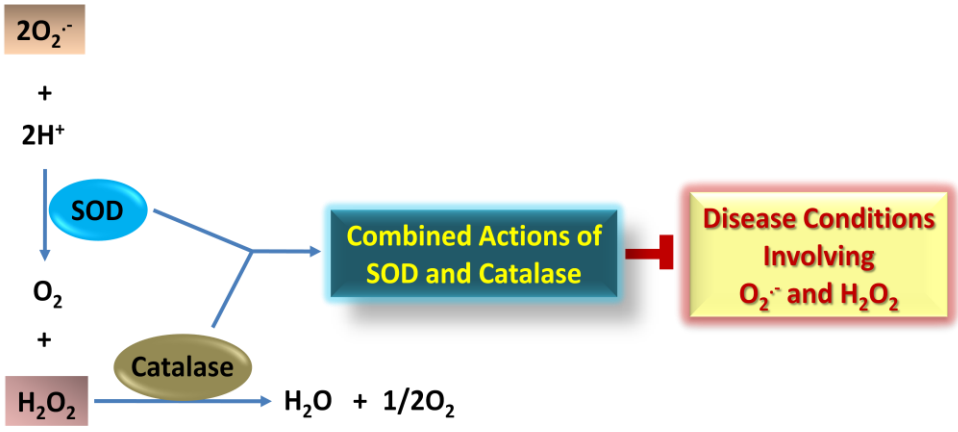

Figure 4-1. SOD- and catalase-catalyzed sequential metabolism of superoxide and hydrogen peroxide in disease intervention. As illustrated, SOD catalyzes the dismutation of superoxide to hydrogen peroxide. The toxic hydrogen peroxide is further decomposed to water by catalase. Combined effects of both SOD and catalase ensure the detoxification of both superoxide and hydrogen peroxide in biological systems. SOD, superoxide dismutase; $O_2^{\cdot-}$, superoxide; H_2O_2, hydrogen peroxide.

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Chapter 5

Glutathione and its Synthesizing Enzymes

Abstract

The reduced form of glutathione (GSH) is the most abundant intracellular antioxidant thiol in mammalian systems. It is synthesized from three amino acids (i.e., glutamate, cysteine, and glycine) via two successive reactions catalyzed by γ-glutamylcysteine ligase and glutathione synthetase, respectively. In mammalian cells and tissues, GSH participates in diverse reactions, including reacting with reactive oxygen and nitrogen species, electrophiles, and other non-enzymatic antioxidant compounds, as well as protein deglutathionylation. Via participating in these various redox reactions, GSH plays an important part in mammalian physiology. Extensive studies in animal models demonstrate crucial protective effects of GSH in a wide variety of disease processes involving oxidative stress. Consistently, studies in human subjects also suggest potential benefits of GSH in human diseases, including hemolytic anemia, hypertension, diabetes, and chronic obstructive pulmonary disease.

1. Overview

1.1. Definition and History

The term glutathione, if not specified, may refer to both the reduced form (GSH) and the oxidized form (GSSG) of glutathione, as well as the protein-bound glutathione moieties. The oxidized form is also known as glutathione disulfide. This chapter focuses on the reduced form of glutathione, i.e., GSH. In this context, the term glutathione is often used loosely to refer to GSH in the literature.

In 1888, M.J. de Rey Paihade discovered a substance, which was proved to be GSH by F.G. Hopkins in 1929 [1]. In 1935, C.R. Harington and T.H. Mead reported the synthesis of GSH [2]. It is now known that GSH is the most abundant non-protein thiol-containing compound in mammalian cells. Although it was initially believed to mainly act as a reductant, over the past two decades, GSH has been demonstrated to play important roles in various biological processes and disease conditions.

1.2. Basic Characteristics

GSH is a tripeptide (γ-glutamylcysteinylglycine) with a molecular mass of 307 (structure shown in Figure 5-1). In mammalian cells, the cytosolic concentrations of GSH are in the range of 1-10 mM. GSH is also present in high concentrations in mitochondria and nuclei. In contrast, the extracellular levels of GSH are much lower, and GSH levels in plasma are usually in the range of low micromolar concentrations. In mammalian cells, GSH synthesis involves two cytosolic enzymes, namely, γ-glutamylcysteine ligase (GCL) and glutathione synthetase (GSS) (Section 2.1). GCL consists of two subunits: the heavy catalytic subunit designated as GCLC with a molecular mass of ~73 kDa and the light modifier subunit designated as GCLM with a molecular mass of ~31 kDa. GCLC and GCLM are encoded by separate genes that are localized on chromosomes 6p12 and 1p22.1, respectively, in humans. Human GSS gene is localized on chromosome 20q11.2.

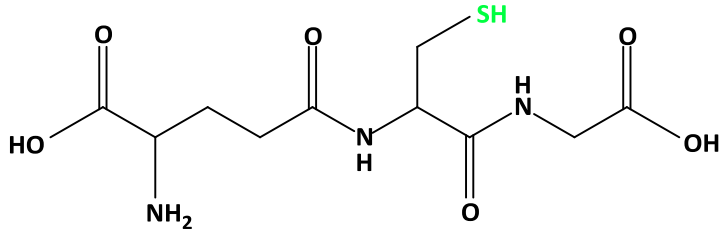

Figure 5-1. Structure of γ-glutamylcysteinylglycine (GSH). As indicated, GSH is a tripeptide, and the sulfhydryl group (-SH) is from the cysteine residue.

2. Biochemistry and Molecular Regulation

2.1. Biochemistry

GSH is synthesized from three amino acids via two successive enzymatic reactions in cytoplasm (Figure 5-2). The first step involves combination of cysteine and glutamate to produce the dipeptide γ-glutamylcysteine. The reaction is catalyzed by GCL, also known as γ-glutamylcysteine synthetase (GCS). This reaction requires coupled ATP hydrolysis. The next step involves the enzyme GSS, which catalyzes the addition of glycine to the dipeptide to form the tripeptide γ-glutamylcysteinylglycine (GSH). Similar to GCL, GSS also requires coupled ATP hydrolysis.

2.2. Molecular Regulation

γ-Glutamylcysteine ligase is the key enzyme of GSH biosynthesis. As stated above, this enzyme consists of two subunits, namely, GCLC and GCLM. As the name indicates, GCLM modulates the activity of the enzyme and affects the steady state levels of GSH in mammalian cells and tissues.

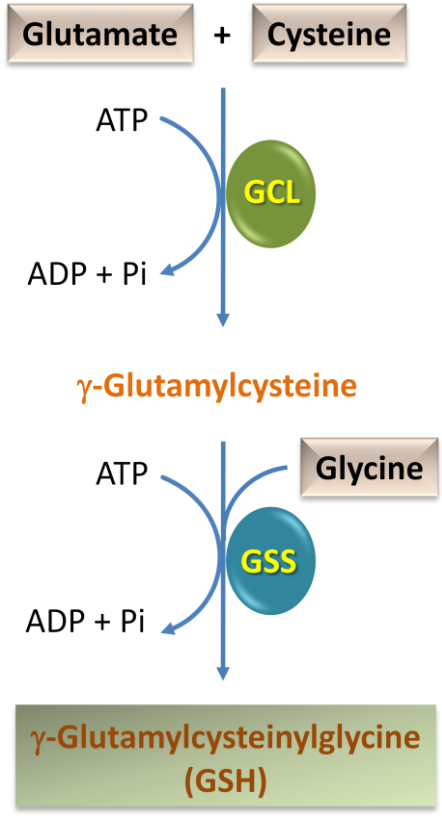

Figure 5-2. Schematic illustration of GSH biosynthesis. As depicted, GSH is synthesized from three amino acids through two successive reactions catalyzed by γ-glutamylcysteine ligase (GCL) and glutathione synthetase (GSS), respectively. Two molecules of ATP are consumed for synthesis of each molecule of GSH. It is also worth noting that GCL is the key enzyme of the GSH biosynthesis pathway with cysteine as the rate-limiting substract.

Both subunits are subject to induction by chemical inducers and oxidative stress conditions, and upregulation of either or both of the subunits leads to increased levels of cellular GSH. Transcriptional and post-transcriptional regulation of both subunits has been described [3, 4]. Post-transcriptional regulation includes mRNA stabilization/destabilization and post-translational modifications.

A number of transcription factors are involved in the regulation of GCLC and GCLM gene expression in mammalian cells. These include AP-1, AP-2, SP1, NF-κB, c-Myc, Nrf1, and Nrf2 [3]. Among these transcription factors, Nrf2 appears to play the most important role in controlling both basal and inducible expression of GCL under various conditions. Regulation of GCL expression by Nrf2 occurs through an antioxidant response element-driven mechanism (Section 3.1 of Chapter 2).

While much is known about GCL regulation, little attention has been paid to GSS. Recent studies have suggested the involvement of AP-1, NF-κB, Nrf1, and Nrf2 in the regulation of mammalian GSS gene expression in several cell types [3].

3. Role in Health and Disease

3.1. General Biological Activities

In mammalian cells and tissues, GSH participates in at least four types of biochemical reactions, as listed below. These reactions form the basis for GSH's involvement in physiology and pathophysiology.

- Reaction with reactive oxygen and nitrogen species (ROS/RNS)
- Reaction with electrophiles
- Reaction with other non-enzymatic antioxidants
- Protein deglutathionylation

3.1.1. Reaction of GSH with ROS/RNS

GSH is a major defense against ROS/RNS. GSH can directly react with ROS/RNS, leading to the detoxification of these reactive species. GSH is also used as a cofactor by glutathione peroxidase (Chapter 6) in the detoxification of hydrogen peroxide and other peroxides, as well as peroxynitrite. In addition, reaction of GSH with RNS results in the formation of S-nitrosoglutathione. S-Nitrosoglutathione acts as a second messenger to transduce nitric oxide (NO) bioactivity [5].

3.1.2. Reaction of GSH with Electrophiles

Electrophiles (electron-deficient species), such as reactive aldehydes are derived from xenobiotic biotransformation as well as lipid peroxidation. GSH reacts with electrophiles, forming less reactive conjugates. Thus, conjugation with GSH represents an important mechanism of detoxification of electrophilic species. The conjugation reactions with GSH may occur spontaneously, but are markedly accelerated by glutathione S-transferase (Chapter 8). Notably, for certain xenobiotics, GSH conjugation may result in their bioactivation [6]. The term bioactivation (also known as toxification) refers to conversion of less toxic or reactive chemicals to more toxic or reactive metabolites.

3.1.3. Reaction of GSH with Other Non-protein Antioxidants

While GSH is the most abundant non-protein thiol antioxidant in mammalian cells, there are also many other cellular non-protein antioxidants, such as α-tocopherol (Chapter 21) and ascorbate (also known as vitamin C; Chapter 20). α-Tocopherol reduces lipid peroxyl radical to form lipid hydroperoxide, and in the reaction α-tocopherol is oxidized to α-tocopherol radical. The α-tocopherol radical can be reduced back to α-tocopherol by ascorbate, which is oxidized to dehydroascorbate. Dehydroascorbate can then be restored to the reduced form by a GSH-dependent reaction catalyzed by dehydroascorbate reductase (Figure 5-3). GSH deficiency results in decreased tissue levels of vitamin C in animal models, pointing to an in vivo role of GSH in vitamin C metabolism [7].

3.1.4. Protein Deglutathionylation

Reversible protein S-glutathionylation (protein-SSG) is an important post-translational modification involved in redox signaling. Analogous to protein dephosphorylation catalyzed by phosphatases, glutaredoxin isozymes (Section 2.1 of Chapter 9) using GSH as a cofactor

catalyze deglutathionylation of proteins. This participates in regulating diverse intracellular signaling pathways.

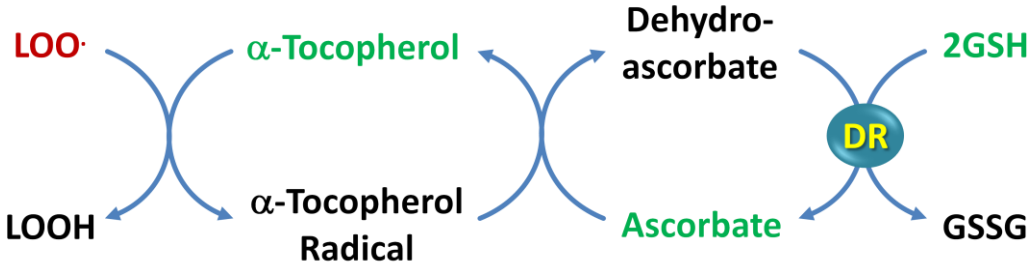

Figure 5-3. Role of GSH in the regeneration of other non-protein antioxidants, including α-tocopherol and ascorbate. See text (Section 3.1.3) for detailed description. LOO·, lipid peroxyl radical; LOOH, lipid hydroperoxide; DR, dehydroascorbate reductase; GSSG, glutathione disulfide.

3.2. Animal Studies

3.2.1. Experimental Approaches

The biological activities of GSH in mammals have been investigated following modulation of its levels in cells or tissues via various approaches. The levels of cellular or tissue GSH can be elevated by employing the following four approaches.

- Delivery of membrane permeable GSH esters (Chapter 25)
- Increase of the availability of cysteine using *N*-acetylcysteine (Chapter 25)
- Induction of GSH synthesis by chemical inducers (Chapter 2)
- Transgenic overexpression of GCL

Likewise, the levels of cellular or tissue GSH can be decreased via utilizing the following two approaches.

- Buthionine sulfoximine (BSO)-mediated inhibition of GCL activity
- Targeted disruption of GCL gene (i.e., the gene knockout model)

It is noteworthy that BSO is a selective inhibitor of GCL. It has been widely used to decrease cellular or tissue GSH so as to determine the role of GSH in various physiological and pathophysiological processes [8].

Homozygous deletion of GCLC gene ($GCLC^{-/-}$) in mice is embryonically lethal, indicating an essential role for GCLC and GSH in development. On the other hand, homozygous deletion of GCLM gene ($GCLM^{-/-}$) in mice has little effects on survival or development. $GCLM^{-/-}$ mice still have the ability to synthesize GSH in limited amounts (~10-20% of normal levels). Similarly, mice with heterozygous deletion of GCLC gene ($GCLC^{+/-}$) are viable and fertile. $GCLC^{+/-}$ mice exhibit a gene-dose dependent decrease in the GCLC protein and GCL activity, but only about a 20% diminution in GSH levels [9].

3.2.2. Role in Cardiovascular Diseases

The role of GSH and GSH synthesizing enzymes in cardiovascular diseases has been investigated by employing BSO treatment or gene knockout animal models. Multiple studies show that depletion of GSH by BSO causes severe hypertension in normal animals, suggesting a critical role for GSH in controlling oxidative stress and blood pressure regulation [10]. Chronic treatment of rats with BSO decreases plasma GSH levels, resulting in modifications of plasma low-density lipoprotein (LDL). The LDL modifications can be prevented by co-treatment of the animals with GSH ethyl ester. Chronic treatment of mice with BSO also aggravates atherosclerosis in mice lacking apolipoprotein E (ApoE). ApoE-deficient mouse is susceptible to atherosclerosis, and as such is a commonly used model for studying atherogenesis and its protection. On the other hand, it is reported that an increase in macrophage GSH content reduces cell-mediated oxidation of LDL and possibly atherosclerosis in ApoE-deficient mice [11].

Depletion of myocardial GSH by BSO results in impaired post-ischemic contractile function after global ischemia in isolated perfused hearts. Oxidation of myocardial GSH also causes mitochondrial depolarization and cardiac arrhythmias in isolated perfused hearts. Recently, it has been shown that mice lacking GCLM ($GCLM^{-/-}$) are more susceptible to in vivo myocardial ischemia-reperfusion injury compared with wild-type animals [12]. Chronic depletion of GSH by BSO also leads to oxidative cardiomyopathy and aggravation of heart failure induced by drugs, including doxorubicin and cyclophosphamide.

S-Nitrosoglutathione has also been suggested to exert protective effects in various cardiovascular disorders. A recent study shows that endogenous S-nitrosoglutathione protects against in vivo ischemic myocardial injury in mice due, at least in part, to S-nitrosylation-mediated stabilization of hypoxia-inducible factor-1α (HIF-1α) and increased angiogenesis [13]. The term angiogenesis refers to growth of new blood vessels from preexisting vessels.

3.2.3. Role in Diabetes

The involvement of GSH in diabetes has been investigated in cultured pancreatic islets and in vivo animal models of diabetes. In vitro studies demonstrate a critical activity for GSH and GCL in protecting against the pancreatic β-cell injury elicited by oxidative stress, inflammatory cytokines, as well as exposure to high concentrations of glucose [14, 15]. Notably, insulin induces GCL and GSH in cell cultures, suggesting a possible interaction between insulin and GSH homeostasis.

There is also evidence supporting a beneficial role for GSH in diabetes in vivo. Chronic depletion of GSH by BSO reduces insulin sensitivity and aggravates diabetes in experimental animals. Conversely, administration of GSH precursors, including GSH ethyl ester and *N*-acetylcysteine ameliorates the pathophysiology of diabetes in animal models [14, 15].

3.2.4. Role in Neurological Diseases

The protective effects of GSH in neurodegenerative disorders have been demonstrated by different approaches in experimental animals. These include the use of BSO to deplete tissue GSH and administration of GSH ethyl ester or *N*-acetylcysteine to augment tissue GSH levels, followed by determination of the changes in the pathophysiology of neurodegeneration. In normal animals, chronic treatment with BSO reduces GSH levels and induces oxidative damage in various tissues or organs, including brain [16]. GSH depletion also sensitizes the

brain to neurotoxin-induced degeneration, which can be protected by co-treatment with either GSH ethyl ester or *N*-acetylcysteine. GSH may also protect against convulsion, aging-associated memory impairment, and noise-induced hearing loss [17]. Another line of evidence supporting an important role for GSH in protecting against neurodegeneration is that GSH decrease usually precedes the neurodegenerative processes in various animal models. Furthermore, deficiency in the transporter for neuronal cysteine uptake in mice has been shown to cause impaired neuronal GSH metabolism, oxidative stress, and aging-dependent neurodegeneration [18].

Consistent with the observations in animal studies, extensive in vitro experiments also demonstrate a critical involvement of GSH in protecting against neuronal cell injury elicited by various neurotoxic insults. It is further shown that GSH is essential for the survival of primary neurons in cultures [19].

3.2.5. Role in Pulmonary Diseases

Reduced levels of GSH are commonly observed in animal models of pulmonary diseases. Administration of BSO to normal animals decreases GSH levels and causes oxidative damage and mitochondrial defects in lung tissue. These deleterious effects can be reversed by co-treatment of the animals with GSH ethyl ester [20], suggesting that GSH is a critical factor for normal physiology of the lung. There is ample evidence showing that GSH also acts as a protective molecule under various pulmonary pathophysiological conditions. For example, inhibition of GSH synthesis by BSO has been reported to potentiate hyperoxia-induced lung injury and mortality in experimental animals. GSH depletion by BSO leads to aggravation of lung injury induced by pulmonary toxins [21]. On the other hand, administration of GSH ethyl ester or other GSH precursors has been shown to attenuate oxidative lung injury, airway inflammation, and allergic asthma in animal models [21, 22].

3.2.6. Role in Hepatic and Gastrointestinal Diseases

Liver is one of the major pools of GSH in mammals. Animal studies along with cell culture experiments have established an important role for GSH in protecting against a number of liver disorders, including drug/xenobiotic-induced hepatotoxicity, alcoholic liver disease, liver ischemia-reperfusion injury, endotoxin-induced liver injury, and nonalcoholic fatty liver disease [23].

Acetaminophen-induced hepatotoxicity remains a major cause of acute liver failure. Studies using GCLC overexpression, GCLC knockdown, and GCLM knockout models demonstrate a crucial involvement of GSH in protecting against this drug-induced liver injury [9]. Although a moderate decrease in hepatic GSH levels caused by BSO doesn't lead to significant liver injury, it markedly sensitizes the animals to hepatotoxicity induced by various liver toxicants, including ethanol. BSO-mediated decrease in hepatic GSH levels also aggravates hepatic ischemia-reperfusion injury as well as endotoxin-induced liver injury, both of which can be ameliorated by co-treatment with GSH ethyl ester or other GSH precursors.

As stated above (Section 3.2.1), global homozygous deletion of GCLC gene is embryonically lethal. This makes it necessary to create models with organ-selective deletion of this gene. In this context, it has been recently shown that hepatocyte-specific GCLC deletion leads to a rapid onset of steatosis with mitochondrial injury and liver failure in mice [24]. This finding suggests that GSH is essential for preserving normal hepatic function and for protecting against the development of nonalcoholic fatty liver disease. In line with this notion, increases

of liver GSH by GSH precursors attenuate the progression of nonalcoholic fatty liver disease in animal models [25].

Multiple animal studies demonstrate beneficial effects of GSH in certain gastrointestinal disorders, including experimental colitis and intestinal inflammation induced by bacterial infections [26, 27]. GSH and GSH-related enzymes serve as an important line of defense against oxidative stress and inflammatory injury as well as toxic chemicals in the gut.

3.2.7. Role in Renal Diseases

In contrast to its well-established benefits in many other organs/tissues, the role of GSH in renal physiology and pathophysiology has been a subject of controversies. Early studies showed that GSH ethyl ester aggravated renal ischemia-reperfusion injury, whereas decrease of renal GSH by BSO afforded a protective effect, suggesting a possible detrimental action of GSH in renal ischemia-reperfusion injury [28]. However, administration of *N*-acetylcysteine, another GSH precursor was shown to attenuate renal ischemia-reperfusion injury [29]. It has further been reported that decrease of renal GSH by BSO leads to a concomitant induction of heme oxygenase, which may function to protect against renal ischemia-reperfusion injury [30]. As discussed in Chapter 15, heme oxygenase is an important antioxidant defense against tissue ischemia-reperfusion injury.

The role of GSH in nephrotoxicity induced by drugs and toxicants also varies with the animal models and the nephrotoxicants examined. For example, multiple studies report a potentiation effect of GSH on cisplatin-induced renal injury though other studies show the opposite. GSH mediates bioactivation of certain xenobiotics, such as polychlorinated alkenes, causing nephrotoxicity. In contrast, renal GSH is involved in protecting against kidney injury induced by various other nephrotoxicants, including heavy metals, radiographic contrast materials, and the anticancer drug ifosfamide.

3.2.8. Role in Skin Diseases

Oxidative stress and dysregulated inflammatory responses are frequently involved in skin disorders, including ultraviolet (UV) light-induced skin injury and contact dermatitis. Administration of BSO decreases GSH levels in skin, and sensitizes the animal to both UV light-induced skin injury and contact dermatitis. Conversely, treatment of the animals with GSH ethyl ester or *N*-acetylcysteine attenuates oxidative injury and inflammation in skin tissue [31]. Moreover, GSH is also found to participate in the regulation of skin wound healing in an animal model [32].

3.2.9. Role in Cancer

GSH plays diverse roles in cancer. Elevation of GSH in tumor cells is a major mechanism of anticancer drug resistance. Depletion of tumor cell GSH by BSO has been shown to potentiate the tumor killing activity of various anticancer drugs, such as cisplatin and doxorubicin. However, BSO co-treatment also potentiates the adverse effects of anticancer drugs on normal tissues, which may become a limiting factor for the combined strategy. In vitro studies suggest that high GSH levels may promote tumor cell growth and invasion.

Both ROS and electrophiles are critically involved in chemical carcinogenesis. Depletion of tissue GSH by BSO has been reported to cause DNA damage in normal experimental animals, and potentiate carcinogenesis induced by chemical carcinogens, including aflatoxin B1

[33]. Aflatoxin B1 is a potent human liver carcinogen. Likewise, elevation of tissue GSH levels by GSH precursors or chemical inducers has been demonstrated to be effective in protecting against chemical- as well as UV light-induced carcinogenesis in experimental animals. In addition to inhibiting chemical carcinogenesis, GSH also plays a protective role in spontaneous tumor development. Recently, it is reported that GSH depletion by BSO results in a 5-fold increase in the incidence of colonic tumors in p53-knockout mice, suggesting that GSH deficiency and loss of p53 function may have a synergistic effect on spontaneous colonic tumor development [34].

3.2.10. Role in Other Diseases and Conditions

In addition to the diseases described above, GSH also has a beneficial role in various other disorders and conditions, including HIV infection, scurvy, cataract, and aging in animal models. Moreover, GSH serves as a regulator of immune cell responses, which may participate in controlling viral replication [35]. Indeed, the effectiveness of the GSH precursor *N*-acetyl-cysteine in protecting against HIV infection has been demonstrated in experimental animals and suggested in certain clinical trials [36].

3.3. Human Studies and Clinical Perspectives

The well-documented biological activities of GSH in diverse animal models of human diseases have prompted translational and clinical research on GSH over the last two decades. Multiple GSH precursors or analogs have been investigated in clinical trials for the intervention of human diseases. Studies have also identified mutations and gene polymorphisms in GSH synthesizing enzymes and their potential role in disease processes. This section begins with a description of hereditary disorders resulting from gene mutations of GSH synthesizing enzymes. It then summarizes the major findings from gene polymorphism studies and interventional clinical trials on GSH precursors.

3.3.1. GCL and GSS Deficiencies due to Gene Mutations

Mutations have been identified in both GCLC and GSS genes. It has been demonstrated that missense mutations in the heavy subunit gene of GCL cause decreased GCL activity and GSH levels in erythrocytes, and the patients with this rare hereditary GCL deficiency suffer from hemolytic anemia [37]. Glutathione synthetase (GSS) deficiency is also a rare hereditary disorder with decreased GSS activity due to mutations in the corresponding gene. Patients with GSS deficiency also suffer from hemolytic anemia as well as 5-oxoprolinuria [38]. For both GCL and GSS deficiency, hemolytic anemia is believed to result from oxidative stress in red blood cells due to decreased cellular GSH levels. 5-Oxoprolinuria in GSS-deficient patients results from the accumulation of γ-glutamylcysteine and its subsequent conversion to 5-oxoproline. As shown in Figure 5-2, GSS catalyzes the reaction between γ-glutamylcysteine and glycine to form the tripeptide GSH. Hence, GSS deficiency causes accumulation of the substrate γ-glutamylcysteine.

3.3.2. Gene Polymorphisms

A variety of gene polymorphisms exist for both GCLC and GCLM. Many observational

epidemiological studies demonstrate that gene polymorphisms of GCLC or GCLM are associated with a wide variety of disease conditions, including ischemic heart disease, atherosclerosis, hypertension, diabetes, schizophrenia, chronic obstructive pulmonary disease, cystic fibrosis, nonalcoholic liver disease, and certain types of cancer [39-43]. Gene polymorphism studies may thus help identify subpopulations at high risk of developing the above disease processes, and such knowledge may serve as a guide for disease risk assessment and early intervention.

3.3.3. Interventional Clinical Trials

Decreased levels of GSH have been observed in a number of human disease conditions, including those listed above in Section 3.3.2. It is rational that repletion of GSH may thus protect against the disease processes. Multiple clinical trials have suggested a potential benefit of using GSH precursors, especially *N*-acetylcysteine in the intervention of certain human diseases, including hypertension, diabetes, chronic obstructive pulmonary disease, and cystic fibrosis [21, 44-46] (more detailed discussion in Chapter 25).

4. Conclusion and Future Directions

As the most abundant non-protein thiol in mammalian cells, GSH participates in numerous cellular processes that are critical for proper physiological functions of organs/systems. In this context, depletion of GSH in animal models aggravates a series of pathophysiological processes, which can be corrected, at least partially, by administration of GSH or GSH precursors. There are also multiple lines of evidence suggesting a beneficial role for GSH in human health and disease. Although significant progress has been made in the research area of GSH and its clinical applications, there is still much to be done to better understand how GSH synthesis and metabolism may be manipulated to therapeutic advantage. In this regard, future studies should focus on developing novel chemical agents (including both GSH enhancing and depleting agents) that can be used to selectively modulate GSH levels in target tissues to achieve therapeutic goals. It may be pertinent to suggest that therapeutic research involving GSH or its precursors must be carefully designed to take into account of the pharmacokinetic and pharmacodynamic profiles as well as the potential adverse effects of the thiol-containing compounds.

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Chapter 6

Glutathione Peroxidase

Abstract

Mammalian glutathione peroxidase (GPx) represents a family of six isozymes, namely, GPx1-6. GPxs are critical antioxidant enzymes catalyzing the decomposition of hydrogen peroxide or organic hydroperoxides. Studies in both transgenic overexpression and gene knockout animal models demonstrate an important function for GPx isozymes, especially GPx1-4 in protecting against a wide range of disease processes. Although being not as convincing as that from animal experiments, evidence from human studies also suggests a beneficial role for GPxs in certain disease conditions, particularly cardiovascular disorders.

1. Overview

1.1. Definition and History

Glutathione peroxidase (GPx) is the general name for a family of multiple isozymes that catalyze the reduction of hydrogen peroxide or organic hydroperoxides to water or corresponding alcohols using the reduced form of glutathione (GSH) as the electron donor [1, 2]. In mammalian tissues, there are six GPx isozymes, namely, GPx1, 2, 3, 4, 5, and 6. GPx1, 2, 3, and 4 are selenoproteins. GPx6 is also a selenoprotein in humans and pigs, but not in rats and mice. GPx5 is a non-selenoprotein. Here the term selenoprotein refers to any protein that contains one or more selenocysteine residues. Selenocysteine, also known as the 21st amino acid, has a structure similar to cysteine, but with an atom of selenium taking the place of the usual sulfur.

Twenty-five selenoproteins are identified in humans, among which are many antioxidant enzymes [3]. These selenium-containing antioxidant enzymes include the aforementioned GPxs (GPx1-4, and 6), thioredoxin reductases (TrxR1, 2, and TGR; Chapter 12), and methionine sulfoxide reductase B1 (MsrB1, also known as selenoprotein R; Chapter 14). The other selenoproteins include iodothyronine deiodinases (DIO1, 2, and 3), selenoprotein H (Sel H), Sel I, Sel K, Sel M, Sept15, Sel N, Sel O, Sel P, Sel S, Sel T, Sel V, Sel W, and selenophosphate synthetase 2 (SPS2) [4]. These selenoproteins have diverse biological functions. Some

of them, such as Sel P, Sel S and Sel W also possess antioxidant activities. Selenium deficiency and mutations or polymorphisms in selenoprotein genes possess a strong correlation with a variety of human diseases. These include muscle and cardiovascular disorders, immune dysfunction, cancer, neurological disorders, male infertility, and endocrine dysfunction [5]. This chapter focuses on GPx isozymes, the most extensively investigated selenoproteins in biology and medicine.

GPx activity was first reported in erythrocytes by G.C. Mills in 1957 [6]. The enzyme was found to protect hemoglobin from oxidative breakdown. This enzyme was further demonstrated to be a selenoenzyme by L. Flohe and coworkers in 1973 [7]. The enzyme was later named GPx1, and has become the most extensively studied isozyme of the GPx family. Later, several additional mammalian GPx isozymes were isolated and characterized. As stated above, in mammals the GPx family now has six members, and the classification is based on their amino acid sequence, substrate specificity, and subcellular localization.

1.2. Basic Characteristics

GPx1, 2, 3, and 6 are homotetrameric proteins with a subunit molecular mass of ~20-25 kDa, whereas GPx4 is a 20-22 kDa monomeric enzyme. GPx5 is a homodimeric enzyme with a subunit molecular mass of 24 kDa. The nomenclature and cellular and chromosomal localization of the various GPx isozymes are described below.

1) GPx1: Also known as classical or cytosolic GPx (cGPx), GPx1 is the first mammalian GPx identified and one of the most abundant and ubiquitously expressed selenoproteins. It is present in cytosol, mitochondria, and nuclei. In humans, GPx1 is localized on chromosome 3p21.3.
2) GPx2: Also known as gastrointestinal GPx (GI-GPx), GPx2 is mainly expressed in gastrointestinal tract. It is also found in liver and lung. GPx2 is present in cytosol and nuclei. In humans, GPx2 is localized on chromosome 14q24.1.
3) GPx3: Also known as plasma GPx (pGPx), GPx3 is a secreted form of enzyme found in plasma. Kidney is the major source of GPx3 in plasma. This enzyme is also expressed in many other tissues, including lung and heart, and is present in cytosol. In humans, GPx3 is localized on chromosome 5q23.
4) GPx4: Also known as phospholipid hydroperoxide GPx (PHGPx), GPx4 enzyme is ubiquitously expressed in a variety of tissues. GPx4 is also a main structural component of the sperm mitochondrial capsule in mature spermatozoa, where it exists as an enzymatically-inactive, oxidatively cross-linked, insoluble protein [8]. Subcellular distribution of GPx4 includes cytosol, nuclei, mitochondria, and membranes. In humans, GPx4 is localized on chromosome 19p13.3.
5) GPx5: Also known as epididymal GPx (eGPx), this GPx isozyme is selectively expressed in epididymis. It is secreted into the epididymal lumen. In humans, GPx5 is localized on chromosome 6p22.1.
6) GPx6: GPx6 is a recently identified isozyme. The expression of GPx6 is believed to be restricted to the developing embryo and in olfactory epithelium in adults, suggesting a possible role in embryonic development and olfactory function. In humans, GPx6 is localized on chromosome 6p22.1.

2. Biochemistry and Molecular Regulation

2.1. Biochemistry

All of the GPx isozymes are able to catalyze the reduction of hydrogen peroxide (H_2O_2) or organic hydroperoxides (LOOH) to water or corresponding alcohols (LOH) using GSH as the electron donor (Figure 6-1). GPx4 is also able to reduce phospholipid hydroperoxides in membranes, and as such is named phospholipid hydroperoxide GPx (PHGPx). As a main structural component of the sperm mitochondrial capsule in mature spermatozoa, GPx4 is also involved in sperm maturation and male fertility [9]. Due to the low levels of GSH in extracellular fluid, GPx3 may also use extracellular thioredoxin and glutaredoxin as electron donors. In addition to decomposing H_2O_2 and LOOH, GPx isozymes can also reduce peroxynitrite in vitro [10].

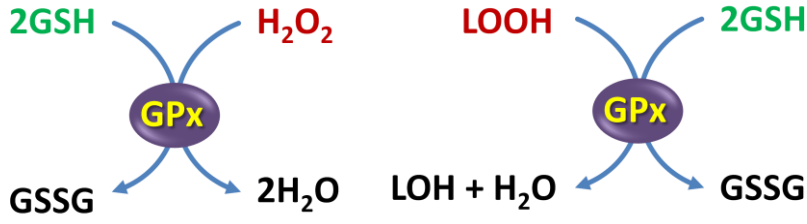

Figure 6-1. Glutathione peroxidase (GPx)-catalyzed decomposition of peroxides. As illustrated, GPx catalyzes decomposition of hydrogen peroxide (H_2O_2) or organic hydroperoxides (LOOH) to form water or the corresponding alcohols (LOH). The reactions require GSH as the electron donor. GSSG stands for glutathione disulfide.

2.2. Molecular Regulation

Although the first member of GPx family was identified more than fifty years ago, regulation of gene expression for many of the GPx isozymes is not well-understood. Regulation of GPxs may occur at both transcriptional and post-transcriptional levels [11]. It has been reported that p53 is a critical transcription factor involved in the modulation of GPx1 gene expression. Indeed, a p53-binding site is mapped to the promoter region of GPx1 gene. GPx1 is also subject to post-translational modifications by c-Abl and Arg tyrosine kinases [12]. Multiple transcription factors participate in controlling GPx2 gene expression. These include Nrf2, β-catenin/TCF, and p63 (a member of the p53 tumor suppressor family) [11, 13]. The promoter region of GPx3 has been characterized, and various transcription factors, including AP-1, SP1, and HIF-1 have been implicated in the transcriptional regulation [14]. The transcriptional regulation of GPx4 gene has recently been extensively investigated. A range of transcription factors are shown to participate in regulating GPx4 gene in cellular systems. These factors include C/EBP-epsilon, NF-κB, SP1, SP3, NF-Y, and Smad [11, 15]. In contrast to the above four isozymes of GPx family, studies on gene regulation of the recently identified members GPx5 and GPx6 are scarce.

3. Role in Health and Disease

3.1. Animal Studies

3.1.1. Experimental Approaches

The biological activities of GPxs have been studied in numerous animal models over the past several decades. Gene knockout or transgenic overexpession studies of the individual GPx isozymes provide the most compelling data on their role in health and disease. Transgenic overexpression of GPx1-5 has been created in animal models. Knockout mouse models for GPx1-5 have also been reported in the literature. Notably, homozygous deletion of GPx4 is embryonically lethal [16]. Primarily based on studies with these gene knockout and transgenic overexpression models, GPx isozymes are found to play important roles in various physiological and pathophysiological processes. In addition to the genetic models, the GPx mimetic ebselen has also been used for disease intervention in both animal models and clinical trials (Chapter 24).

3.1.2. Role in Cardiovascular Diseases

Cardiovascular diseases are among the most extensively studied disease conditions with respect to the role of GPxs in animal models of human diseases. Among the various GPx isozymes, GPx1 has received the most attention. Both gene overexpression and knockout studies have demonstrated a critical protective role for GPx1 in hypertension, myocardial ischemia-reperfusion injury, atherosclerosis, and heart failure [17-19]. It has been also found that GPx1 overexpression ameliorates virus-induced myocarditis [20]. In addition to GPx1, there are reports showing beneficial effects of GPx4 in atherosclerosis and myocardial ischemia-reperfusion injury in animal models [21, 22].

3.1.3. Role in Diabetes

In vitro studies have conclusively demonstrated a role for GPxs in protecting the pancreatic β-cells from glucose toxicity and oxidative stress. However, the involvement of GPx1 in diabetes in animal models has been a subject of controversies. For example, one study reports that mice with global transgenic overexpression of GPx1 spontaneously develop insulin resistance and obesity [23]. In contrast, another study shows that the pancreatic β-cell-selective overexpression of GPx1 reverses diabetes in db/db mice [24]. Similarly, GPx3 overexpression in adipocytes results in improvement of high glucose-induced insulin resistance and attenuation of proinflammatory gene expression, whereas GPx3 neutralization in adipocytes promotes expression of proinflammatory genes [25]. In addition, reduced plasma levels of GPx3 are found in diabetic mice, further suggesting a protective role for this GPx isozyme in experimental diabetes.

3.1.4. Role in Neurological Diseases

Studies using both transgenic overexpression and gene knockout animal models convincingly show GPx1 as an important cellular factor in protecting against various neurological disorders, including Parkinson's disease, Alzheimer's disease, and cerebral ischemia-reperfusion injury [26-28]. Recently, the role of GPx4 in neuroprotection has also been investigated. As stated above in Section 3.1.1, GPx4 is essential for embryonic development as

GPx4 knockout ($GPx4^{-/-}$) mice die in utero by midgestation. Heterozygous ($GPx4^{+/-}$) mice are born and able to reproduce. However, these mice spontaneously develop neurodegeneration, suggesting a critical involvement of GPx4 in normal physiology of the brain. Brains of $GPx4^{+/-}$ mice are found to have increased lipid peroxidation, which leads to increased amyloidogenesis in experimental Alzheimer's disease [29]. Selective depletion of GPx4 in hippocampus is also shown to result in neurodegeneration, and GPx4 is directly involved in proapoptotic signaling, leading to neuron death [30]. In vitro studies demonstrate that the neuroprotective effects of GPx4 result from its function in removal of lipid hydroperoxides [31]. In this context, GPx4 is associated with cell membrane and acts a major defense against membrane lipid peroxidation. Collectively, these findings support GPx4 acting as an important protective molecule in neurodegenerative diseases.

3.1.5. Role in Pulmonary Diseases

GPx1-deficient mice develop normally and show no increased sensitivity to hyperoxia-induced lung injury [32]. However, as compared with wild-type mice, GPx1-deficient mice are more susceptible to lung injury and mortality induced by lethal amounts of paraquat, a redox-cycling compound [33]. GPx2 in lung tissue is induced by exposure to cigarette smoke, and the induction occurs via an Nrf2-dependent mechanism [34]. The Nrf2-dependent upregulation of GPx2 may be a potential mechanism against cigarette smoke-induced lung injury. Targeted disruption of GPx2 gene in mice sensitizes the animals to allergen-induced airway inflammation, suggesting that GPx2 may also be an important defense against inflammation in the lung [35]. GPx3 is found to be induced in asthmatic lungs via a redox-regulated mechanism; however, the exact role of this isozyme in protecting against oxidative and inflammatory lung injury remains unclear [36].

3.1.6. Role in Hepatic and Gastrointestinal Diseases

GPx1 has been shown to protect liver from oxidative and inflammatory injury induced by diaquat, ethanol, and endotoxin in animal models [37-39]. However, knockout of GPx1 in mice renders the animals more resistant to acetaminophen-induced hepatotoxicity, and GPx1 overexpression sensitizes the animals to this drug-induced liver injury [40, 41]. As noted earlier, GPx1 is an intracellularly expressed protein. In contrast, either overexpression of the plasma GPx3 or intravenous administration of GPx protein is shown to protect against acetaminophen liver toxicity in mice [41]. Under either condition, the GPx protein is mainly present in extracellular fluids. It remains unclear why intracellular overexpression of GPx1 increases acetaminophen-induced liver injury in animal models. It is suggested that this sensitivity may be due to the inability of the GPx1-overexpressing mice to efficiently recover GSH depletion as a result of acetaminophen metabolism. Transgenic overexpression of GPx4 in mice is shown to protect against diaquat-induced lipid peroxidation and apoptosis in liver tissue [42].

Multiple studies demonstrate that GPx isozymes, particularly GPx2 play an important part in protecting against gastrointestinal inflammation. Mice with combined disruption of GPx1 and GPx2 have been shown to spontaneously develop chronic colitis, with symptoms and pathological alterations consistent with inflammatory bowel disease [43]. This finding suggests GPx2 as an integral part of gut homeostasis. As aforementioned, GPx2 is mainly expressed in gastrointestinal tract, and thus it may also serve as an important line of antioxidant defense against peroxides either generated in the gut or introduced via diet.

3.1.7. Role in Renal Diseases

GPx3 is secreted primarily by renal tubular cells. Recently, GPx3 is found to bind to the basal membranes of mouse renal cortex tubular cells [44]. It is further shown that GPx3 overexpression protects against renal ischemia-reperfusion-elicited inflammatory response and kidney injury in mice [45]. Mice overexpressing GPx1 are also more resistant than wild-type littermates to renal ischemia-reperfusion injury [45]. However, GPx1 knockout in mice does not sensitize the animals to streptozotocin-induced diabetic nephropathy [46]. Streptozotocin is a compound that causes cytotoxicity to the pancreatic β-cells, leading to subsequent development of type 1 diabetes in experimental animals.

3.1.8. Role in Skin Diseases

GPx4 exhibits high specific activity in reducing lipid hydroperoxides, and thus may play a critical role in protecting skin from ultraviolet (UV) irradiation-triggered long-term effects like premature skin aging and cancer. Indeed, overexpression of GPx4 in human dermal fibroblasts suppresses UV irradiation-induced lipid peroxidation, inflammation, and activation of interstitial collagenase [47]. Skin cancer cells are shown to have a reduced GPx activity and an elevated peroxidative burden. In vivo knockout of GPx2 predisposes the mice to UV irradiation-induced formation of squamous cell carcinoma [48]. In addition to skin cancer, GPx isozymes also play a critical role in protecting against other types of cancer (see Section 3.1.9 below).

3.1.9. Role in Cancer

ROS, including hydroperoxides are implicated in multistage carcinogenesis. Thus, by decomposition of hydroperoxides, GPxs may function to protect against spontaneous cancer development [49]. In cell cultures, overexpression of GPxs inhibits tumor cell growth and invasiveness, whereas inactivation of GPxs results in increased oxidative DNA damage. Double-knockout of GPx1 and GPx2 causes intestinal inflammation and an increased incidence of intestinal cancer in mice [50]. Deficiency in selenoproteins, including GPxs accelerates prostate carcinogenesis in a transgenic mouse model [51]. Conversely, overexpression of GPx3 in prostate cancer cells is found to suppress the cancer cell growth and metastasis in a xenograft animal model [52]. Together, these observations support a protective role for GPxs in cancer development.

3.1.10. Role in Other Diseases and Conditions

GPxs are implicated in various other physiological and pathophysiological processes, including reproduction, eye disorders, immune regulation, and sepsis. As stated earlier in Section 1.2, GPx4 is not only an antioxidant enzyme, but also a structural protein in spermatozoa. Depletion of GPx4 in spermatocytes causes male infertility in mice [9]. GPx5 is a non-selenoprotein that is selectively expressed in epididymidis, and secreted in significant amounts into the epididymal lumen. This enzyme is suggested to act as an important antioxidant defense in the epididymal lumen to protect spermatozoa from oxidative stress [53]. GPxs may also be an important antioxidant defense of lens in vivo. GPx1 deficiency leads to increased cataract in mice [54]. Recently, it is reported that increased expression of GPx4 in photoreceptors of mice strongly protects retina from oxidative damage [55]. Transgenic mice overexpressing GPx1 and GPx3 are more resistant than wild-type counterparts to endotoxem-

ic conditions, as evidenced by a decreased inflammation and hypotension, and an increased survival [56].

3.2. Human Studies and Clinical Perspectives

The potential involvement of GPxs in human health and disease has been investigated over the past two decades primarily by two approaches: (1) detection of the changes of GPx isozymes in human tissues under various disease conditions and (2) observational epidemiological studies on the association between GPx gene polymorphisms and altered risk of disease development. While these studies in human subjects provide important information on GPx isozymes in human health and disease, it should be borne in mind that neither approach is able to establish a causative role for GPxs in a particular disease condition.

In line with the well-established role for GPx1 in protecting against cardiovascular disorders in animal models, a low activity of red blood cell GPx1 has been demonstrated to be independently associated with an elevated risk of cardiovascular events in humans [57]. Gene polymorphisms have been identified in human GPx1, and individuals possessing one or two ALA6 alleles have been shown to be at a modest increased risk of coronary artery disease [58]. The presence of Pro197Leu substitution of the GPx1 gene may also play a crucial role in determining the genetic susceptibility to coronary atherosclerosis in type 2 diabetes [59]. Recently, multiple studies have shown that the promoter polymorphisms in GPx3 gene are associated with an increased risk of arterial ischemic stroke as well as cerebral venous thrombosis [60, 61], though one study has reported no association of GPx3 gene with cerebral venous thrombosis in a German population [62]. In addition to cardiovascular diseases, several population studies also demonstrate an association between GPx gene polymorphisms and increased risk of developing various types of cancer, including breast cancer, prostate cancer, and colorectal carcinoma [63-66].

4. Conclusion and Future Directions

As a major defense in the detoxification of peroxides, mammalian GPxs play a critical role in protecting against various pathophysiological conditions that involve oxidative stress and inflammation. Both gene knockout and overexpression animal models have contributed greatly to our current understanding of the biological activities of various GPx isozymes in mammals. Such knowledge has facilitated the studies on the role of GPxs in human health and disease. In this context, multiple observational epidemiological studies in human subjects suggest a potential beneficial function for GPxs in certain human disorders, especially cardiovascular diseases. Future studies should focus on investigating the involvement of GPxs in other human disease conditions, and emphasize both basic and translational research on the newly identified GPx isozymes, GPx5 and 6. Such studies will provide novel insights into the complementary actions of various GPx isozymes in disease protection. Future research should also be aimed at the development of pharmacological approaches to augmenting tissue GPx expression and the potential applications of GPx manipulation in the prevention and treatment of common human disorders.

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Chapter 7

Glutathione Reductase

Abstract

Glutathione reductase (GR) catalyzes the formation of GSH from glutathione disulfide (GSSG) at the expense of NADPH. As part of the glutathione redox cycle, GR plays an important role in the detoxification of reactive oxygen and nitrogen species and other reactive intermediates. In addition, by maintaining the high GSH/GSSG ratios, GR is also involved in regulating intracellular redox homeostasis. Studies using GR-overexpressing or deficient animal models demonstrate a role for GR in protecting against certain pathophysiological conditions involving oxidative stress. In humans, hereditary GR deficiency has been reported, and the clinical manifestations include shortened lifespan of red blood cells, cataract, and favism. The exact role of GR in common disease conditions in both experimental animals and humans warrants further investigations.

1. Overview

As described in Chapter 6, upon reaction with reactive oxygen and nitrogen species (ROS/RNS), GSH is oxidized to GSSG (also known as glutathione disulfide). In mammalian cells, the ratios of intracellular GSH to GSSG are high usually in the range of 10:1 to 100:1. Maintenance of such high ratios of intracellular GSH to GSSG is critical for normal cellular activities, including redox signaling. Glutathione reductase (GR) reduces GSSG to GSH, and is crucial for maintaining the high ratios of intracellular GSH to GSSG [1].

Mammalian GR is a homodimeric flavoprotein with a molecular mass of 104 kDa. It is widely expressed in various tissues. GR activity is present in both cytosol and mitochondria. The cytosolic and mitochondrial isozymes of GR are biochemically indistinguishable and encoded by a single gene. In humans, GR gene is localized on chromosome 8p21.1.

2. Biochemistry and Molecular Regulation

GR catalyzes the reduction of one molecule of GSSG to form two molecules of GSH using NADPH as the reducing cofactor (Figure 7-1).

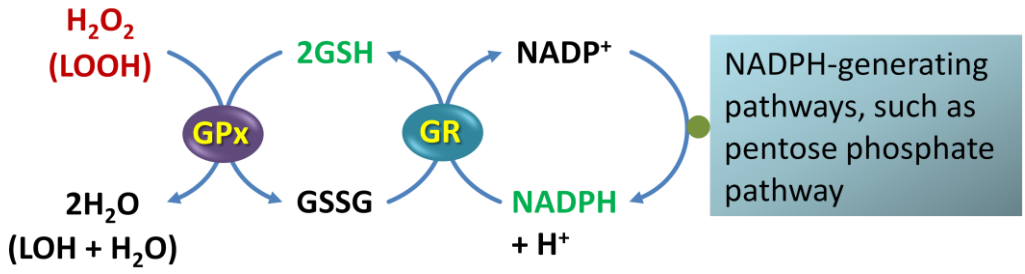

Figure 7-1. Glutathione reductase (GR)-catalyzed regeneration of GSH from glutathione disulfide (GSSG). As illustrated, GR catalyzes the reduction of GSSG to GSH using NADPH as the electron donor. NADPH is primarily provided by the pentose phosphate pathway with glucose-6-phosphate dehydrogenase as a key enzyme. Deficiency of glucose-6-phosphate dehydrogenase causes decreased formation of NADPH, compromising GR-catalyzed regeneration of GSH. This may eventually lead to decreased levels of GSH in cells and increased cellular susceptibility to oxidative stress. Deficiency of glucose-6-phosphate dehydrogenase has been identified in humans.

As stated above, mammalian GR is a homodimer. The binding sites for NADPH and GSSG are at the opposite sides of each subunit of the dimer, and FAD is bound to the center of each subunit. NADPH reduces the FAD, which then passes its electrons onto a disulfide bridge in the active site of the enzyme. The two sulfhydryl groups so formed then interact with GSSG and reduce it to two molecules of GSH, and the protein disulfide of the enzyme is reformed [1, 2].

The cloning and sequencing of mammalian GR were reported in 1990 [3]. However, the molecular regulation of mammalian GR expression is poorly understood. Studies suggest a possible role for Nrf2 signaling in the regulation of the constitutive expression of GR in mammalian cells, including cardiomyocytes, macrophages, and bone marrow stromal cells [4-6]. In yeast, GR is regulated by the transcription factor yAP-1 [7]. In both cultured cells and in vivo animal models, GR is induced by various chemical agents, including dithiolethiones. Induction of GR by dithiolethiones occurs via an Nrf2-dependent mechanism. Increased expression of GR is also reported following exposure of cells to oxidative stress conditions.

3. Role in Health and Disease

3.1. Animal Studies

3.1.1. Experimental Approaches

There are multiple studies on the biological activities of GR in animal models. A GR mutant mouse strain ($Gr1^{a1Neu}$) with decreased tissue GR activities has been reported and used to investigate the effects of GR deficiency on oxidative tissue injury [8, 9]. Transgenic overexpression of GR has been created in *Drosophila melanogaster* [10]. However, to date, transgenic overexpression and gene knockout mouse models of GR have not been reported in the literature. Inhibition of GR activity by pharmacological inhibitors, especially *N*,*N*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) has been frequently employed to study the biological

functions of this antioxidant enzyme in both cellular systems and experimental animals. BCNU is an alkylating anticancer agent that may exert many other cellular effects in addition to inhibition of GR. Recently, a selective irreversible inhibitor of GR, namely, 2-acetylamino-3-[4-(2-acetylamino-2 carboxyethylsulfanylthiocarbonylamino)phenylthiocarbamoylsulfanyl] propionic acid (2-AAPA) has been reported [11]. At concentrations that markedly inhibit GR activity, 2-AAPA has no effects on γ-glutamylcysteine ligase, glutathione synthetase, superoxide dismutase, and catalase, but shows minimal inhibition of glutathione S-transferase and glutathione peroxidase [11].

3.1.2. Role in Disease Conditions

By maintaining the high ratios of GSH to GSSG, GR plays an important part in the detoxification of ROS/RNS as well as the maintenance of cellular redox homeostasis. GR-deficient mice ($Gr1^{a1Neu}$) are shown to be more susceptible to diaquat-induced oxidative stress and renal proximal tubular injury [12]. The GR-deficient mice are also more sensitive to hyperoxia-induced lung injury after the thioredoxin reductase is inhibited pharmacologically [13]. Similarly, inhibition of GR activity in animals or cultured cells by chemical inhibitors or siRNA (small interfering RNA used for knockdown of gene expression) results in increased sensitivity to oxidative injury. On the other hand, transgenic overexpression of GR in *Drosophila melanogaster* extends survival under hyperoxic conditions [10]. Viral-vector mediated increased expression of GR in macrophages attenuates atherosclerotic lesion formation in low-density lipoprotein (LDL) receptor-deficient mice [14]. In vitro, overexpression of GR protects macrophages from oxidized LDL (oxLDL)-induced cytotoxicity and mitochondrial damage [15]. Although GR is found to protect against oxidative injury in various cellular and animal models, selective expression of GR in type II cells has been shown to provide no benefits on hyperoxic lung injury in mice [16]. It has also been reported that local expression of GR gene in mouse brain is causally associated with anxiety-like behavioral phenotypes [17]; however, the significance of this finding remains unclear.

3.2. Human Studies and Clinical Perspectives

Hereditary GR deficiency has been documented in humans though it is a rare disorder. The first familial deficiency of GR in human red blood cells was reported in 1976 [18]. The GR activity could not be restored by in vivo riboflavin administration or in vitro FAD addition, suggesting a mutation in the gene encoding GR. Recently, a nonsense and a missense mutations of the GR gene have been identified, which cause clinical GR deficiency in patients [19]. The clinical symptoms of GR deficiency include reduced lifespan of red blood cells, cataract, and favism. Favism refers to acute hemolytic anemia caused by ingestion of fava beans, which contain compounds that are capable of inducing oxidative stress in cells, including red blood cells. A recent observational epidemiological study suggested a correlation between GR activity and a series of cardiovascular risk factors, including systolic arterial pressure, total cholesterol levels, and LDL cholesterol levels [20]. GR may also play a role in the pathogenesis of malaria. In this regard, it has been recently reported that GR deficiency and drug-induced GR inhibition may protect against malaria by inducing enhanced ring stage phagocytosis [21]. Thus, in humans GR may play a protective or detrimental role depending on the disease conditions.

4. Conclusion and Future Directions

In mammalian cells, GR is a major enzyme for maintaining the high ratios of GSH to GSSG. In contrast to other GSH-related enzymes, such as glutathione peroxidase (Chapter 6) and glutathione S-transferase (Chapter 8), studies on the biological activities of GR in mammalian models using gene knockout or transgenic overexpression approaches have been scarce. Extensive cell culture studies and several animal experiments demonstrate a role for GR in protecting against oxidative injury. Hereditary GR deficiency due to gene mutations has been reported in humans. However, the role of GR in common human diseases, such as cardiovascular disorders and diabetes warrants further investigations. In this context, continued basic research on GR in animal models of human diseases will provide important insights into the biological significance of this antioxidant enzyme in health and disease, and lay a foundation for translational and clinical research.

5. References

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Chapter 8

Glutathione S-Transferase

Abstract

Glutathione S-transferase (GST) refers to a superfamily of enzymes that catalyze the conjugation of GSH with a wide variety of xenobiotics. GSTs also play an important role in cell signal transduction and redox regulation. GSTs are major cellular defenses against oxidative and electrophilic stress, and protect against many disease processes in experimental animals. Deficiency of GSTM1 and GSTT1 due to homozygous gene deletions has been identified in human populations. These two null genotypes as well as gene polymorphisms in other members of the GST superfamily are associated with increased risk of developing various diseases, including cardiovascular disorders, diabetes, neurodegeneration, and cancer.

1. Overview

1.1. Definition and History

Glutathione S-transferase (GST), which is now formally called glutathione transferase, is a general name for a superfamily of enzymes that catalyze the conjugation of GSH with a wide variety of xenobiotics. These enzymes play an important role in protecting mammalian cells and tissues from electrophilic and oxidative stress. Some GST isozymes also regulate a number of cellular processes via non-enzymatic reactions [1, 2]. As listed below, this superfamily of enzymes consists of three main families, which are widely distributed in mammalian tissues.

- Cytosolic GSTs
- Mitochondrial GSTs
- Microsomal GSTs, also known as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG)

Among the three main families, cytosolic GSTs are the most extensively studied enzymes involved in the detoxification of electrophilic xenobiotics and reactive oxygen species (ROS)

in mammalian systems [3].

As stated in Section 1.1 of Chapter 5, M.J. de Rey Paihade in 1888 discovered a substance, which was proved to be GSH by F.G. Hopkins in 1929. In 1959, the role of GSH in the formation of mercapturic acid from bromobenzene in experimental animals was suggested in studies by H.G. Bray and coworkers [4]. The enzymatic catalysis of GSH conjugation reactions with xenobiotics was subsequently reported by J. Booth and coworkers in 1961 [5]. More than a decade later in 1974, GSTs, the enzymes responsible for catalyzing the conjugation reactions were eventually purified and characterized by W.B. Habig and coworkers as the first enzymatic step leading to the formation of mercapturic acid [6]. The GSH-dependent conjugation reactions have been widely used to determine GST activity in biological systems, including cultured cells and animal tissues.

1.2. Basic Characteristics

1) Cytosolic GSTs: Mammalian cytosolic GSTs are all dimeric with a molecular mass of ~50 kDa. They are classified into seven classes, namely, alpha (A), mu (M), pi (P), sigma (S), theta (T), omega (O), and zeta (Z). Table 8-1 lists the human and mouse cytosolic GST genes.

Table 8-1. Human and mouse cytosolic glutathione S-transferase genes

Class (human)	Gene (human)	Class (mouse)	Gene (mouse)
Alpha (A)	GSTA1 GSTA2 GSTA3 GSTA4 GSTA5	Alpha (A)	Gsta1 Gsta2 Gsta3 Gsta4 Gsta5
Mu (M)	GSTM1 GSTM2 GSTM3 GSTM4 GSTM5	Mu (M)	Gstm1 Gstm2 Gstm3 Gstm4 Gstm5 Gstm6 Gstm7
Pi (P)	GSTP1	Pi (P)	Gstp1 Gstp2
Sigma (S)	GSTS1	Sigma (S)	Gsts1
Theta (T)	GSTT1 GSTT2	Theta (T)	Gstt1 Gstt2 Gstt3
Omega (O)	GSTO1	Omega (O)	Gsto1 Gsto2
Zeta (Z)	GSTZ1	Zeta (Z)	Gstz1

2) Mitochondrial GSTs: Mammalian mitochondrial class kappa GST isozymes are dimeric. Mouse, rat, and human possess only a single kappa GST (GSTK). This enzyme catalyzes GSH conjugation reactions with certain xenobiotics.
3) Microsomal GSTs: Most of the mammalian MAPEG enzymes are involved in the production of eicosanoids. Six human MAPEG isozymes have been identified. Some of the isozymes are involved in the detoxification of electrophilic xenobiotics via GSH conjugation.

2. Biochemistry and Molecular Regulation

2.1. Biochemistry

Cytosolic GSTs are among the most extensively studied enzymes involved in the detoxification of xenobiotics and ROS. As illustrated in Figure 8-1, these enzymes catalyze the conjugation reactions of GSH with electrophilic xenobiotics, including reactive aldehydes and quinone compounds to form less reactive conjugates (xenobiotic-GS). Some GST isozymes also exhibit glutathione peroxidase activity, catalyzing reduction of organic hydroperoxides (LOOH) to form alcohols (LOH) [1].

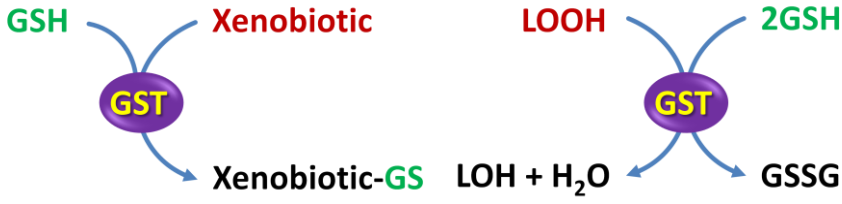

Figure 8-1. Glutathione S-transferase (GST)-catalyzed biochemical reactions**.** As illustrated, GST-catalyzed conjugation reactions between electrophilic xenobiotics and GSH form less reactive xenobiotic-GSH conjugates (Xenobiotic-GS). GST-catalyzed decomposition of organic hydroperoxides (LOOH) results in the formation of the corresponding alcohols (LOH). In this reaction, GSH is oxidized to glutathione disulfide (GSSG).

2.2. Molecular Regulation

The molecular regulation of GST expression has been a subject of extensive studies during the past decades. GSTs are subject to both transcriptional and post-transcriptional regulation. They are inducible by a wide variety of conditions/factors, including oxidative stress, electrophilic xenobiotics, and chemoprotective agents (Section 3 of Chapter 2). A range of transcription factors have been identified to participate in regulating GST gene expression. Among them are AP-1, NF-κB, AhR, and Nrf2. The importance of each of these transcription factors in modulating GST gene expression varies with the GST isozymes and the types of cells or tissues examined. Among the various transcription factors, Nrf2 appears to play the most important role in regulating many members of GSTs via an antioxidant response ele-

ment (ARE)-driven mechanism. Indeed, ARE is identified in the promoter region of the genes for many GST isozymes [1, 7].

3. Role in Health and Disease

3.1. General Biological Activities

The biological activities of mammalian GST isozymes can be summarized into the following three categories. These biological activities are the basis for GST's involvement in health and disease.

- Protection against cytotoxicity of electrophilic xenobiotics and ROS via enzymatic reactions
- Protection against carcinogenesis
- Regulation of cell signaling

3.1.1. Protection against Cytotoxicity of Electrophiles and ROS via Enzymatic Reactions

GSTs play a central role in phase 2 biotransformation, in which they catalyze the GSH conjugation reactions with various substrates containing electrophilic moieties. The resulting more water-soluble conjugates are excreted via the multidrug resistant protein efflux pumps or undergo further metabolism to mercapturic acids. Substrates of GST isozymes include a wide range of endogenous metabolites, environmental xenobiotics, as well as alkylating and free radical-generating anticancer drugs. Some GST isozymes also function as glutathione peroxidases, catalyzing the reduction of organic hydroperoxides, including those derived from lipid peroxidation. In this regard, certain GST isozymes are reported to protect against ROS-induced injury [8]. Thus, GSTs play an important role in the detoxification of both electrophilic species and ROS. It should be noted that although GST-catalyzed glutathione conjugation is generally regarded as a detoxification mechanism, GSH conjugation of certain xenobiotics, such as halogen-containing compounds may lead to their bioactivation, resulting in increased toxicity [9].

3.1.2. Protection against Carcinogenesis

Many carcinogens are electrophilic compounds and also substrates for GSTs. Thus, GST-catalyzed GSH conjugation of carcinogenic chemicals or their reactive metabolites represents an important detoxification mechanism for chemical carcinogens. As discussed below, GSTs, especially GSTP may also regulate cell signaling, and as such play a protective role in the development of spontaneous tumors.

3.1.3. Regulation of Cell Signaling

In addition to the enzymatic activities, some GST isozymes, particularly GSTP also possess non-enzymatic functions, regulating a number of cellular processes (e.g., kinase cascades) that are involved in the intrinsic ability of mammalian cells to survive genotoxic, metabolic, and oxidative stress [10]. These non-enzymatic reactions may contribute to the

protective activities of GST isozymes in disease conditions, especially cancer development. Moreover, recent studies suggest that GSTs may participate in S-glutathionylation [11]. As noted earlier (Section 3.1.4 of Chapter 5), S-glutathionylation is an important mechanism of post-translational regulation involved in cell redox signaling.

3.2. Animal Studies

3.2.1. Experimental Approaches

Animal studies provide much of our current knowledge on the role of GSTs in health and disease. The major experimental approaches in animal models include the following.

- Gene knockout of the individual GSTs
- Transgenic overexpression of the individual GSTs
- Pharmacological induction or inhibition of endogenous GST activity

Both transgenic and gene knockout animal models generate the most convincing evidence on the biological activities of the individual GSTs. On the other hand, although pharmacological approaches also provide important information, the specificity issues associated with the approaches deserve caution when drawing a conclusion on the role of a particular GST in biological systems. In this context, sulfasalazine has been used as a chemical inhibitor of general GST activity, whereas dichloroacetic acid has been reported to deplete GSTZ in experimental animals [12].

3.2.2. Role in Cardiovascular Diseases

Both oxidative and electrophilic stress contribute to the development of cardiovascular disorders, including atherosclerosis, hypertension, myocardial ischemia-reperfusion injury, and xenobiotic/drug-induced cardiotoxicity. By detoxifying oxidative and electrophilic species, GSTs may play a protective role in the above disease processes. In addition, the role of GSTs in redox signaling may also contribute to their beneficial effects in cardiovascular system. In cultured cells, overexpression of GSTs protects cardiovascular cells from oxidative and electrophilic injury. GSTs also regulate vascular smooth muscle cell proliferation and migration, both of which are critical events in atherogenesis [13]. Viral vector-mediated GST overexpression is shown to mitigate transplant atherosclerosis and inflammatory responses in rabbit carotid allografts [14]. Studies using spontaneous hypertension rats also suggest a possible protective role for GSTM1 in vascular oxidative stress [15]. Although the role of GSTs in myocardial ischemia-reperfusion injury has not been reported, GSTs are found to detoxify aldehydes generated during myocardial ischemia-reperfusion in animal models [16]. Aldehydes have been shown to participate in the pathogenesis of myocardial ischemia-reperfusion injury [17].

3.2.3. Role in Neurological Diseases

GSTs are detoxification enzymes that can inhibit oxidative and chemical stress underlying neurodegeneration. In this regard, dopamine-derived quinone metabolites as well as environmental chemicals are implicated in neurodegenerative disorders, especially Parkinson's

disease. In vitro studies demonstrate a critical role for GSTs in protecting against dopamine-quinone toxicity. In line with the in vitro studies, it has been reported that increased GST activity rescues dopaminergic neuron loss in a Drosophila model of Parkinson's disease [18]. In a mouse model, it has been demonstrated that GSTP is the only GST family member expressed in substantia nigra neurons, and depletion of this GST isozyme sensitizes the animals to experimental parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [19]. MPTP is known to cause parkinsonism in human subjects, and has been widely used as a model chemical for inducing parkinsonism in experimental animals.

3.2.4. Role in Pulmonary Diseases

Lung is a major target organ of environmental pollutants, including both electrophilic and oxidative species, and GSTs serve as a principal defense against these noxious species. In vitro, GSTs play a critical part in protecting lung cells from injury elicited by oxidants and other reactive species, including tobacco smoke. In experimental animals, inhalation of either GST or GSH ameliorates oxidative stress and airway inflammation induced by ovalbumin, and the combination of GST and GSH affords a synergistic protective effect [20]. Studies with GSTP-null mice suggest that GSTP1 is an endogenous inhibitor of allergic responses in an experimental model of asthma [21]. Together, these in vivo studies support an important role for GSTs in protecting against airway oxidative stress and inflammation.

3.2.5. Role in Hepatic Diseases

Various GST gene knockout models have been used to study the involvement of GST isozymes in liver disorders, including acetaminophen overdose-induced hepatotoxicity [22]. The hepatotoxicity associated with acetaminophen overdose results from liver GSH depletion and the subsequent accumulation of the toxic metabolite, *N*-acetyl-*p*-benzoquinoneimine. *N*-Acetyl-*p*-benzoquinoneimine is a potent electrophile that causes liver cell injury. It was initially thought that conjugation of *N*-acetyl-*p*-benzoquinoneimine with GSH was predominately catalyzed by GSTP in liver cells, and that deficiency of GSTP would aggravate acetaminophen-induced hepatotoxicity. However, GSTP knockout mice are found to be more resistant to acetaminophen-induced liver toxicity than wild-type mice [23]. While the mechanism involved in this unexpected resistance is unclear, GSTP-null mice are shown to be able to rapidly regenerate hepatic GSH, which may be associated with the tolerance to acetaminophen-induced liver toxicity. In contrast to the GSTP-null mice, GSTZ1-deficient mice are very sensitive to acetaminophen toxicity, and show significant hepatotoxicity when exposed to doses that have limited effects in normal mice [24]. Although there is a possibility that GSTZ1 contributes directly to the normal detoxification of acetaminophen, it seems more likely that the relative low level of hepatic GSH in GSTZ1-null mice is the major contributing factor. These observations suggest differential roles for various GST isozymes in a disease condition, and also point to the confounding factors involved in the gene knockout animal models (see Section 5.2 of Chapter 2 for more discussion of caveats on genetic models).

Studies using knockout animal models have shown beneficial effects of GSTA3 and GSTA4 in liver injury induced by other hepatotoxicants, including carbon tetrachloride, ethanol, and aflatoxin B1 [25-27]. Notably, mice deficient in GSTZ (also known as maleylacetoacetate isomerase) exhibit a range of pathological changes in the absence of insults, including hepatitis as well as kidney injury and splenic atrophy. This suggests a role for GSTZ in the detoxification of endogenous toxic metabolites [28].

3.2.6. Role in Cancer

GSTs play multiple roles in cancer, which can be summarized as follows.

- Protection against chemical carcinogenesis
- Protection against spontaneous cancer development
- Induction of GSTs as a mechanism of anticancer drug resistance

Many carcinogens are either direct DNA-reacting electrophiles or metabolized to form electrophilic metabolites. By catalyzing GSH conjugation with the DNA-reacting electrophilic species, GSTs protect cellular DNA as well as other critical targets from damage by chemical carcinogens. Targeted disruption of GST isozymes causes increased susceptibility to chemical carcinogenesis in various animal models. On the other hand, induction of GSTs by pharmacological inducers is an important mechanism for chemoprotection against chemical carcinogenesis [29].

In addition to protecting against chemical carcinogenesis, GSTs also play a beneficial role in spontaneous carcinogenesis. For example, a gene knockout study shows that GSTP deficiency leads to markedly enhanced colon tumorigenesis in APC^{Min} mice [30]. Knockout of GSTP in mice also results in inflammatory responses in colon. As compared with wild-type and heterozygous mice, GSTP homozygous knockout ($GSTP^{-/-}$) mice spontaneously develop various types of tumors with a significantly higher frequency and with a median tumor free lifespan 20 weeks shorter. In addition, one third of the $GSTP^{-/-}$ mice develop lung adenomas, while less than 10% of $GSTP^{+/-}$ and $GSTP^{+/+}$ mice present such tumors [31]. These observations suggest that GSTP may suppress the development of spontaneous tumors. In addition to tumor development, GSTP deficiency in mice also causes myeloproliferation possibly via a deregulation of JNK and Janus kinase/STAT pathways [32]. As discussed above (Section 3.1.3), GSTP regulates cell signaling and controls cell proliferation via a process independent of the enzymatic activity.

Another scenario related to GSTs in cancer is the anticancer drug resistance due to upregulation of GSTs in cancer cells. Many anticancer drugs, especially the alkylating agents are good substrates for GSTs. Treatment with these anticancer agents can lead to upregulation of GSTs in tumor cells, which in turn renders the tumor cells more resistant to cytotoxicity of these drugs. This has been shown as a major mechanism of anticancer drug resistance [33]. In this context, inhibition of GSTs in tumor cells is desirable as this will enhance the tumor-killing activity of the anticancer drugs.

3.2.7. Role in Other Diseases and Conditions

GSTs may also play a role in several other disease conditions. For example, GSTP-null mice exhibit an increased sensitivity to cyclophosphamide-induced urinary bladder injury [34], a significant side effect associated with the use of cyclophosphamide in patients. As stated in Section 3.2.7 of Chapter 5, GSH may be involved in the bioactivation of the anticancer drug cisplatin, leading to nephrotoxicity. It is believed that GSTs catalyze the reaction between GSH and cisplatin, forming a GSH conjugate. This cisplatin-GSH conjugate is subsequently metabolized to a nephrotoxicant via γ-glutamyl transpeptidase and a cysteine S-conjugate β-lyase in the kidney [35].

GSTZ1 is identical to maleylacetoacetate isomerase and catalyzes a significant step in the

catabolism of phenylalanine and tyrosine. Exposure of GSTZ1-deficient mice to high dietary phenylalanine causes a rapid loss of white blood cells, likely due to the accumulation of maleylacetoacetate or maleylacetone in the circulation [12]. Recently, a protective role for GSTA4 in insulin resistance and diabetes has been suggested. Targeted disruption of adipose GSTA4 leads to increased protein carbonylation, oxidative stress, and mitochondrial dysfunction, which may contribute to insulin resistance and the development of diabetes. In addition, GSTA4 expression is found to be selectively downregulated in adipose tissue of obese insulin resistant mice and in human obesity-linked insulin resistance [36].

3.3. Human Studies and Clinical Perspectives

Gene polymorphisms exist for many GSTs, especially the cytosolic GST isozymes. These polymorphisms may have a significant influence on the structure and function of corresponding GSTs. Two GST gene (GSTM1, GSTT1) deletions (also known as null polymorphisms) have been identified in human populations. The association of these two gene deletions with disease susceptibility, drug effects, and chemical toxicity has been extensively investigated over the past decade. This section begins with a discussion of these two gene deletions, followed by a description of other GST gene polymorphisms and their potential involvement in human health and disease.

3.3.1. GST Deficiencies due to Gene Deletions

Deletion of either GSTM1 or GSTT1 gene results in a null genotype characterized by a general deficiency in the individual GST isozyme activity. The homozygosity for the deficiency occurs at a high frequency in many population groups. Individuals homozygous for either deletion are thought to be at increased risk for tumors as a consequence of decreased capacity to detoxify possible carcinogens. Indeed, extensive studies demonstrate that either null genotype is associated with increased risk of developing various types of cancer [37-39]. Since GSTM1 and GSTT1 are critical enzymes responsible for the detoxification of a range of xenobiotics, including certain anticancer drugs, these enzymes are also involved in anticancer drug resistance. In this context, studies have shown that individuals with the null genotype respond more favorably to certain cancer chemotherapy [40].

Either GSTM1 or GSTT1 deficiency is also associated with elevated risk of developing a number of other diseases. These include hyperlipidemia, atherosclerosis, coronary heart disease, diabetes, neurodegeneration, asthma, chronic obstructive pulmonary disease, and alcoholic liver injury [41-47].

3.3.2. GST Gene Polymorphisms

In addition to the above null genotype for GSTM1 and GSTT1, other gene polymorphisms have also been identified for these two isozymes as well as many other members of the GST superfamily. Numerous observational epidemiological studies in various population groups have suggested an association between GST gene polymorphisms and risk of developing many pathophysiological conditions, including cardiovascular disorders, neurodegenerative diseases, carcinogenesis, and cancer drug resistance [1, 40, 48]. These observations in human subjects are consistent with the established biological activities of GSTs in experimental animals.

4. Conclusion and Future Directions

GSTs play a critical role in the detoxification of oxidative and electrophilic species. GSTs also participate in cell signaling and growth regulation. Via these diverse activities, GSTs provide protection against various disease processes involving oxidative and electrophilic stress as well as growth dysregulation in animal models. Consistent with the findings in experimental animals, extensive population studies on gene polymorphisms also implicate a beneficial role for GSTs in human health and disease. Continued genetic variation studies in human subjects will further enhance our understanding of the association between GSTs and human disease conditions, and facilitate our ability to identify the susceptible subpopulations. Such studies will also contribute to the development of effective strategies for disease intervention in high risk human subpopulations.

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Chapter 9

Glutaredoxin

Abstract

Glutaredoxin (Grx) refers to a family of GSH-dependent enzymes that catalyze the reduction of glutathionylated proteins. Grxs also participate in many other cellular processes, including cell signaling, growth regulation, anti-inflammation, and iron homeostasis. Animal studies demonstrate a critical involvement of various Grx isozymes in protecting against cardiovascular disorders, such as myocardial ischemia-reperfusion injury, cardiac hypertrophy, heart failure, and drug-induced cardiotoxicity. Grxs also exert beneficial effects on other disease processes, including neurodegeneration, anemia and iron overload, diabetes, airway inflammation, and cancer in animal models. A mutation that results in Grx5 gene splicing defects has been identified as the cause of anemia and iron overload in a human patient, but the role of Grxs in other human disease conditions remains to be elucidated.

1. Overview

1.1. Definition and History

The term glutaredoxin (Grx) refers to a family of GSH-dependent oxidoreductases. The primary function of Grxs is to catalyze the reduction of glutathionylated proteins, and thereby maintain the cellular redox homeostasis [1]. This reduction reaction is known as deglutathionylation. Protein glutathionylation refers to covalent attachment of glutathione to a protein cysteine residue via a disulfide bridge. Protein glutathionylation increases during oxidative stress. It represents a post-translational modification of proteins. Analogous to protein phosphorylation and dephosphorylation, protein glutathionylation and deglutathionylation play an important role in cell signal transduction. Because of the reversible nature of protein glutathionylation, it is also considered a transient protective mechanism for critical cysteine residues of proteins. This notion means that without glutathionylation the critical cysteine residues of proteins may become irreversibly oxidized or modified during oxidative stress. However, once the cysteine residues first become glutathionylated, they will no longer be irreversibly oxidized or modified. Importantly, the original cysteine residues can be reformed

upon Grx-catalyzed deglutathionylation.

The first Grx was identified in 1970's as a glutathione-dependent reductase of the disulfide formed in ribonucleotide reductase during its catalytic cycle in *Escherichia coli* [2, 3]. It was shown that Grx was able to restore the growth of *E. coli* in a mutant lacking thioredoxin (Trx; Chapter 10). As discussed in Chapter 10, Trxs and Grxs share a number of similar activities. However, it soon became obvious that Grxs, compared with Trxs, are more versatile with respect to the choice of substrates and reaction mechanisms. Moreover, in addition to the early discovered dithiol Grxs that contain the characteristic Cys-Pro-Tyr-Cys active site motif, subsequent studies over the past years have revealed a second group of Grxs. This group, commonly known as monothiol Grxs, lacks the C-terminal active site thiol in its Cys-Gly-Phe-Ser active site motif but contains all structural and functional elements to bind and utilize GSH as the substrate. Monothiol Grxs are further categorized into (1) single domain monothiol Grxs that consist of only one Grx domain and (2) multidomain monothiol Grxs that contain an N-terminal thioredoxin (Trx)-like domain and one to three C-terminal monothiol Grx domains (also known as PICOT homologous domains) [1].

1.2. Basic Characteristics

In addition to the classification described above in Section 1.1, Grx isozymes are usually named by numbers in the order of their discovery in various species. In mammals, there are multiple Grx isozymes, namely, Grx1, 2, 3, and 5 with Grx1 and Grx2 being most extensively investigated. Grx4 is present in lower eukaryotes. Grxs have a small molecular mass of ~10-12 kDa. Both Grx1 and Grx2 are widely distributed in mammalian tissues. Grx1 is mainly located in cytosol. Grx2 is primarily localized in mitochondria, and it can also be present in nuclei. In humans, Grx1 and Grx2 are localized on chromosomes 5q14 and 1q31.2-q31.3, respectively. In mammals, Grx3 is also known as protein kinase C interacting cousin of thioredoxin (PICOT), and is a multidomain monothiol cytosolic Grx. Grx5 is also a monothiol Grx, which is mainly present in mitochondria. In humans, Grxs 3 and 5 are localized on chromosomes 10q26 and 14q32.13, respectively.

2. Biochemistry and Molecular Regulation

2.1. Biochemistry

Grxs contain active cysteine residues and catalyze the reduction (deglutathionylation) of glutathionylated proteins. During the reaction, Grxs are oxidized with the formation of a disulfide bridge, which is then reduced back to the active cysteine groups by utilizing GSH (Figure 9-1). Thus, Grxs are GSH-dependent oxidoreductases. Grxs also catalyze the reduction of disulfide formed in ribonucleotide reductase as well as the reduction of dehydroascorbate to ascorbate. In addition, Grxs are shown to catalyze glutathionylation of proteins [4]. In summary, the biochemical reactions catalyzed by mammalian Grxs include: (1) deglutathionylation, (2) reduction of ribonucleotide reductase, (3) reduction of dehydroascorbate, and (4) glutathionylation.

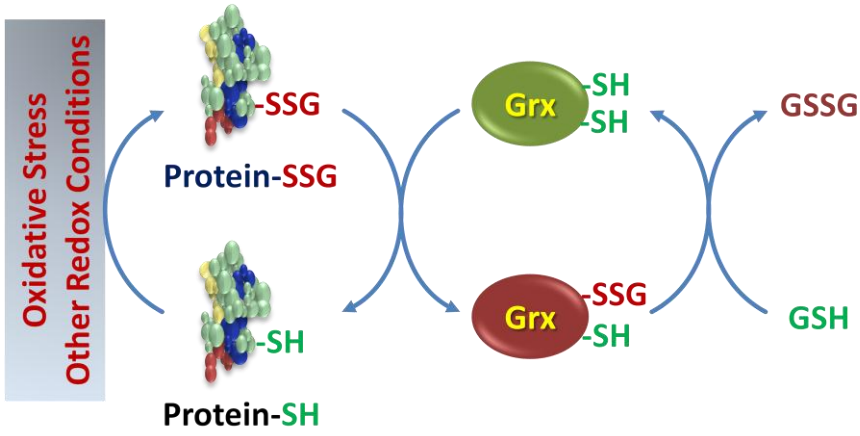

Figure 9-1. Glutaredoxin (Grx)-catalyzed protein deglutathionylation**.** As illustrated, protein glutathionylation occurs under oxidative stress and other redox conditions. Grxs catalyze reduction of protein-SSG to protein-SH, and during the reaction Grxs become glutathionylated. The glutathionylated Grxs are reduced back to Grxs by GSH with the concomitant formation of glutathione disulfide (GSSG). GSSG can be reduced back to GSH by glutathione reductase (Chapter 7) utilizing NADPH as the electron donor (not shown).

2.2. Molecular Regulation

Our understanding of the transcriptional regulation of Grx expression in mammalian cells is very limited. The AP-1 binding site has been found in the promoter region of mammalian Grx gene. Mammalian Grxs are inducible by oxidative stress as well as certain chemicals, including 17-β-estradiol. Induction of Grxs by oxidative stress stimuli may occur via an AP-1-dependent mechanism. There is also evidence showing that under certain experimental conditions Grx activity may be enhanced in response to an oxidative stimulus without a concomitant increase in Grx protein expression; however, the exact mechanism of such Grx activation remains to be elucidated [4].

3. Role in Health and Disease

3.1. General Biological Activities

The general biological activities of Grxs can be summarized into the following four categories [1, 5].

- Protection against oxidative injury via mechanisms including maintenance of cellular redox homeostasis
- Regulation of cell growth and apoptosis via participating in cell signaling
- Anti-inflammatory effects via downregulating the expression of proinflammatory cytokines and adhesion molecules
- Regulation of iron homeostasis by acting as iron-sulfur proteins

These biological activities are the molecular basis for the involvement of Grxs in health and disease. Figure 9-2 illustrates the major biological activities of Grx isozymes in mammalian systems.

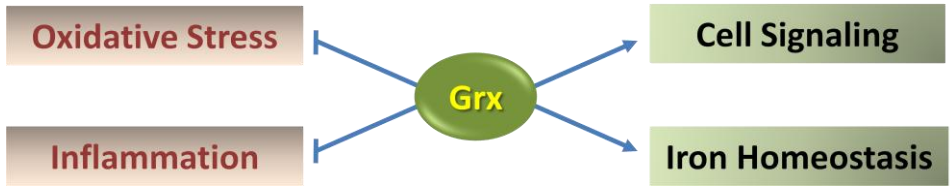

Figure 9-2. Glutaredoxin (Grx)-mediated biological activities. As illustrated, Grxs protect against oxidative stress and inflammation. In addition, these redox active proteins also participate in cell signaling and regulating iron homeostasis.

3.2. Animal Studies

3.2.1. Experimental Approaches

Both transgenic overexpression and gene knockout animal models have been created to determine the role of Grx isozymes in disease conditions. Homozygous knockout of Grx3 in mice is embryonically lethal, indicating an essential role for Grx3 in embryonic development [6]. In contrast to Grx3, homozygous knockout of other Grxs in mice does not result in embryonic lethality. In addition to the genetically manipulated models, the changes of Grxs in various disease processes have been observed in animal models. However, such observations do not establish any causal relationship between the changes in Grxs and the disease pathophysiology [1, 4].

3.2.2. Role in Cardiovascular Diseases

Cardiovascular disorders are the most extensively studied disease conditions with respect to the role of Grxs in disease pathophysiology. Recently, two studies have reported the effects of Grx1 knockout on myocardial ischemia-reperfusion injury in mice. The first study demonstrated that deficiency of Grx1 did not sensitize the adult mice to myocardial ischemia-reperfusion injury, though the embryonic fibroblasts derived from the knockout mice were more susceptible to oxidative stress in vitro [7]. In contrast, the second study showed that Grx1 knockout exacerbated myocardial ischemia-reperfusion injury and enhanced the formation of reactive oxygen species (ROS), indicating a beneficial role for Grx1 in myocardial ischemia-reperfusion injury [8]. The protective role for Grx1 in myocardial ischemia-reperfusion injury is further supported by subsequent studies with Grx1-overexpressing mice. Mouse hearts overexpressing Grx1 exhibit significantly improved post-ischemic ventricular recovery and reduced myocardial infarct size compared with wild-type hearts. Grx1 overexpression also attenuates the ROS formation due to myocardial ischemia-reperfusion [8]. More recently, an adenoviral vector-mediated Grx1 gene therapy is shown to increase Grx1 expression in myocardium and protect against myocardial ischemia-reperfusion injury in diabetic mice [9].

Transgenic overexpression of Grx2 is demonstrated to reduce myocardial ischemia-reperfusion injury by preventing both apoptosis and necrosis of cardiomyocytes. Isolated hearts from these transgenic mice display significantly improved contractile performance and reduced infarct size [10]. Transgenic overexpression of Grx2 also ameliorates doxorubicin-induced cardiotoxicity [11]. Doxorubicin-induced cardiotoxicity occurs via an oxidative stress mechanism.

The role of Grx3 in cardiovascular diseases has recently been extensively investigated using Grx3 transgenic overexpression mice. Grx3 in cardiac cells is upregulated in response to hypertrophic stimuli both in vitro and in vivo. Studies using transgenic mice with cardiac-specific overexpression of Grx3 demonstrate that Grx3 is a potent inhibitor of cardiac hypertrophy induced by pressure overload [12]. Cardiac-specific overexpression of Grx3 also results in increased ventricular function and cardiomyocyte contractility [12]. Further studies show that Grx3 attenuates cardiac hypertrophy by disrupting calcineurin-NFAT signaling, a pathway critically involved in cardiac hypertrophy [13]. Based on these findings, Grx3 has been suggested as an efficient modality for the treatment of cardiac hypertrophy and heart failure [14].

3.2.3. Role in Other Diseases and Conditions

In addition to a critical protective role in cardiovascular disorders, Grxs are also implicated in many other disease processes. Grx1 downregulation is suggested to result in mitochondrial dysfunction in cultured neuronal cells and in animal models of neurodegenerative diseases, such as Parkinson's and motor neuron diseases [15]. Studies using Grx1 knockout mice show that absence of Grx1 increases lens susceptibility to oxidative stress and cataract induced by ultraviolet light [16]. Consistently, the lens epithelial cells derived from Grx1-deficient mice exhibit increased sensitivity to oxidative stress in vitro [17].

Both Grxs 3 and 5 are required for iron-sulfur cluster assembly and heme biosynthesis [18, 19]. As such, inactivation or dysfunction of Grx3 or 5 has been implicated in disorders involving iron homeostasis, such as anemia. Other disease conditions, including diabetes, airway inflammation, and cancer may also involve the alterations of Grxs; however, the exact role of Grxs in these disease processes remains to be further elucidated [1, 4].

3.3. Human Studies and Clinical Perspectives

The involvement of Grxs in human health and disease is largely unknown. Although there are reports on the changes of Grx levels in tissues or fluids of human subjects with such disease conditions as lung disorders and cancer, the causal role for Grxs in the disease pathophysiology remains unclear. As stated above, Grx5 is a recently identified Grx isozyme in mammals, and is primarily present in mitochondria. Gene knockout animal models demonstrate an important role for Grx5 in iron homeostasis [20]. A mutation that results in Grx5 gene splicing defects has also been identified as the cause of a sideroblastic-like microcytic anemia in a human patient [21]. The patient had moderate anemia, hepatosplenomegaly, low numbers of ringed sideroblasts, and iron overload [21]. These observations suggest that Grx5 function is highly conserved, and its defect in human subjects may lead to anemia and iron overload. Currently, there have been no reports on gene polymorphisms of Grxs in human populations.

4. Conclusion and Future Directions

Grxs are multifunctional proteins that participate in various cellular processes, including antioxidant defense, cell signaling, cell growth and apoptosis, anti-inflammation, and iron homeostasis. Grxs function to protect against disease processes, especially cardiovascular disorders in experimental animals. Currently, the involvement of Grxs in human health and disease is largely unknown though there has been a single case study demonstrating a role for Grx5 defect in anemia and iron overload in a human subject. Apparently, more basic and clinical research are warranted to further elucidate the role of Grxs in both physiology and disease processes.

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Chapter 10

Thioredoxin

Abstract

Thioredoxin (Trx) refers to a family of small redox proteins that undergo NADPH-dependent reduction by thioredoxin reductase and in turn reduce the disulfide bridge in target proteins. Trxs participate in a variety of cellular processes, including scavenging reactive oxygen and nitrogen species, anti-inflammation, and regulation of cell signaling. Ample experimental evidence demonstrates an important function for Trxs in protecting against various pathophysiological conditions involving oxidative stress and dysregulated inflammation. These include cardiovascular diseases, diabetes, and neurodegeneration. In contrast, the role of Trxs in cancer development remains a subject of controversies in animal models. Studies in human subjects also suggest a possible involvement of Trxs in certain disease conditions, such as neural tube defects, diabetes, and cancer.

1. Overview

Thioredoxin (Trx or Trn) was first described in 1964 in *Escherichia coli* as an electron donor for ribonucleotide reductase, which is an essential enzyme for DNA synthesis [1]. Trx is conserved from *E. coli* to mammals. The mammalian Trxs are a family of small redox active proteins with a molecular mass of ~12 kDa. There are at least three Trxs in mammals: Trx1, Trx2, and sperm Trx (Sp-Trx, also designed as $p32^{TrxL}$). Trxs 1 and 2 are the most extensively studied Trx isozymes in biology and medicine. They are ubiquitously distributed in mammalian tissues. Trx1 is predominantly located in cytosol and can be excreted into extracellular space or translocate into nuclei, whereas Trx2 is present in mitochondria. In humans, Trx1 and Trx2 are localized on chromosomes 9q13 and 22q13.1, respectively.

2. Biochemistry and Molecular Regulation

2.1. Biochemistry

Trxs are redox proteins that undergo NADPH-dependent reduction by thioredoxin reductase (Chapter 12) and in turn reduce oxidized cysteine groups in proteins [2]. Trxs have a

conserved Cys-Gly-Pro-Cys redox catalytic site that is responsible for reducing the protein disulfide bridge. As a result, the two cysteine residues of the Trxs are oxidized to form disulfide (i.e., the oxidized form of Trxs). The oxidized Trxs are then reduced back to Trxs via thioredoxin reductase using NADPH as the electron donor (Figure 10-1).

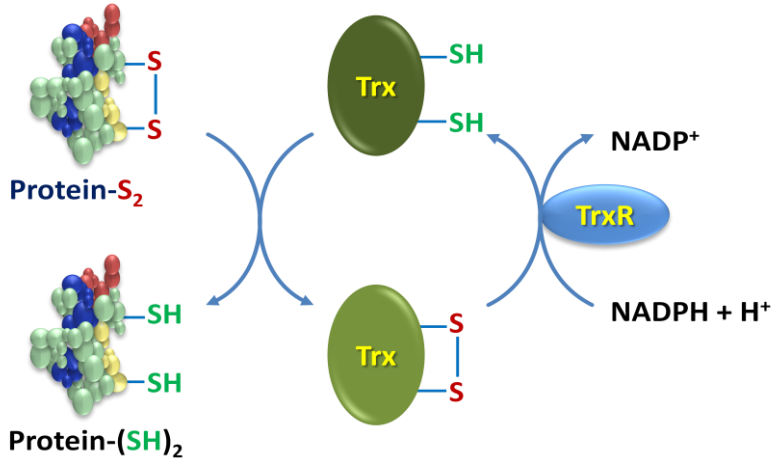

Figure 10-1. Thioredoxin (Trx)-mediated reduction of protein disulfide bridge**.** During the reduction reaction, the sulfhydryl groups on Trx are oxidized to form a disulfide bridge. The oxidized Trx is then reduced back to Trx by thioredoxin reductase (TrxR) using NADPH as the electron donor.

Trxs serve as a cofactor for many mammalian enzymes, including ribonucleotide reductase, peroxiredoxins, and glutathione peroxidase-3. In addition, Trx1 can reduce and activate a number of transcription factors (e.g., NF-κB, AP-1, SP1) that regulate various cellular processes. Trxs also bind to and modify the activities of other cellular proteins, including those involved in apoptosis. Recently, both Trx1 and Trx2 have been shown to regulate protein denitrosylation [3]. Protein denitrosylation, the removal of nitroso (-NO) groups primarily from cysteine residues in proteins, is an important aspect of nitric oxide-based signaling that regulates protein activity and protein-protein interactions [4].

Trxs possess antioxidant activities via various mechanisms, as listed below.

- Trxs may directly scavenge reactive oxygen species (ROS).
- Trxs act as cofactors for peroxiredoxins (Section 2.1 of Chapter 11), which are important enzymes for the detoxification of hydrogen peroxide, organic hydroperoxides, and possibly peroxynitrite.
- Trxs act as cofactors for methionine sulfoxide reductases (Section 2.1 of Chapter 14), enzymes involved in repairing oxidative protein damage and protecting against oxidative injury.
- Trxs may also serve as electron donors for glutathione peroxidase-3 (plasma glutathione peroxidase), an enzyme involved in the detoxification of ROS in extracellular environment (Chapter 6). However, due to the low concentrations of Trxs in plasma, this reaction may not contribute much to the antioxidant activities of Trxs.

2.2. Molecular Regulation

Trx activities are regulated at multiple levels, including transcriptional regulation, subcellular localization (e.g., nuclei, cytosol, mitochondria, extracellular space), interaction with other molecules, and post-translational modifications [2, 5]. Trxs are responsive to a variety of stimuli, such as oxidative stress and inflammatory cytokines. At the transcriptional level, Trx expression is regulated by multiple transcription factors, including AP-1, AP-2, NF-κB, SP1, and Nrf2. On the other hand, as stated above in Section 2.1, a growing number of transcription factors (e.g., NF-κB, AP-1, SP1) also require Trx-mediated reduction for efficient DNA binding.

Trx activities are also modulated by interacting proteins. For example, the activities of Trx1 are negatively regulated by thioredoxin interacting protein (Txnip). Txnip is also known as thioredoxin binding protein-2 (TBP-2). In addition to negatively regulating Trx1, Txnip may possess other biological activities, such as acting as a metabolic control protein and a tumor suppressor [6]. Various biochemical and molecular pathways are implicated in post-translational modifications of Trxs, which include oxidation and nitrosylation of the cysteine groups, glutathionylation, and tyrosine nitration. Notably, nitrosylation of certain cysteine residues of Trx1 is found to enhance Trx1 activities, whereas tyrosine nitration leads to Trx1 inhibition [5].

3. Role in Health and Disease

3.1. Animal Studies

3.1.1. Experimental Approaches

The biological activities of Trxs have been a subject of extensive studies over the past few years. Both transgenic overexpression and gene knockout animal models along with other genetic models have been created and used for understanding the functions of Trxs in health and disease. In contrast to Trx1 gene knockout, homozygous deletion of Trx2 gene has been shown to cause embryonic lethality, indicating an essential role for Trx2 in development [7]. In addition to the above genetic approaches, recombinant Trx protein has also been used as therapeutics for various pathophysiological conditions in experimental animals [8]. Primarily based on findings from animal studies, the biological activities of mammalian Trxs can be summarized into the following two categories.

- Protection against disease conditions involving oxidative and inflammatory stress
- Involvement in cancer development and anticancer drug resistance

3.1.2. Role in Cardiovascular Diseases

Trxs play an important protective role in various forms of cardiovascular disorders in animal models, including hypertension, atherosclerosis, myocardial ischemia-reperfusion injury, heart failure, autoimmune myocarditis, and drug-induced cardiotoxicity. It has been reported that transgenic overexpression of Trx2 attenuates angiotensin II-induced vascular dysfunction, oxidative stress, and hypertension in mice [9]. Trx2 overexpression also results in im-

provement of endothelial function and reduced atherosclerotic lesions in ApoE-deficient mice [10]. Studies with genetic animal models demonstrate a beneficial role for Trxs in myocardial ischemia-reperfusion injury [11]. In addition, endothelial-specific expression of Trx2 is found to promote ischemia-mediated arteriogenesis and angiogenesis, leading to enhanced functional recovery from ischemic injury [12]. In a hypertensive rat model, viral vector-mediated Trx1 gene delivery attenuates the heart failure after a chronic myocardial infarction, and the Trx1 gene delivery also results in increased expression of heme oxygenase-1 [13], a critical antioxidant protein in cardiovascular protection (Section 3.1.2 of Chapter 15). The role of Trx1 in protecting against heart failure is also evidenced by its attenuation of cardiac hypertrophy and oxidative stress in myocardium [14]. In addition, Trx1 overexpression ameliorates myosin-induced autoimmune myocarditis via inhibiting chemokine expression and leukocyte chemotaxis, suggesting a critical role for Trx1 in suppressing inflammation in myocardium [15]. Trx1 is found to be induced by doxorubicin, a cardiotoxic anticancer drug. Transgenic overexpression of Trx1 protects against doxorubicin-induced cardiotoxicity and oxidative stress in mice [16].

3.1.3. Role in Diabetes

Trxs have a protective role in both type 1 and type 2 diabetes in animal models. Transgenic overexpression of Trx1 suppresses streptozotocin-induced pancreatic inflammation, oxidative stress, and diabetes [17]. A lentiviral vector-mediated Trx1 overexpression in islets prolongs graft survival in autoimmune diabetic NOD mice, suggesting a beneficial function for Trx1 in islet transplantation for the treatment of type 1 diabetes in experimental animals [18]. These results also support the notion that oxidative stress and inflammation are key events in type 1 diabetes. Transgenic overexpression of Trxs not only protects against the development of type I diabetes, but also prevents progression of type 2 diabetes [19]. Trxs have been shown to also protect against diabetic complications in animal models, including diabetic cardiomyopathy, nephropathy, and osteopenia [20-22]. In addition, transgenic overexpression of Trx1 inhibits the development of experimental chronic pancreatitis induced by repeated administration of cerulean and lipopolysaccharide in mice [23]. In the same experimental model, Trx1 is observed to attenuate pancreatic fibrosis via suppressing oxidative stress and monocyte chemoattractant protein-1 (MCP)-1-mediated chronic inflammation [23]. These findings suggest that Trx1 not only protects against the pancreatic β-cell injury, but also serves as an important defense against pancreatitis and associated fibrosis.

3.1.4. Role in Neurological Diseases

Overexpression of Trxs in transgenic mice has been shown in multiple studies to protect against ischemic brain injury as well as excitotoxic brain damage [24-26]. Similarly, intravenous administration of recombinant Trx protein is also found to decrease brain damage following transient focal cerebral ischemia in mice [27]. In *Drosophila melanogaster*, overexpression of Trxs suppresses Parkin-associated endothelin receptor-like receptor-induced neurotoxicity and extends longevity, indicating a potential protective effect of Trxs in Parkinson's disease and aging [28]. Homozygous knockout of mitochondrial Trx2 has been shown to cause massive apoptosis, failure of neural tube closure, exencephaly, and early embryonic lethality in mice, suggesting that Trx2 possesses a particularly important function in brain development [7].

3.1.5. Role in Pulmonary Diseases

Numerous studies using transgenic mice as well as recombinant Trx protein have demonstrated a beneficial involvement of Trxs in various pulmonary disorders, including airway inflammation, asthma, emphysema, and drug/toxicant-induced pulmonary toxicity [29-35]. In addition, Trxs also play a role in protecting against pulmonary ischemia-reperfusion injury and influenza virus-induced pneumonia in animal models [36-38]. The above pulmonary protective activities of Trxs are believed to result from both antioxidant and anti-inflammatory functions associated with these redox-active proteins.

3.1.6. Role in Hepatic and Gastrointestinal Diseases

Either transgenic overexpression of Trxs or administration of recombinant Trx protein has been shown to ameliorate hepatic injury induced by ethanol, thioacetamide, and lipopolysaccharide [39-41]. Trx2 haploinsufficiency ($Trx2^{+/-}$) in mice also results in mitochondrial injury and increased oxidative stress in liver. In addition, $Trx2^{+/-}$ mice are more susceptible to the ROS-generator diaquat-induced liver injury than wild-type littermates [42].

Multiple studies also demonstrate a protective role for Trxs in gastrointestinal disorders in animal models. For example, either transgenic overexpression of Trx1 or administration of recombinant Trx protein attenuates indomethacin-induced gastric mucosal injury in mice [43]. The *Helicobacter felis*-induced gastritis and oxidative stress are also suppressed in mice overexpressing Trx1 [44]. Either transgenic overexpression of Trx1 or administration of recombinant human Trx protein results in amelioration of experimental colitis in mice, and the colonic protective effects are associated with diminished formation of macrophage migration inhibitory factor [45].

3.1.7. Role in Renal Diseases

Trx1 has a beneficial role in certain renal disorders, including ischemia-reperfusion injury and diabetic nephropathy. The endogenous Trx1 protein is rapidly depleted from the cytosol in the cortical proximal tubular cells and detected in the tubular fluid following renal ischemia-reperfusion in mice, pointing to a possible involvement of Trx1 in renal ischemia-reperfusion injury. Indeed, transgenic overexpression of human Trx1 in mice leads to increased expression of the Trx in kidney tissue and amelioration of ischemia-reperfusion injury, as assessed by blood creatinine and urea nitrogen levels [46]. Transgenic overexpression of Trx1 in mice also suppresses the development of diabetic nephropathy and oxidative renal tissue injury following induction of type 1 diabetes by streptozotocin [20].

3.1.8. Role in Skin Diseases

The anti-inflammatory and antioxidative activities of Trxs are implicated in dermal protection. A recent study using Trx transgenic mice shows that Trx1 overexpression suppresses contact hypersensitivity response by inhibiting leukocyte recruitment during the elicitation phase of the contact dermatitis [47]. The study also suggests that Trx1 may be a potential target for treating contact dermatitis [47]. In addition to exerting the anti-inflammatory and antioxidative effects, Trxs are also found to reduce the disulfide bonds of allergens and thereby mitigate the allergenicity of commercial wheat preparations in multiple animal models [48]. This finding opens the door to the testing of Trxs in the production of hypoallergenic, more-digestible foods.

Trxs may play a role in protecting against skin aging. In cultured normal human fibroblasts, suppression of Trx1 results in premature senescence [49]. Trx1 is also shown to suppress ultraviolet irradiation-induced oxidative stress, proinflammatory responses, and activation of matrix metalloproteinase-1 in human skin cells [50]. Although Trxs may exert a beneficial effect in skin aging, transgenic overexpression of Trx1 in keratinocytes is reported to augment chemically-induced skin carcinogenesis in a two-stage mouse model using topical 7,12-dimethylbenz(a)antracene as an initiator and 12-*O*-tetracecanoylphorbol-13-acetate as a promoting agent [51]. The potential involvement of Trxs in cancer is further discussed in Section 3.1.9 below.

3.1.9. Role in Cancer

While there is ample evidence supporting the protective effects of Trxs in tissue injury induced by oxidative and inflammatory stress, the role of Trxs, especially Trx1 in cancer has been a subject of controversies, and the results are inconsistent among different animal models. Numerous studies have shown an elevated Trx expression in various types of cancer. Trx1 may contribute to many of the hallmarks of cancer, including increased cell proliferation, resistance to cell death, and increased angiogenesis [52]. Some data suggest that Trx expression may also contribute to anticancer drug resistance while others indicate the opposite. As described above in Section 3.1.8, transgenic overexpression of Trx1 in keratinocytes of mice augments chemically-induced skin carcinogenesis [51]. However, Trx1 overexpression in mice is demonstrated to attenuate oxidative stress and prevent benzene-induced hemato-lymphoid toxicity and thymic lymphoma [53]. Currently, there is no evidence showing that transgenic overexpression of Trxs in animals is associated with an increased incidence of cancer. Administration of recombinant Trx protein has not been shown to promote tumor growth or induce anticancer drug resistance in animal models [6]. Thus, the exact role of Trxs in cancer remains to be further elucidated.

3.1.10. Role in Other Diseases and Conditions

Trxs have been implicated as protectors in many other diseases and related conditions, including arthritis, sepsis, eye disorders, male infertility, and aging. Transgenic overexpression of Trx1 is shown to protect against joint destruction and oxidative stress in a murine arthritis model, in which arthritis is induced by administering a mixture of anti-type II collagen monoclonal antibody (mAb) and lipopolysaccharide (LPS) [54]. Administration of recombinant Trx protein also suppresses the mAb/LPS-induced joint swelling in wild-type mice [54]. Either transgenic overexpression of Trx1 or administration of recombinant Trx protein attenuates LPS-induced inflammatory cell activation and septic shock in animal models [55, 56]. These observations are consistent with the ability of Trxs to suppress inflammation and oxidative stress. Oxidative stress and inflammation are important mechanisms underlying eye disorders, such as retinal photic injury. A protective role for Trxs in retinal photooxidative damage has been demonstrated in animal models by using both Trx-transgenic overexpression and administration of exogenous recombinant Trx protein [57, 58]. Transgenic overexpression of Trx1 inhibits germ cell apoptosis induced by experimental cryptorchidism in mice, suggesting that Trx1 intensification may be a useful therapeutic strategy for male infertility associated with heat stress [59]. As discussed above in Section 3.1.8, Trxs may protect against cell senescence in cell cultures. The potential role for Trxs in aging has been investigated using transgenic overexpression models. Trx-overexpressing mice exhibit extended

median and maximum lifespans compared with wild-type counterparts. Telomerase activity in spleen tissues of Trx-overexpressing mice is higher than that in wild-type littermates [60]. Tissue oxidative stress is also attenuated in Trx-overexpressing mice. As aforementioned, overexpression of Trxs also extends the longevity of *Drosophila melanogaster* [28] (Section 3.1.4). Taken together, these findings support an antiaging activity of Trxs in experimental animals.

3.2. Human Studies and Clinical Perspectives

The diverse biological activities of Trxs as demonstrated by extensive animal studies make these redox-active proteins also an attractive subject of investigations in the context of human health and disease. The involvement of Trxs in human diseases has been studied primarily via (1) examining the changes of these proteins in accessible tissue samples of patients with various disease conditions and (2) gene polymorphism studies. As discussed in prior chapters, neither of the above approaches establishes a causal relationship between the changes in antioxidant proteins or genes and the altered risk or incidence of disease conditions in the human subjects. But, these studies do provide important information regarding the significance of Trxs in human health and disease, and facilitate identification of risk factors and genetic biomarkers for human diseases.

Animal studies suggest that Trx2 is critical for neural tube closure during embryonic development [7]. This observation has prompted a recent investigation of the association between genetic polymorphisms in Trx2 gene and increased risk for neural tube defects. It has been observed that a novel Ins/Del polymorphism in the proximal promoter region of human Trx2 gene reduces the transcriptional activity of the gene, and is associated with an increased risk of spina bifida [61]. The gene polymorphisms of Trxs are also found to be associated with an increased risk of diabetes [62]. In addition, genetic variations in the thioredoxin interacting protein (Txnip) are demonstrated to be associated with hypertriglyceridemia and increased blood pressure in type 2 diabetes [63]. The involvement of Trxs in human cancer development has been reported in multiple studies. Increased expression of Trx1 is documented in several types of human cancer, including lung, pancreatic, colon, gastric, and breast cancer [52]. Recently, elevated expression of Trx1 has been identified as an independent poor prognosis factor in patients with liver metastasis from colorectal cancer [64]. Although the exact role for Trxs in human cancer remains to be determined, the association of Trx1 expression with aggressive tumor growth, resistance to standard therapy, and decreased patient survival makes it a potential target for cancer drug development [52].

4. Conclusion and Future Directions

Trxs are protective molecules in various disease processes, including cardiovascular injury, diabetes, and neurodegeneration in animal models. However, the exact role of Trxs in cancer remains inconclusive as both suppressive and promoting activities of Trxs with regard to cancer development have been reported in experimental animals. Data on the involvement of Trxs in human diseases are relatively scarce. Limited studies in human subjects suggest a

possible protective role for Trxs in spina bifida and diabetes, and a potential detrimental effect on the development of certain types of cancer. Future efforts should be devoted to the identification of novel genetic variations of Trx genes and their influence on tissue Trx expression and susceptibility to disease pathophysiology. As the involvement of Trxs in cancer is controversial, more basic research is warranted to define the exact functions of these redox-active proteins in multistage carcinogenesis as well as anticancer drug resistance.

5. References

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Chapter 11

Peroxiredoxin

Abstract

Peroxiredoxin (Prx) refers to a family of thiol-dependent peroxidases that reduce hydrogen peroxide and organic hydroperoxides to water and alcohols, respectively. There are six known mammalian isozymes (Prx1-6), classified as typical 2-Cys, atypical 2-Cys, or 1-Cys Prxs based on the mechanism and the number of cysteines involved during catalysis. In addition to their well-established peroxide-scavenging activity, Prxs also participate in the regulation of various cell signaling pathways. Extensive animal studies provide substantial evidence supporting a critical protective role for Prxs in various disease processes involving oxidative and inflammatory stress. In contrast, studies on the involvement of Prxs in human health and disease are much limited.

1. Overview

1.1. Definition and History

Peroxiredoxin (Prx) is a general term that refers to a family of small (22-27 kDa) nonseleno peroxidases currently known to possess six mammalian isozymes, namely, Prx1, 2, 3, 4, 5, and 6. These isozymes are able to reduce hydrogen peroxide, organic hydroperoxides, and possibly peroxynitrite, and thus represent a class of important antioxidants in mammals [1]. As discussed in Chapter 6, glutathione peroxidase represents another family of enzymes that detoxify peroxides and possibly peroxynitrite as well.

The first Prx was discovered by E.R. Stadtman and coworkers in 1988 in *Saccharomyces cerevisae*, where it protected the enzyme glutamine synthase from oxidation in a system that serendipitously included thiols to generate hydrogen peroxide. The enzyme was first named thiol-specific antioxidant (TSA) due to its dependence on thiol compounds [2]. The name was then changed to thioredoxin peroxidase as the protein was found to be oxidized by hydrogen peroxide and reduced back by thioredoxin (see Chapter 10 for discussion of thioredoxin). Subsequent studies showed homologous proteins in other species that shared the peroxidase activity, and the term peroxiredoxin was introduced, which has been widely adopted in the literature.

1.2. Basic Characteristics

The six Prxs expressed in mammalian cells are classified into three subgroups: (1) typical 2-Cys Prxs including Prx1-4, (2) atypical 2-Cys Prx (Prx5), and (3) 1-Cys Prx (Prx6). Prxs are widely distributed in mammalian tissues, and the subcellular localization varies with the isozymes. Prx 1, 2, and 6 are mainly located in cytosol. Prx3 is restricted to mitochondria. Prx4 is present in endoplasmic reticulum and also secreted into extracellular environment. Prx5 is localized intracellularly to cytosol, mitochondria, nuclei and peroxisomes. In humans, Prx1, 2, 3, 4, 5, and 6 are localized on chromosomes 1p34.1, 19p13.2, 10q25-q26, Xp22.11, 11q13, and 1q25.1, respectively. Table 11-1 summarizes the basic characteristics of the six Prx isozymes in mammals.

Table 11-1. Basic Characteristics of the six isozymes of mammalian peroxiredoxin

Isozyme	Subgroup	Cellular Location	Chromosomal Localization
Prx1	Typical 2-Cys	Cytosol	1p34.1
Prx2	Typical 2-Cys	Cytosol	19p13.2
Prx3	Typical 2-Cys	Mitochondria	10q25-q26
Prx4	Typical 2-Cys	Endoplasmic reticulum, extracellular space	Xp22.11
Prx5	Atypical 2-Cys	Cytosol, mitochondria, nuclei, peroxisomes	11q13
Prx6	1-Cys	Cytosol	1q25.1

2. Biochemistry and Molecular Regulation

2.1. Biochemistry

Prxs catalyze the reduction of hydrogen peroxide and various organic hydroperoxides to form water and alcohols, respectively through the reactive cysteine (Cys) residues of the enzymes (Figure 11-1). They may also be able to reduce peroxynitrite under certain experimental conditions [3].

The typical 2-Cys Prxs (Prx1, 2, 3, and 4) are homodimers and contain both the N- and C-terminal conserved Cys residues and require both of them for catalytic function. Atypical 2-Cys Prx (Prx5) is a monomer and contains only the N-terminal Cys but requires one additional nonconserved Cys residue for catalytic activity. On the other hand, 1-Cys Prx (Prx6) is a homodimer and contains only the N-terminal Cys and requires no additional Cys for catalytic function.

During reaction with oxidant substrates, the Cys residues of Prxs are oxidized. Thioredoxins provide the electrons for reducing the oxidized Prx1-5, whereas GSH is likely to be employed to reduce the oxidized Prx6. The molecular and biochemical mechanisms underlying the differential use of thioredoxins and GSH by different Prx isozymes remain to be further elucidated.

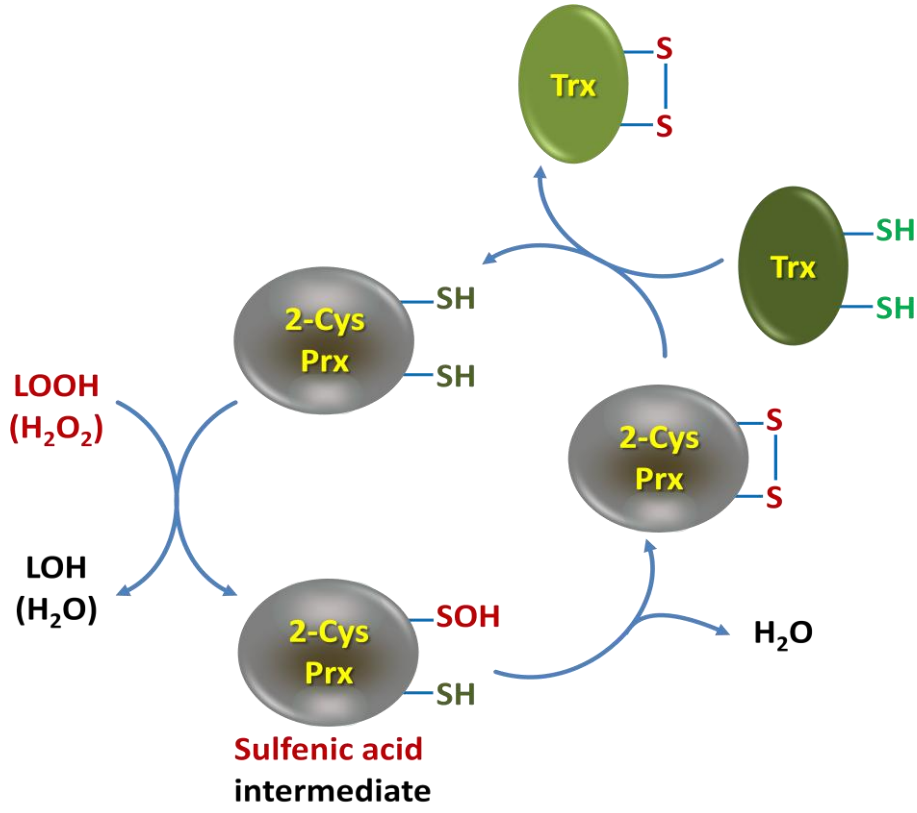

Figure 11-1. Peroxiredoxin (Prx)-mediated reduction of hydrogen peroxide (H_2O_2) and organic hydroperoxides (LOOH). Shown in the scheme is a 2-Cys Prx-catalyzed reduction of H_2O_2 and LOOH to form water and alcohol (LOH), respectively. During the reaction, one sulfhydryl group of the 2-Cys Prx is oxidized to sulfenic acid intermediate, followed by the formation of a disulfide bridge (i.e., the oxidized form of Prx). The disulfide bridge is reduced by thioredoxin (Trx), and the original Prx is regenerated.

2.2. Molecular Regulation

Prxs are regulated at multiple levels, including both transcriptional regulation and post-translational modifications. At the transcriptional level, NF-κB has been shown to suppress the transcriptional expression of Prx6, whereas increased transcription of both Prx1 and Prx6 has been reported to be mediated by Nrf2 via an antioxidant response element-driven mechanism [4-6]. The B cell-specific activator protein, Pax5 is also shown to function as a trans-activator of 1-Cys Prx (Prx6) gene expression [7]. In the promoter of human Prx5 gene, the binding sites for both nuclear respiratory factor 1 and nuclear respiratory factor 2 have been predicted. Both of these transcription factors are shown to largely control the basal expression of Prx5 gene [8]. As described in Section 2.2 of Chapter 4, the forkhead transcription factor Foxo3a plays an important role in the regulation of catalase gene expression in mammalian cells. A recent study also demonstrates a critical role for Foxo3a in regulating the gene ex-

pression of Prx3 in human cardiac fibroblasts [9].

The post-translational modifications of Prxs have been extensively studied over the past few years. Substantial evidence demonstrates a critical involvement of oxidative modifications of cysteine residues in regulating the activities of various Prx isozymes [10]. As described in Section 2.1 of Chapter 13, hyperoxidation of Prx cysteine residues to sulfinic acid results in irreversible enzyme inactivation. In addition to cysteine thiol modifications, protein phosphorylation and acetylation have also been implicated in the regulation of Prx enzymatic activities [10].

3. Role in Health and Disease

3.1. General Biological Activities

Prxs play an important part in the detoxification of hydrogen peroxide and various organic peroxides, and possibly peroxynitrite. This antioxidant function is the basis for the protective effects of Prxs in various types of diseases involving an oxidative stress mechanism. In addition to their antioxidant function, Prxs are reported to participate in other cellular processes, such as regulation of activities of transcription factors (e.g., NF-κB, AP-1, p53), growth factor and ROS signaling, cell proliferation, differentiation, and apoptosis [1, 11]. ROS here stands for reactive oxygen species. Regulation of the above cellular processes or signaling pathways may also be an important mechanism underlying the biological activities of Prxs in health and disease.

3.2. Animal Studies

3.2.1. Experimental Approaches

Both transgenic overexpression and gene knockout models have been created to investigate the biological functions of Prxs in experimental animals. Viral vector-based gene delivery and recombinant Prx protein have also been employed to understand the biological activities of Prxs in experimental models. Largely based on studies using these models, Prxs have been demonstrated to be critical protectors in numerous disease conditions, among which are various forms of cardiovascular disorders.

3.2.2. Role in Cardiovascular Diseases

As stated in prior chapters, oxidative stress is a crucial mechanism underlying various types of cardiovascular disorders. Prxs have been found to undergo complex modifications during cardiovascular oxidative stress, suggesting that Prxs may be targets of oxidative stress in cardiovascular system [12, 13]. In this context, multiple transgenic overexpression and gene knockout animal studies demonstrate a critical protective role for different Prx isozymes in cardiovascular disorders, including atherosclerosis, myocardial ischemia-reperfusion injury, and heart failure.

Homozygous deletion of Prx1 ($Prx1^{-/-}$) in mice causes excessive endothelial activation and inflammation. Deletion of Prx1 also results in early atherosclerosis in ApoE-deficient

mice fed normal chow, suggesting an important protective effect of Prx1 in vascular dysfunction and atherogenesis [14]. Platelet-derived growth factor (PDGF) plays a critical role in atherosclerosis and vascular remodeling. It is reported that Prx2 suppresses PDGF signaling and restenosis in a mouse model [15]. The involvement of Prx6 in atherosclerosis remains controversial. Some data suggest a beneficial role for Prx6 in vascular oxidative stress and atherogenesis [16], but others do not show a protective effect [17].

Prxs become oxidatively modified during myocardial ischemia-reperfusion injury. Targeted disruption of Prx6 in mice has been demonstrated to sensitize the hearts to global ischemia-reperfusion injury, as evidenced by reduced recovery of left ventricular function, increased myocardial infarction, and higher amounts of apoptotic cardiomyocytes, as well as increased oxidative stress compared with wild-type mouse hearts [18]. Similarly, overexpression of mitochondrial Prx3 has been found to prevent left ventricular remodeling and failure after myocardial infarction in mice [19]. Overexpression of Prx3 also protects against myocardial mitochondrial oxidative stress and mitochondrial DNA damage [20]. These results point to an important role for Prxs in protecting against myocardial remodeling and heart failure. They also suggest that Prxs may serve as potential therapeutic targets for the intervention of heart failure.

3.2.3. Role in Diabetes

Prxs are expressed in pancreatic tissue, including the β-cells [21]. Prx isozymes are induced in animal models of both type 1 and type 2 diabetes [22, 23]. In cultures, Prxs in the pancreatic β-cells are also inducible by oxidative stress and proinflammatory cytokines [24]. These observations suggest a possible protective role for Prxs in diabetes. Indeed, transgenic overexpression of Prx3 in mice results in decreased mitochondrial ROS production and increased resistance to stress-induced pancreatic β-cell injury [25]. These animals show improved glucose homeostasis, as evidenced by reduced levels of blood glucose and increased glucose clearance. In addition, the Prx3-overexpressing mice are protected from hyperglycemia and glucose intolerance induced by high-fat diet feeding [25].

3.2.4. Role in Neurological Diseases

Prxs are widely distributed in mammalian tissues, including the central nervous system. The basal expression of the six Prxs shows distinct profiles in different brain regions and different cell types. Prx1 and 6 are expressed in glial cells but not in neuron, whereas Prx2, 3, 4, and 5 are expressed in neurons [26]. Multiple studies suggest that Prxs may play an important part in protecting against neurological disorders in animal models, including parkinsonism, cerebral ischemic injury, age-associated hippocampal oxidative damage and cognitive dysfunction, and drug/toxicant-induced neurotoxicity [27-33]. In addition, neuronal overexpression of Prx2 in *Drosophila melanogaster* is shown to attenuate oxidative stress and prolong longevity, suggesting an overall protective function of Prx2 in neurodegeneration and aging [34]. In line with the animal studies, in vitro experiments also show beneficial effects of Prxs on oxidative and inflammatory injury in cultured neuronal cells.

3.2.5. Role in Pulmonary Diseases

A number of animal studies have documented a protective role for Prxs in several pulmonary disorders, including hyperoxia- and paraquat-induced lung injury, asthma, and endotox-

in-elicited lung inflammatory injury. Prx6 is probably the most extensively studied Prx isozyme with respect to protection against lung injury [35]. Homozygous knockout of Prx6 sensitizes the mice to hyperoxia-induced lung oxidative stress and inflammation as well as mortality [36]. These knockout mice also show increased pulmonary injury following exposure to paraquat, a redox-cycling compound [37]. On the other hand, either transgenic overexpression or adenovirus-mediated gene transfer of Prx6 results in increased resistance to hyperoxia-induced lung injury and mortality in mice [38, 39]. Prx6 also possesses a phospholipase A2 activity and has an important role in lung surfactant turnover [40]. Recently, it is reported that Prx1 knockout sensitizes the animals to airway inflammation and hyperresponsiveness in a Th2-dominant allergic asthma model [41]. Targeted disruption of Prx3 in mice also leads to increased susceptibility of the animals to lipopolysaccharide-induced lung inflammation, airway wall thickening, and oxidative DNA and protein damage [42]. These animal studies strongly suggest a critical involvement of Prxs in protecting against lung inflammation and oxidative stress.

3.2.6. Role in Hepatic Diseases

The role of Prxs in liver ischemia-reperfusion injury and alcoholic liver injury has been recently studied. Hepatic ischemia-reperfusion is found to alter the cellular localization of Prx6 in mice, and targeted disruption of Prx6 sensitizes the animals to liver ischemia-reperfusion injury, which is associated with increased mitochondrial ROS formation and dysfunction [43]. Chronic consumption of alcohol is reported to cause liver injury and oxidative stress as well as decreased expression of Prx6, suggesting a possible protective role for Prx 6 in alcoholic liver injury [44]. However, neither transgenic overexpression nor targeted disruption of Prx6 in mice results in attenuation or exacerbation of alcoholic liver injury [45]. It is further shown that ethanol administration causes significant induction of catalase, glutathione S-transferase, and glutathione peroxidase in both Prx6 transgenic overexpression and knockout mice. The concomitant upregulation of the above antioxidant defenses by ethanol in both genetic models may explain the failure of genetic alterations of Prx6 to modulate alcoholic liver injury [45].

3.2.7. Role in Skin Diseases

Several recent studies have examined the possible involvement of Prx6 in skin disorders. Overexpression of Prx6 in keratinocytes is shown to protect the skin cells from injury elicited by oxidative stress and ultraviolet (UV) irradiation [46]. In experimental animals, targeted disruption of Prx6 gene leads to increased sensitivity to UV irradiation-induced skin injury and oxidative stress [47]. Knockout animal studies also suggest that Prx6 is required for blood vessel integrity in wounded skin [47].

3.2.8. Role in Cancer

It has been known for decades that ROS are involved in multistage carcinogenesis. Prxs via scavenging peroxides appear to have tumor preventive activities as loss of Prx1 in mice leads to premature death from multiple malignancies, including lymphomas, sarcomas, and carcinomas [48]. Prx1 ablation also increases the susceptibility of mice to Ras-induced breast cancer, suggesting a possible function for Prx1 in regulating oncogenic Ras effector pathways [49]. Indeed, Prx1 is shown to inhibit Ras-induced tumorigenesis via regulating PTEN/AKT

activity [49]. PTEN is a tumor suppressor, and AKT is a protein kinase. In vitro transformation assays demonstrate that the enhanced Ras-mediated transformation by loss of Prx1 can be inhibited by wild-type Prx1 but not by a catalytically inactive mutant of Prx1, in which peroxide catalyzing cysteines are replaced by serines. This suggests that the tumor suppressive properties of Prx1 depend on its antioxidant function [50].

There are studies suggesting that Prxs may also have tumor supportive activities. Increased expression of Prxs is documented in various types of cancer. In a cell culture model, overexpression of Prx6 is shown to promote the in vitro proliferation and invasion of breast cancer cells [51]. In an in vivo study, Prx6-overexpressing breast cancer cells are found to grow much faster and exhibit more pulmonary metastases than control cells [51]. Obviously, the exact role of Prxs in cancer warrants further investigations by using additional animal models of carcinogenesis.

3.2.9. Role in Other Diseases and Conditions

A protective role for Prxs has been suggested for various other pathophysiological conditions, including anemia, sepsis, spermatogenic cell injury, and aging. Prx1 knockout mice are shown to develop hemolytic anemia characterized by an increase in red blood cell ROS, which leads to protein oxidation, hemoglobin instability, Heinz body formation, and decreased red blood cell lifespan [48]. It has also been reported that Prx2 functions as an efficient scavenger of hydrogen peroxide in red blood cells, and may thus act as an important defense against oxidative stress in these cells [52].

Prx2 possesses an important function in regulating proinflammatory responses to lipopolysaccharide (LPS) and protecting against endotoxin-induced lethal shock in experimental animals. LPS is found to induce substantially enhanced inflammatory responses in Prx2-deficient macrophages. Mice with targeted disruption of Prx2 show an increased sensitivity to LPS-induced septic shock and lethality, whereas intravenous injection of the adenovirus-encoding Prx2 gene into Prx2-deficient mice significantly rescues the animals from LPS-induced lethal shock as compared with the injection of a control viral vector [53]. Recently, a beneficial role of Prx4 in spermatogenic cells has been suggested as Prx4 knockout mice show elevated spermatogenic cell death via oxidative stress [54]. ROS are believed as a major contributor to aging. By scavenging ROS, Prxs may play a protective role in aging process. In cell cultures, Prx2 is demonstrated to function as an enzymatic antioxidant to prevent cellular senescence [55]. Recently, it is reported that Prx5 confers protection against oxidative stress and promotes longevity in *Drosophila melanogaster* [56].

3.3. Human Studies and Clinical Perspectives

The potential involvement of Prxs in human disease conditions have been investigated, but the evidence is far less convincing than that obtained from animal studies. The levels of Prxs are shown to be altered in tissue samples of patients with various diseases, including cardiovascular disorders, neurodegeneration, autoimmunity, and cancer [57-63]. Notably, increased levels of Prxs are reported in most of the above disease conditions. Downregulation of Prx3-6 is observed in patients with dilated cardiomyopathy [58]. Although a causal role for Prxs in human diseases cannot be established solely based on the changes of Prxs in tissue samples, under certain conditions, levels of Prxs may serve as markers for disease prognosis.

For example, elevated Prx1 in lung tissue is shown to be an independent prognostic factor for disease recurrence and reduced survival in stage I non-small cell lung cancer [62]. In contrast, a study in patients with renal cell carcinoma demonstrates that Prx2 is associated with tumors of a lower grade and with a lower frequency of distant metastasis. Patients with tumors expressing Prx2 are further shown to have better prognosis [63]. In view of the controversial findings, the role of Prxs in human cancer development warrants further investigations.

4. Conclusion and Future Directions

Prxs are a family of important antioxidant enzymes that protect against pathophysiological conditions involving oxidative and inflammatory stress in various animal models. These antioxidant proteins also participate in other cellular processes, including regulation of cell signaling, cell proliferation, and apoptosis. Both direct antioxidant and non-antioxidant activities of Prxs form the basis of the beneficial functionality of Prxs in health and disease. In contrast to animal studies, studies in human subjects aiming to establish a causal involvement of Prxs in disease protection are scarce. To date, gene polymorphisms in Prxs and their association with disease risk have not been reported in humans. Future studies should focus on translational and clinical research to better define the role of Prxs in human health and disease. These studies will lay a foundation for developing therapeutic modalities for the intervention of human diseases in which Prxs play a role.

5. References

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Chapter 12

Thioredoxin Reductase

Abstract

Thioredoxin reductase (TrxR) refers to a class of redox-active enzymes that catalyze the reduction of oxidized thioredoxins using NADPH as the electron donor. TrxRs together with thioredoxins and peroxiredoxins form the mammalian thioredoxin system, which is critically involved in antioxidant defenses as well as many other cellular processes. Gene knockout animal studies suggest a critical involvement of TrxRs in the development and function of certain organs, including heart and brain. TrxRs may also play a role in cancer development, either preventing or promoting carcinogenesis depending on the experimental conditions. Limited observational studies in human subjects point to a potential involvement of TrxRs in certain diseases, such as neurodegeneration and cancer.

1. Overview

Thioredoxin reductase (TrxR) refers to a class of enzymes that catalyze the reduction of oxidized thioredoxins (see Chapter 10 for discussion of thioredoxin) with NADPH as the reducing equivalent [1]. TrxRs belong to a pyridine nucleotide disulfide oxidoreductase family. There are three major isozymes of TrxR in mammals: TrxR1, TrxR2, and thioredoxin glutathione reductase (TGR). Similar to thioredoxin-1 and -2, TrxR1 is present in cytosol and nuclei, and TrxR2 is localized in mitochondria. Both TrxR1 and TrxR2 are widely distributed in mammalian tissues.

The third isozyme TGR is so called because it possesses both thioredoxin reductase and glutathione reductase activities. Hence, TGR can be viewed as a dual functioning enzyme that is involved in keeping both thioredoxins and glutathione at reduced state. TGR is also known as TrxR3. TGR is abundant in testis, suggesting a potential role for this protein in male reproduction. It is also expressed in other tissues, including lung, kidney, heart, and brain. Compared with TrxR1 and TrxR2, TGR has received less attention. It should be noted that in mammals the genes for TrxR1, TrxR2, and TGR are denoted as Txnrd1, Txnrd2, and Txnrd3, respectively. In humans, Txnrd1, Txnrd2, and Txnrd3 are localized on chromosomes 12q23-q24.1, 22q11.21, and 3q21.3, respectively.

2. Biochemistry and Molecular Regulation

2.1. Biochemistry

Mammalian TrxRs exist as homodimers. Each subunit is approximately 54-58 kDa in size and contains one FAD binding domain, one NADPH binding domain, and one interface domain that is required for dimerization. TrxRs are selenoproteins that catalyze the reduction of oxidized thioredoxins using NADPH as the electron donor. As stated above, TGR also catalyzes the reduction of glutathione disulfide (GSSG) to GSH, and thus possesses the glutathione reductase activity (Figure 12-1). TGR is found to have an additional protein disulfide isomerase function in sperm cells, and as such may play a role in sperm maturation [2]. In addition to thioredoxins and GSSG, TrxRs also reduce several other substrates, such as glutaredoxin-2 and protein disulfide isomerase.

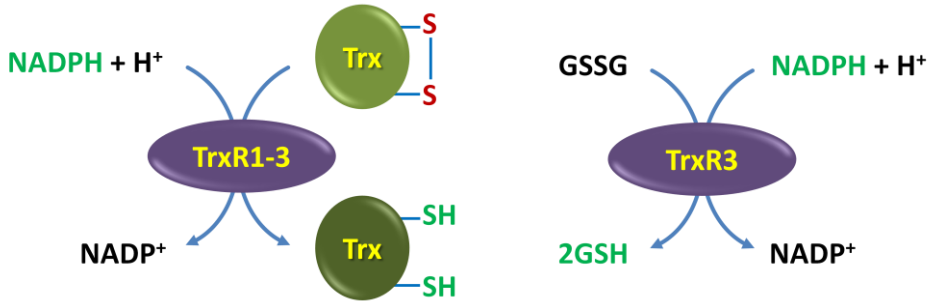

Figure 12-1. Thioredoxin reductase (TrxR)-mediated reduction of oxidized thioredoxins and glutathione disulfide (GSSG). As illustrated, all three isozymes of mammalian TrxR are able to catalyze the reduction of oxidized thioredoxins using NADPH as the electron donor. In addition, TrxR3 (TGR) also catalyzes the reduction of GSSG to GSH using NADPH as the electron donor.

TrxRs possess antioxidant functions that occur via several potential mechanisms, as described below.

- TrxRs reduce the oxidized form of thioredoxins to the active form. As discussed in Section 2.1 of Chapter 10, thioredoxins play an important role in antioxidant defenses via serving as a cofactor for various antioxidant enzymes.
- TrxRs may also be involved in the regulation of heme oxygenase-1 (HO-1). HO-1 is a critical antioxidant and anti-inflammatory enzyme in mammalian tissues (Section 2.1 of Chapter 15).
- TrxRs may support the antioxidant functions of various small molecule antioxidants, including ascorbate, α-tocopherol, and ubiquinone (also known as coenzyme Q).

Although the antioxidant activities of TrxRs have been well-established in various cellular systems, it should be borne in mind that TrxRs may also participate in other cellular processes (e.g., cell proliferation, DNA repair, angiogenesis) that are not directly related to

their antioxidant activities [1]. These non-antioxidant activities may also contribute to the role of these redox-active proteins in health and disease.

2.2. Molecular Regulation

Regulation of TrxR1 expression occurs via various pathways, including transcriptional control and post-translational modifications [1, 3]. At the transcriptional level, Oct-1, SP1, SP3, and Nrf2 are reported to participate in regulating TrxR1 gene expression. TrxR1 is shown to be induced by certain stimuli, such as oxidants and heavy metal ions via an Nrf2-dependent mechanism [4, 5]. Several post-translational modifications of TrxR1 have been suggested, which include glycosylation, phosphorylation, and oxidative modifications [1]. In contrast to TrxR1, little has been reported on the regulation of TrxR2 and TGR expression in mammalian systems.

3. Role in Health and Disease

3.1. Animal Studies

3.1.1. Experimental Approaches

Because of the efficient reduction of oxidized thioredoxins by TrxRs, it is conceivable that many of the biological activities of TrxRs are mediated by thioredoxins. Indeed, the biological functions of thioredoxins and TrxRs overlap. Our current understanding of the biological effects of TrxRs largely results from studies using gene knockout animal models [6]. In this context, homozygous targeted disruption of either Txnrd1 or Txnrd2 in mice causes embryonic lethality, indicating their essential function in embryonic development [7-9]. Mice with heterozygous deficiency of either Txnrd1 or Txnrd2, however, do not exhibit any gross abnormalities.

In addition, various chemical inhibitors have been used to study the biological functions of TrxRs. These pharmacological inhibitors include auranofin, triphenyl phosphine gold chloride, aurothioglucose, and 1-chloro-2,4-dinitrobenzene [10-13]. Although these chemical agents potently inhibit TrxR activity in biological systems, they lack specificity as do many pharmacological inhibitors for other enzymes and pathways.

3.1.2. Role in Cardiovascular Diseases

As stated above, both Txnrd1 and Txnrd2 genes are essential for murine embryonic development. However, homozygous deletion of Txnrd1 or Txnrd2 yields different phenotypes. Homozygous deletion of Txnrd1 in mice is shown to cause early embryonic lethality around embryonic day 9, with severe growth retardation and lack of primitive mesoderm formation. Of note, cardiac development is not affected by a conditional heart-specific deletion of Txnrd1, suggesting that the cytosolic TrxR1 is essential for embryogenesis but dispensable for cardiac development [7]. In contrast, ubiquitous inactivation of Txnrd2 gene results in embryonic death at embryonic day 13, with severe disruption of hematopoiesis and heart development as well as extensive apoptosis in liver [9]. Cardiac tissue-restricted ablation of

Txnrd2 leads to fatal dilated cardiomyopathy, a condition reminiscent of that in Keshan disease and Friedreich's ataxia [9]. The cardiomyopathy in Keshan disease (and possibly also in Friedreich's ataxia) is associated with selenium deficiency. The above experimental findings suggest that in contrast to TrxR1, TrxR2 plays a pivotal role in heart development and cardiac function.

3.1.3. Role in Neurological Diseases

The roles of TrxR1 and TrxR2 in brain development and function have been studied using mice with nervous system-specific deletion of Txnrd1 and Txnrd2 [14]. Mice lacking Txnrd1 in nervous system are significantly smaller than control littermates, and display ataxia and tremor. These mice show cerebellar hypoplasia, which appears to be responsible for ataxia and tremor. In contrast, mice with nervous system-selective deletion of Txnrd2 develop normally, do not show any histological deterioration in the brain, and have a similar lifespan as do control littermates [14]. These results indicate that TrxR1, but not TrxR2 plays a critical role in brain development and function in mice. Recently, it has been reported that overexpression of a human chromosome 22q11.2 segment containing Txnrd2, COMT (catechol-*O*-methyltransferase), and ARVCF (armadillo repeat gene deleted in velocardiofacial syndrome) in mice affects incentive learning and working memory; however, the exact contribution of Txnrd2 to the phenotype is unclear [15].

3.1.4. Role in Cancer and Other Conditions

The exact role of TrxRs in cancer remains unclear, and the results are inconsistent among different studies [16, 17]. On the one hand, TrxR1 is reported to activate the p53 tumor suppressor and be selectively targeted by carcinogenic electrophilic compounds, implicating a role in protecting against carcinogenesis [18]. On the other hand, TrxR1 is shown to be overexpressed in many types of cancer and involved in the facilitation of cancer cell growth, pointing to a cancer promoting activity [16, 17, 19]. In this context, various anticancer drugs are reported to target TrxR1 in killing cancer cells, and inhibition of TrxRs has been proposed as a novel strategy for cancer intervention [20, 21].

There is also evidence from animal studies in combination with cell culture experiments showing that TrxRs may have a protective role in male infertility, anemia, oxidative lung injury, cellular senescence and aging, and viral infections [1, 12, 22-24].

3.2. Human Studies and Clinical Perspectives

The potential involvement of TrxRs in human health and disease has been investigated largely via observing the changes of these proteins in disease conditions, such as atherosclerosis, neurodegeneration, and cancer [16, 25, 26]. These observational studies, however, do not establish a causative role for TrxRs in the disease processes. Multiple gene polymorphisms have been identified for human Txnrd1, and among them two intronic single-nucleotide polymorphisms are found to be significantly associated with the development of familial amyotrophic lateral sclerosis [27]. Gene polymorphism studies also suggest an association between Txnrd1 variation and risk of developing advanced colorectal adenoma [28], pointing to the possibility of using Txnrd gene variations as biomarkers for certain disease risk assessment in human populations.

4. Conclusion and Future Directions

TrxRs along with thioredoxins and peroxiredoxins form an important line of antioxidant defenses in mammalian cells and tissues. Although studies using gene knockout animal models suggest a critical role for TrxR1 or TrxR2 in the development and function of brain or heart, the involvement of Trxs in the pathophysiological processes of many common diseases remains to be determined. Similarly, human studies on the role of Trxs in health and disease are much limited and inconclusive. Future studies should focus on creating novel genetic animal models (including both transgenic overexpression and gene knockout) and using these models to further elucidate the biological activities of all three TrxR isozymes and their role in disease processes. Future efforts should also be directed to the identification of novel genetic variations of TrxRs in human subjects and the investigations of the impact of the genetic variations on tissue TrxR expression and activity as well as individual's susceptibility to disease development.

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Chapter 13

Sulfiredoxin

Abstract

Sulfiredoxin (Srx or Srx1) is a newly identified redox-active enzyme that catalyzes the ATP-dependent reduction of hyperoxidized peroxiredoxins (Prxs) in the presence of thiol equivalents, such as thioredoxins or glutathione. In addition, Srx catalyzes protein deglutathionylation and participates in regulating cell redox homeostasis and cell proliferation. Multiple in vitro cell experiments and limited in vivo animal studies demonstrate a role for Srx in protecting against oxidative stress. However, the involvement of Srx in common disease processes remains largely unknown.

1. Overview

Sulfiredoxin (Srx or Srx1) is a recently discovered small molecular mass (~13 kDa) antioxidant enzyme that catalyzes the reduction of sulfinic peroxiredoxins (Prxs) in the presence of ATP. Srx was first identified in yeast by B. Biteau and coworkers in 2003 [1]. It is only found in eukaryotic cells. This is thought to be due to the role of Srx in the restoration of hyperoxidized Prxs, whose counterparts in proeukaryotes are not sensitive to oxidative inactivation [2, 3]. Srx is ubiquitously expressed in mammalian tissues [4]. It is primarily present in cytosol and also found to undergo translocation to mitochondria [5]. In humans, Srx is localized on chromosome 20p13.

2. Biochemistry and Molecular Regulation

2.1. Biochemistry

Srx interacts with and binds to 2-Cys Prxs, forming a complex, as evidenced by their coimmunoprecipitation and structural studies [6]. As described in Chapter 11, Prxs are antioxidant enzymes that exert cytoprotective effects in many models of oxidative stress. However, under highly oxidizing conditions Prxs can be inactivated through hyperoxidation of their

active site cysteine residue to form sulfinic acid (-SO_2H). This hyperoxidized form of Prxs cannot be reduced by thioredoxins, and is thus considered as an inactivated state. Srx acts by catalyzing the ATP-dependent formation of a sulfinic acid phosphoric ester on Prxs, which is then reduced by thiol equivalents, such as thioredoxins or glutathione, thereby reactivating the hyperoxidized Prxs [2, 3, 7] (Figure 13-1).

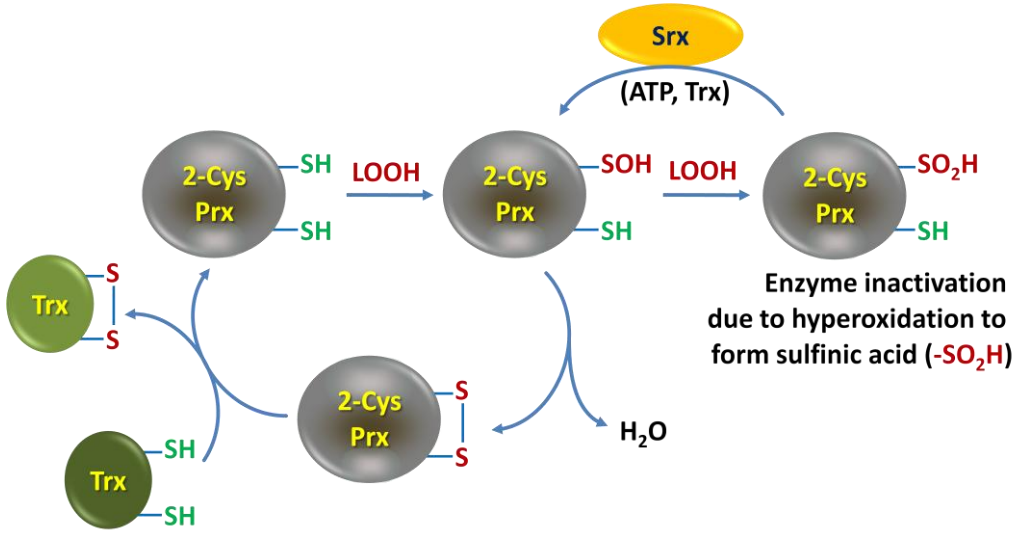

Figure 13-1. Hyperoxidation of peroxiredoxin (Prx) to form sulfinic acid, and sulfiredoxin (Srx)-mediated reduction of the Prx sulfinic acid to sulfenic acid. During normal reduction of peroxides (LOOH), the cysteine residue of Prx is oxidized to sulfenic acid (-SOH), which then leads to the formation of disulfide bridge. However, under excessive oxidative conditions, the sulfenic acid can be further oxidized to sulfinic acid (-SO_2H), leading to inactivation of the enzyme. Srx catalyzes reduction of sulfinic acid to sulfenic acid. This reaction is dependent on ATP and utilizes thioredoxin (Trx) or GSH (not shown) as the electron donor.

Srx has been shown to protect against oxidative injury primarily via reactivating Prxs. In addition, Srx has been found to catalyze deglutathionylation of proteins, including 2-Cys Prxs [8, 9]. Deglutathionylation is an important mechanism of post-translational modifications of proteins, including signaling molecules, and has been implicated in cell redox homeostasis and growth regulation [10].

2.2. Molecular Regulation

Srx is inducible by stress conditions, including hyperoxia. It has been demonstrated that Nrf2 regulates Srx expression via an antioxidant response element (ARE)-driven mechanism, which appears to be a major signaling event underlying the inducible expression of Srx under oxidative stress [11, 12]. Indeed, in silico analysis of the 5'-promoter-flanking region of Srx has identified multiple AREs that are highly conserved [13]. In addition, Srx expression has been shown to be regulated by the transcription factor AP-1 [12]. The proximal AP-1 binding site of the Srx promoter is found to be embedded within the ARE. Regulation of Srx may also

involve histone acetylation. It is reported that expression of Srx can be induced by enhancing histone acetylation through treatment of neurons with the histone deacetylase inhibitor trichostatin A [14]. Furthermore, inhibition of histone deacetylase by trichostatin A is associated with decreased formation of hyperoxidized Prxs in neurons exposed to oxidative stress [14].

3. Role in Health and Disease

Since its identification in 2003, Srx has been investigated in both cellular systems and animal models. Srx undergoes translocation into mitochondria in response to oxidative stress, and plays a crucial role in reducing hyperoxidized Prx3 within mitochondria [5]. As stated in Section 1.2 of Chapter 11, Prx3 is mainly localized in mitochondria. Overexpression of mitochondria-targeted Srx in a human embryonic kidney cell line also leads to decreased formation of mitochondrial reactive oxygen species (ROS) and increased resistance to apoptosis elicited by rotenone [5]. Rotenone is a pesticide that selectively blocks the complex I of mitochondrial electron transport chain, leading to increased ROS formation. Similarly, in cultured lung epithelial cells, overexpression of Srx protects cells from hydrogen peroxide (H_2O_2)-induced toxicity, whereas attenuation of Srx expression by RNA interference potentiates the toxicity of H_2O_2 [13].

Srx is markedly induced in the lungs of mice exposed to hyperoxia. This hyperoxic induction of Srx is shown to be mediated by Nrf2 signaling [11]. Hyperoxia also elicits the accumulation of the sulfinic form of the mitochondrial Prx3 in lung tissue, likely due to mitochondria being the major site of hyperoxia-induced production of ROS. Hyperoxia induces the degradation of Prx3 in the lung tissue of Nrf2-deficient mice but not in that of wild-type animals, suggesting that in the absence of a sufficient amount of Srx, sulfinic Prx3 is converted to a form that is susceptible to proteolysis [11]. Exposure of mice to cigarette smoke is also found to induce Srx expression in the lung tissue via an Nrf2-dependent mechanism, and such an Nrf2-dependent upregulation of Srx has been proposed to protect against cigarette smoke-elicited oxidative stress in lungs [13].

In addition to its involvement in protecting against oxidative stress, Srx is suggested to also participate in controlling cell proliferation and death pathways [15]. However, its role in cancer remains unclear. On the one hand, elevated levels of Srx in epithelial cancer cell lines are shown to promote cell proliferation. On the other hand, increased Srx levels sensitize the cancer cells to cisplatin-induced killing [15]. In a mouse epidermal JB6 cell model, knockdown of Srx is reported to abolish tumor promoter-induced transformation, suggesting that Srx is required for JB6 cell transformation [16]. Furthermore, screening of patient tissues by tissue microarray demonstrates elevated Srx expression in several types of human skin cancer [16]. The exact role of Srx in carcinogenesis awaits further investigations.

4. Conclusion and Future Directions

Srx, a newly identified redox-active enzyme appears to protect against oxidative stress in cellular systems and certain animal models. In addition to its antioxidant function, Srx also plays a role in reversal of protein glutathionylation as well as in cell growth regulation. The

role of Srx in health and disease currently remains largely unknown and thus warrants further investigations both in animal models and in human subjects.

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Chapter 14

Methionine Sulfoxide Reductase

Abstract

Mammalian methionine sulfoxide reductase (Msr) refers to two structurally unrelated classes of thiol-dependent enzymes, namely, MsrA and MsrB that catalyze the reduction of methionine *S*-sulfoxide and methionine *R*-sulfoxide, respectively. Cell experiments and animal studies demonstrate that Msrs play an important part in protecting against oxidative stress and oxidative stress-associated pathophysiological processes. Observational studies with human tissues and in human populations also suggest a possible role for Msrs in certain disease conditions, including neurological diseases and aging, skin disorders, cancer, and obesity.

1. Overview

Oxidative modifications of proteins have been implicated in various degenerative disease conditions and aging. As compared with most other amino acid residues in proteins, methionine is particularly susceptible to oxidation by reactive oxygen species (ROS) and other related species. The products of such oxidation are a diastereomeric mixture of methionine sulfoxides, as listed below.

- Methionine *S*-sulfoxide
- Methionine *R*-sulfoxide

These two oxidized forms of methionine can be reduced back to methionine by methionine sulfoxide reductase (Msr).

In mammals, Msr refers to two structurally unrelated classes of monomeric enzymes, namely, MsrA and MsrB. MsrA and MsrB catalyze the reduction of methionine *S*-sulfoxide and methionine *R*-sulfoxide, respectively, using thioredoxin as the electron donor [1, 2]. Mammals have a single MsrA gene, and the encoded Msr enzyme can exist in two forms, one in cytosol and the other in mitochondria. In contrast, there are three MsrB isozymes in mammals, namely, MsrB1, MsrB2, and MsrB3. MsrB1 is a selenoprotein and present in cytosol and nuclei. MsrB2 resides in mitochondria. MsrB3 can be localized in mitochondria or en-

doplasmic reticulum. Msr isozymes are ubiquitously expressed in mammalian tissues, but their expression levels vary in different tissues. The highest expression levels of MrsA have been reported in liver, kidney, and brain. MsrB1, like MsrA, is highly expressed in liver and kidney, whereas the highest expression levels of MsrB2 and MsrB3 are found in heart and skeletal muscle.

Among the four Msr isozymes (MsrA, MsrB1, MsrB2, and MsrB3) only MsrB1 is a selenoprotein, and is shown to have the highest catalytic activity due to the selenocysteine in the active site. MsrB1 is also known as selenoprotein R (Sel R; Section 1.1 of Chapter 6). The other Msr isozymes have cysteine residues in the active sites instead of selenocysteine. Msrs are low molecular mass proteins with molecular masses of approximately 25 kDa for MsrA, 12 kDa for MsrB1, and 20 kDa for MsrB2 and MsrB3. In humans, the genes for MrsA, MsrB1, MsrB2, and MsrB3 are localized on chromosomes 8p23.1, 16p13.3, 10p12, and 12q14.3, respectively.

2. Biochemistry and Molecular Regulation

2.1. Biochemistry

As stated above, MsrA and MsrB catalyze the reduction of methionine *S*-sulfoxide and methionine *R*-sulfoxide, respectively, to form the normal methionine using thioredoxin as the electron donor (Figure 14-1).

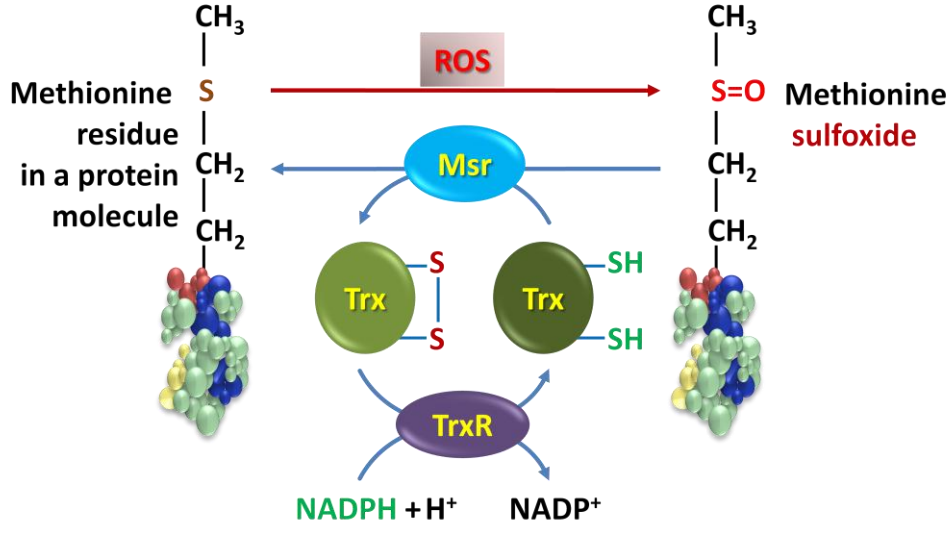

Figure 14-1. A simplified schematic illustration of methionine sulfoxide reductase (Msr)-catalyzed reduction of protein methionine sulfoxide. As depicted, thioredoxin (Trx) is used as the electron donor for the Msr-catalyzed reaction. Thioredoxin reductase (TrxR) regenerates the Trx from its oxidized form using NADPH as the reducing equivalent. See text (Section 2.1) for detailed description of the biochemical mechanisms underlying Msr-catalyzed reduction of protein methionine sulfoxide.

Mammalian MsrA has three conserved cysteine residues, which participate in the reaction as catalytic and resolving cysteines. A sulfenic acid intermediate at the catalytic cysteine is generated when this cysteine residue attacks the sulfur of methionine *S*-sulfoxide. Then, thiol-disulfide exchange involving the two resolving cysteine residues results in the formation of a disulfide bond on the enzyme surface, which is finally reduced by thioredoxin [2].

Mammalian MsrB1 has one conserved cysteine residue in the N-terminal portion and the catalytic selenocysteine in the C-terminal region. During catalysis, the selenocysteine is converted to selenenic acid intermediate upon interacting with the sulfur of methionine *R*-sulfoxide, and the selenenic acid intermediate rearranges to form seleneylsulfide with the help of the resolving cysteine residue. The seleneylsulfide is reduced to selenocysteine by thioredoxin. In contrast, mammalian MsrB2 and MsrB3 have only one conserved cysteine residue and seem to have evolved a different catalytic mechanism for the direct reduction of the sulfenic acid intermediate by thioredoxin [2].

Although thioredoxin is the most important cellular cofactor for mammalian Msr isozymes, other thiol equivalents may also be utilized as reductants under certain in vitro experimental conditions. These include dithiothreitol, thionein, and selenocystamine [1-3]. However, it remains unclear if these thiol compounds also support Msr activity in vivo.

2.2. Molecular Regulation

Some progress has recently been made regarding the regulation of mammalian Msr isozymes. However, the exact transcriptional and post-transcriptional mechanisms remain largely unknown. Studies in non-mammalian cells, including yeast demonstrate an important role for thioredoxin and calcium phospholipid-binding protein in controlling MsrA gene expression [4, 5]. In addition, yeast glutathione peroxidase-3 is shown to directly interact with MsrA and may modulate the MsrA activity in a redox state-dependent manner [6]. In *Caenorhabditis elegans*, MsrA expression is regulated by the DAF-16/FOXO pathway [7]. The transcriptional regulation of MsrA gene in mammalian cells may occur via two different promoters, whose activity is partially regulated by all-trans retinoic acid via retinoic acid receptors [8]. The transcriptional regulation of human MrsB1 has recently been investigated. Human MsrB1 gene promoter is shown to contain multiple SP1 binding sites, and the expression of MsrB1 is tightly controlled by the transcription factor SP1. In addition, the promoter activity of human MsrB1 gene is also subject to epigenetic modifications, such as methylation [9]. In MsrA-null mice, MsrB1 expression is diminished as compared with that in wild-type animals, suggesting that MsrA may have a role in modulating the expression of MsrB1 [10].

3. Role in Health and Disease

3.1. Animal Studies

3.1.1. Experimental Approaches

Similar to other antioxidant enzymes, both gene knockout and transgenic overexpression animal models have been created to understand the biological functions of Msrs. In this re-

gard, gene knockout of MsrA or MsrB1 in mice does not cause embryonic lethality, indicating that neither gene is essential for murine development. However, both MsrA-null mice and MsrB1-null mice develop phenotypes of oxidative stress and aging-related tissue degeneration, suggesting an antioxidant function for Msrs in mammals [11, 12]. In addition to the animal models, cellular systems have also been utilized to study the biological activities of Msrs. In this context, overexpression of Msrs in cultured cells is shown to protect against oxidative stress, whereas decreased Msr expression results in exacerbation of oxidative cell injury [13-15].

3.1.2. Role in Cardiovascular Diseases

A role for MsrA in ischemic cardiac injury has been suggested by the observation that both the cytosolic and mitochondrial Msr activities are reduced during myocardial ischemia-reperfusion injury [16]. Overexpression of MsrA in primary cardiomyocytes leads to attenuation of cell injury induced by hypoxia and reoxygenation [17]. Further studies using MsrA-null mice show that MsrA deficiency exaggerates protein oxidation, myocardial apoptosis, impaired cardiac function, and increased mortality after myocardial infarction, supporting a protective role for MsrA in ischemic heart disease in experimental animals [18].

There is also evidence for an involvement of Msrs in protecting against vascular degeneration and atherogenesis. For instance, overexpression of MrsA in vascular smooth muscle cells protects these cells from oxidative damage [19]. It is reported that methionine oxidation impairs reverse cholesterol transport by apolipoprotein A-I, a major component of high-density lipoprotein (HDL) [20]. HDL-mediated reverse cholesterol transport in the arterial wall is an important mechanism of suppressing atherogenesis. Reversing methionine oxidation with Msr is found to restore HDL's ability to transport cholesterol in vitro [20]. The exact role of Msrs in protecting against atherosclerosis and other vascular disorders warrants further studies in animal models, such as ApoE-null mice.

3.1.3. Role in Aging and Neurological Diseases

Early studies by E.R. Stadtman and coworkers demonstrated that as compared with wild-type mice, MsrA knockout ($MsrA^{-/-}$) mice exhibit enhanced sensitivity to hyperoxia-induced injury, and a shorter lifespan under both normal and hyperoxic conditions [11]. These $MsrA^{-/-}$ mice also develop an atypical (tip-toe) walking pattern after six months, and have higher tissue levels of oxidized protein under hyperoxia [11]. These results suggest that MsrA may play an important role in aging and neurological disorders. In line with these observations in mice, overexpression of MrsA in *Drosophila melanogaster* is found to markedly extend the lifespan. The MsrA-overexpressing flies are also more resistant to paraquat-induced oxidative stress, and the onset of senescence-induced decline in the general activity level and reproductive capacity is delayed markedly [21]. Overexpression of MsrA in yeast also prolongs lifespan. Although substantial evidence supports a critical protective role for MsrA in aging process in various models, a recent study reports that lack of MsrA in mice increases sensitivity to oxidative stress but does not diminish lifespan [22]. The effect of MsrB in aging has been recently examined. Overexpression of MsrB in yeast extends lifespan only under calorie restriction conditions. However, overexpression of MsrB has no influence on aging in *Drosophila melanogaster* [23].

There is evidence showing that MsrA is an important protector in certain neurodegenerative disorders in animal models. As compared with wild-type animals, MsrA knockout mice

exhibit lower locomotor activity and altered gait that exacerbate with age. Dopamine regulation and signaling pathways are impaired in these $MsrA^{-/-}$ mice, suggesting a possible involvement of MsrA in experimental parkinsonism [24]. Recently, it is further reported that dopamine D2 receptor function is compromised in the brain of $MsrA^{-/-}$ mice [25]. In *Drosophila melanogaster*, MsrA and S-methyl-L-cysteine (a substrate of MsrA) are shown to prevent parkinsonism caused by ectopic expression of human α-synuclein in the flies' nervous system [26]. In cultured cells, MsrA blocks dopaminergic cell death and protein aggregation induced by rotenone and mutant α-synuclein [27]. Both rotenone and mutant α-synuclein are implicated in the pathophysiology of parkinsonism.

Methionine oxidation has been suggested to play a role in β-amyloid toxicity, an important mechanistic event in Alzheimer's disease. $MsrA^{-/-}$ mice exhibit enhanced neurodegeneration in brain hippocampus as compared with wild-type mice. In addition, a loss of astrocyte integrity, elevated levels of β-amyloid deposition, and tau phosphorylation are dominant in various regions of $MsrA^{-/-}$ hippocampus but not in those of wild-type mice [28]. The cultured brain slices of the hippocampus region of $MsrA^{-/-}$ mice also show increased sensitivity to oxidative stress [28]. These findings indicate that a deficiency of MsrA activity may foster oxidative stress, accumulation of faulty proteins via methionine oxidation, deposition of aggregated proteins, and eventually premature brain cell death.

3.1.4. Role in Other Diseases and Conditions

Msrs have also been implicated in the protection of various other disease conditions involving an oxidative stress mechanism. As stated above in Section 1, MsrB1 is widely distributed throughout different tissues, but the highest levels are found in liver and kidney. MsrB1 knockout mouse model has recently been created, and studies using this model demonstrate that liver and kidney are most affected in terms of susceptibility to oxidative damage of proteins [12]. These observations suggest a role for MsrB1 in protecting against oxidative stress in liver and kidney

Msrs are expressed in eye tissues including lens and retina, and ample evidence supports a beneficial role for Msrs in eye disorders, especially cataract [29, 30]. Deletion of MsrA in mice results in hyperbaric oxygen-induced cataract possibly due to failure in repairing mitochondrial cytochrome c whose methionine residues are oxidized to methionine sulfoxides [30]. Targeted silencing of MsrA in cultured retinal pigmented epithelium or lens cells sensitizes these cells to oxidative injury [31, 32]. MsrB isozymes are also found to be expressed in retinal and lens cells, and protect these cells from oxidative stress [33, 34].

There is also evidence from both in vitro and in vivo models, showing beneficial effects of Msrs in skin disorders, immunity, and infections [35-39].

3.2. Human Studies and Clinical Perspectives

Although being not as convincing as that from animal experiments, evidence from studies in human subjects does exist pointing to a possible role for Msrs in human health and disease. Our current knowledge on the involvement of Msrs in human disease conditions results primarily from determining the changes of Msrs in tissue samples of patients with disease conditions as well as population studies aiming to identify the genetic loci or genetic variations of

Msrs in regard to disease risk. Largely based on these studies, a potential involvement of Msrs has been suggested in several disease conditions, including aging and neurological diseases, skin disorders, cancer, and obesity.

3.2.1. Role in Human Aging and Neurological Diseases

Gene polymorphism studies suggest an association between MsrA (-402C/T) polymorphism and longevity in a population of 1534 ethnic Tartars [40]. Studies with brain tissues of Alzheimer's patients demonstrate a significant decrease in MsrA activity compared with control subjects. mRNA analysis further suggests that the decreased MsrA activity is likely due to a post-transcriptional modification [41]. A recent study has identified a novel 4-base pair MsrA deletion associated with schizophrenia. It is shown that the 4-base pair deletion within the MsrA gene is under-transmitted to schizophrenia patients in two different populations, and causes reduced gene expression in individuals homozygous for the deletion [42]. The findings suggest that there may be a link between the MsrA polymorphism and risk of developing schizophrenia. Further studies on the mechanisms by which MsrA is involved in schizophrenia pathophysiology may provide important insights into the biological underpinnings of this neurological disorder.

3.2.2. Role in Human Skin Diseases

Both Msrs A and B are expressed in human skin tissue, including melanocytes. Patients with the depigmentation disorder vitiligo are shown to have low catalase expression and constantly accumulate millimolar concentrations of hydrogen peroxide (H_2O_2) in their skin tissues. Such high concentrations of H_2O_2 can readily oxidize methionine residues in proteins. Indeed, in vivo FT-Raman spectroscopy studies have revealed the presence of methionine sulfoxides in the depigmented skin of patients with active vitiligo [43]. Expression of MsrA and MsrB is also markedly decreased in the epidermis of patients with vitiligo compared with healthy controls. In vitro studies demonstrate that the recombinant human MsrA and MsrB enzymes are readily inactivated by H_2O_2 at concentrations relevant to those seen in the depigmented skin tissues of patients with vitiligo [43]. Together, these observations suggest an important protective role for Msrs in human vitiligo.

Senile hair graying may also involve loss of Msr activity in the hair follicles. An in vivo study with FT-Raman spectroscopy shows that human gray/white scalp hair shafts accumulate H_2O_2 in millimolar concentrations [44]. An almost absent protein expression for catalase and MsrA and MsrB in association with a functional loss of methionine sulfoxide repair is demonstrated in the entire gray hair follicles. Furthermore, oxidation of the methionine residues, including Met 364 in the active site of tyrosinase, the key enzyme in melanogenesis, leads to enzyme inactivation [44]. Collectively, these results point to a causal involvement of Msr inactivation in senile hair graying.

3.2.3. Role in Human Cancer and Obesity

MsrA gene is linked to carcinogenesis in humans. It is reported that chromosome 8p deletions contribute to metastasis of hepatocellular carcinoma. Since human MsrA is localized to chromosome 8p23.1, MsrA is suggested to be a candidate gene. Further studies using oligonucleotide microarrays have identified MsrA gene on chromosome 8p as a possible metastasis suppressor for human hepatitis B virus-positive hepatocellular carcinoma [45]. In a hepatocel-

lular cancer cell line, overexpression of MsrA is shown to inhibit cell colony formation and invasion [45]. The protective role of MsrA as well as other Msr isozymes in carcinogenesis warrants further investigations.

The accumulation of abnormal amounts of intra-abdominal fat (central adiposity) is associated with serious adverse metabolic and cardiovascular outcomes, including type 2 diabetes and atherosclerotic coronary heart disease. A recent genome-wide association scan meta-analysis involving data from 38,580 individuals has identified three loci influencing adiposity and fat accumulation, among which is MsrA. This locus of MsrA is strongly associated with central adiposity [46]. Further mechanistic studies will provide novel insights into the role of MsrA in human physiology and development of obesity.

4. Conclusion and Future Directions

Msr isozymes protect cells from oxidative stress via catalyzing the reduction of methionine sulfoxides to methionine. Studies using genetically manipulated animal models have provided convincing evidence supporting a beneficial role for Msrs in various pathophysiological conditions involving an oxidative and inflammatory stress mechanism. Evidence from studies in human subjects also suggests a potential involvement of Msrs in modulating the risk of developing certain diseases. There is a great need to understand the effects (either protective or detrimental) of various Msr isozymes in pathophysiology of other common diseases in animal models, including diabetes and metabolic syndrome, cancer, as well as disorders affecting lung, liver, and kidney. Future studies should also emphasize translational and clinical research to further explore the biological functions of Msr isozymes in relation to human health and disease.

5. References

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Chapter 15

Heme Oxygenase

Abstract

Mammalian heme oxygenase (HO) refers to a family of two major isozymes, namely, HO-1 and HO-2 that catalyze the first and rate-limiting step in the oxidative degradation of heme to eventually produce bilirubin along with the release of carbon monoxide and iron. The antioxidant and anti-inflammatory activities of HO enzymes and their products bilirubin and carbon monoxide are well-recognized. Extensive animal studies have established a critical role for HO enzymes, especially HO-1 in protecting against various disease conditions, ranging from cardiovascular diseases to skin disorders. However, studies also suggest a promoting activity for HO-1 in cancer growth and progression in animal models, which is believed to result from the proproliferative and proangiogenic effects of HO-1. A growing body of evidence from studies in human subjects indicates a beneficial role for HO-1 in various disease processes, which is consistent with findings from animal experiments. On the other hand, studies in cancer patients suggest that HO-1 may play either an inhibitory or a promoting role in human cancer development depending on the types of cancer.

1. Overview

Heme oxygenase (HO) was originally identified in 1968 and 1969 by R. Tenhunen and coworkers in seminal papers where they characterized the enzyme HO and its cellular localization [1, 2]. HO is the rate-limiting enzyme in heme catabolism. In mammals, there are two major isozymes of HO: the inducible form HO-1 and the constitutively expressed form HO-2. Expression of HO-1 occurs at low levels in most tissues under normal conditions with the exception of spleen (the site of red blood cell hemoglobin turnover) and several other unique cell types (e.g., renal inner medullary cells, Kupffer cells in the liver, Purkinje cells in the cerebellum, $CD4^+/CD25^+$ regulatory T cells). HO-2 is constitutively expressed in mammalian tissues under physiological conditions, with the highest levels found in brain and testes. The molecular masses for HO-1 and HO-2 are 32 and 36 kDa, respectively. In mammalian cells, HO-1 and HO-2 are mainly associated with endoplasmic reticulum. HO-1 is also reported to localize to distinct subcellular compartments, including plasma membrane caveolae, mitochondria, and nuclei. A putative third isozyme of HO, namely, HO-3 is reported to be ex-

pressed in rat tissues. However, the in vivo significance of this putative isozyme in mammalian systems is unclear. In humans, HO-1 and HO-2 are localized on chromosomes 22q13.1 and 16p13.3, respectively.

2. Biochemistry and Molecular Regulation

2.1. Biochemistry

Both HO-1 and HO-2 catalyze the first and rate-limiting step in the oxidative degradation of heme to form the open-chain tetrapyrrole biliverdin with concurrent release of ferrous ion (Fe^{2+}) and carbon monoxide (CO). This reaction requires molecular oxygen as well as reducing equivalents derived from NADPH cytochrome P450 reductase. The HO-catalyzed reaction displays regiospecificity for the heme molecule such that only the α-isomer of biliverdin is produced. The biliverdin formed is subsequently reduced to bilirubin by biliverdin reductase (Figure 15-1) [3, 4].

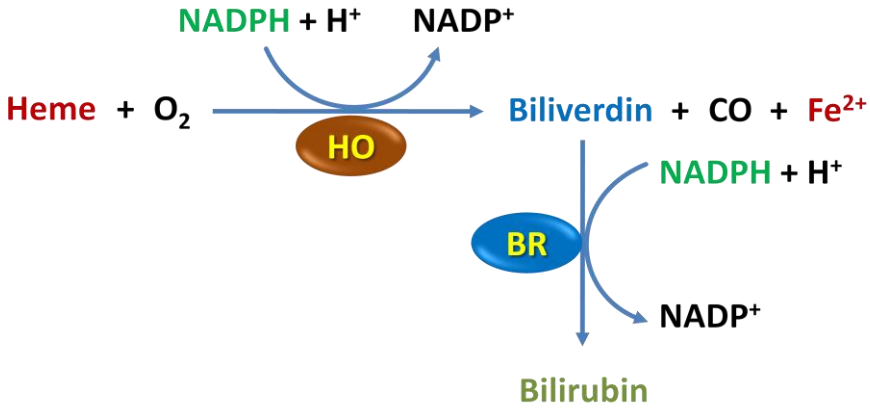

Figure 15-1. Heme oxygenase (HO)-catalyzed degradation of heme. As illustrated, HO catalyzes the degradation of heme to form biliverdin along with the release of carbon monoxide (CO) and iron. Biliverdin is further converted to bilirubin by biliverdin reductase (BR). NADPH serves as the electron donor for both HO- and BR-catalyzed reactions.

The biological activities of HO isozymes are largely determined by their biochemical properties, which include the follows.

- Degradation of heme
- Formation of biliverdin
- Formation of bilirubin
- Production of carbon monoxide
- Release of iron

Since heme is a pro-oxidant, its degradation by HO minimizes oxidative stress induced by excess heme. Both biliverdin and bilirubin possess antioxidant activities as well as other cytoprotective functions, such as anti-inflammation and antiproliferation (see Chapter 19 for discussion of bilirubin). Carbon monoxide is also known to exert anti-inflammatory, antiproliferative, and vasodilatory activities [5]. Due to the pro-oxidant activity of iron (e.g., participation in Fenton reaction, leading to the formation of hydroxyl radical, the most reactive species formed in biological systems), the iron released from HO-catalyzed reaction would seem to lead to detrimental effects. However, elevation of HO activity and release of iron are usually associated with concurrent induction of ferritin, an iron-chelating protein (see Chapter 18 for discussion of ferritin). Induction of ferritin thus minimizes the pro-oxidant potential of the released iron. The reaction products biliverdin, bilirubin, and carbon monoxide primarily mediate the antioxidative and anti-inflammatory activities of the HO isozymes under various disease conditions. Recent work has identified HO-1 and its gaseous product, carbon monoxide to also possess potent proangiogenic properties [6, 7]. There is also evidence suggesting that HO-2 may act as an intracellular sensor of oxygen, nitric oxide, and carbon monoxide in mammalian systems [8].

2.2. Molecular Regulation

Regulation of Mammalian HO-1 expression occurs primarily at the transcriptional level though post-transcriptional modifications may also be involved. HO-1 gene expression is highly inducible by diverse stimuli, including heme, reactive oxygen and nitrogen species (ROS/RNS), hyperoxia, hypoxia, electrophiles, heavy metals, ultraviolet light, and inflammatory cytokines [3, 4].

A number of signaling pathways have been implicated in the regulation of HO-1 gene expression in mammalian cells. These include mitogen activated protein kinases (MAPKs), tyrosine kinases, phosphatidylinositol 3-kinase (PI3K) as well as protein kinases A, G, and C. Consistent with the involvement of diverse signaling cascades in the induction of HO-1 gene expression, the promoter region of HO-1 contains a wide variety of regulatory elements for transcription factors, among which are AP-1, NF-κB, Nrf2, and hypoxia-inducible factor-1 (HIF-1). Evidence also suggests that HO-1 protein localized in nuclei may activate transcription factors (e.g., AP-1) important in oxidative stress [9]. It is thought that nuclear localization of HO-1 protein may serve to upregulate genes that promote cytoprotection against oxidative stress. The exact molecular mechanisms underlying HO-1-mediated transcriptional regulation remain to be elucidated.

In contrast to the structure of the HO-1 gene, which has a simple composition of 5 exons and 4 introns, that of mammalian HO-2 is among the most complex gene structures. Five or more transcripts for HO-2 are present in mammalian tissues, with tissue-dependent patterns of display and abundance. However, studies on transcriptional regulation of HO-2 gene have revealed that the glucocorticoid response element (GRE) is the only functional responsible element in the promoter region of HO-2 gene [8]. Although HO-2 is constitutively expressed, it expression is subject to redox regulation. In addition, there is a direct protein-protein interaction between HO-2 and HO-1 [10]. Furthermore, it has been shown that HO-2 can downregulate the expression of HO-1, thereby directing the coordinated expression of HO-1 and HO-2 [11].

3. Role in Health and Disease

3.1. Animal Studies

3.1.1. Experimental Approaches

Various experimental approaches have been utilized to investigate the biological functions of both HO-1 and HO-2 enzymes. These include: (1) transgenic overexpression and gene knockout mouse models, (2) viral vector-mediated HO gene transfer, and (3) use of chemical inhibitors (e.g., metalloporphyrins) or chemical inducers (e.g., hemin) to modulate HO activity in experimental animals. Among these various approaches, genetically manipulated mouse models have been extensively employed, and have provided the most convincing evidence on the role of HO-1 and HO-2 enzymes in particular disease conditions. In contrast, although the above chemical inducers or inhibitors of HO exhibit certain selectivity for HO activity, the results should be interpreted with caution due to other potential effects of these chemical agents.

3.1.2. Role in Cardiovascular Diseases

HO-1 is one of the best characterized antioxidants proven to be effective in ameliorating cardiovascular pathophysiology associated with oxidative and inflammatory stress in animal models. The cardiovascular disorders in which HO-1 plays a beneficial role include hypertension, atherosclerosis, myocardial ischemia-reperfusion injury, cardiac transplantation, and heart failure. These well-documented cardiovascular protective effects of HO-1 in experimental animals have been reviewed extensively [12-16].

HO-2 is constitutively expressed in myocardium and vasculature. Multiple studies also suggest a potential protective role for HO-2 in cardiovascular pathophysiology. HO-2 is shown to regulate vascular tone, and deletion of HO-2 causes endothelial cell dysfunction and increased blood pressure in experimental models [17-19]. However, a recent study reports that targeted disruption of HO-2 in mice does not aggravate hypertension induced by either chronic infusion of angiotensin II or inhibition of nitric oxide by N(G)-nitroarginine methyl ester [20]. Hypoxemia is found to induce HO-2 protein expression in myocardium of mice [21]. But, the role of HO-2 in myocardial ischemia-reperfusion injury as well as atherosclerosis has not been documented in the literature. Hence, the function of HO-2 in cardiovascular diseases remains to be further determined.

3.1.3. Role in Diabetes and Metabolic Syndrome

Upregulation of HO-1 by chemical inducers (e.g., hemin, tin chloride, cobalt protoporphyrin) attenuates hyperglycemia and insulin resistance, and protects against diabetic complications in various animal models of diabetes [22-25]. In line with these in vivo observations, overexpression of HO-1 is found to protect cultured pancreatic β-cells from apoptosis caused by prodiabetic stimuli, including inflammatory cytokines [26]. There is also evidence supporting a beneficial role of HO-2 in diabetes and metabolic syndrome in experimental animals. For instance, HO-2-null mice are shown to be obese, display insulin resistance, and have high blood pressure [19]. HO-2-deficient mice are also more susceptible to streptozotocin-induced diabetes, diabetic nephropathy, and oxidative stress. The diabetic nephropathy in these HO-2-deficient mice can be ameliorated by induction of HO-1 activity via treatment

with cobalt protoporphyrin, suggesting a complementary function of HO-1 and HO-2 in protecting against experimental diabetes [27].

3.1.4. Role in Neurological Diseases

HO-2 is highly expressed in brain and cerebral vessels, and has been extensively studied in regard to neuroprotection. Substantial evidence supports that HO-2 plays a protective role in various neurological disorders, including focal cerebral ischemic injury, injury due to intracerebral hemorrhage, traumatic brain and spinal cord injury, and neurodegenerative disorders in animal models. In addition, HO-1 is also found to be neuroprotective.

3.1.4.1. Cerebral Injury due to Ischemia and Hemorrhage

Targeted disruption of HO-2 in mice leads to exacerbation of infarct volume after middle cerebral artery occlusion [28]. Infusion of tin protoporphyrin IX, an HO inhibitor, increases infarct volume in wild-type mice but not in HO-2-null mice. It is further shown that the greater infarct volume in HO-2-null mice is not attributable to a larger volume at risk, lower intraischemic blood flow, or poor reflow, but rather to a neuroprotective effect of HO-2 activity [28]. Indeed, overexpression of HO-2 in neurons protects these cells from oxidative injury [29]. Transgenic overexpression of HO-1 in mice has been demonstrated to be neuroprotective in a model of permanent middle cerebral artery occlusion [30]. Stimulation of HO-1 activity by pharmacological agents also suppresses ischemic injury during the acute period of stroke in experimental animals. Consistently, overexpression of HO-1 in neuronal cells is found to protect these cells from toxicity elicited by oxidative insults, including ROS and heme.

Genetic deletion of HO-2 renders cultured neurons more vulnerable to hemin-induced cytotoxicity. Consistently, HO-2 knockout mice exhibit increased brain injury volume and worsened neurological functions following exposure to free heme liberated from hemorrhage [31]. While these in vitro and in vivo findings demonstrate that HO-2 is a crucial neuroprotective enzyme in detoxifying high levels of heme in the brain following intracerebral hemorrhage, other studies suggest a detrimental effect of HO-2 as well as HO-1 in intracerebral hemorrhage [32, 33]. Thus, further work is needed to determine the exact role of HO-2 in intracerebral hemorrhage-elicited brain injury.

3.1.4.2. Traumatic Brain and Spinal Cord Injury

Increased formation of ROS/RNS and oxidative stress are implicated in the pathogenesis of traumatic brain injury. The role of HO-2 in attenuating traumatic brain injury has been studied in HO-2-deficient mice. Gene knockout of HO-2 in mice aggravates early pathogenesis after traumatic injury to the immature brain, as evidenced by markedly increased apoptosis in the hippocampal dentate gyrus and a greater cortical lesion volume in the knockouts relative to wild-type counterparts within the first week post-injury [34]. In a controlled cortical impact traumatic brain injury model of adult mice, targeted disruption of HO-2 is found to exacerbate lipid peroxidation-mediated neuron loss and impaired motor recovery. This suggests that the constitutively expressed neuronal HO-2 may protect neurons against traumatic brain injury by reducing lipid peroxidation via the catabolism of free heme [35]. Studies using heterozygous HO-1-deficient ($HO\text{-}1^{+/-}$) mice also demonstrate that HO-1 can stabilize the blood-spinal cord barrier and limit oxidative stress and white matter damage in acutely injured murine spinal cord [36].

3.1.4.3. NEURODEGENERATIVE DISEASES

There is ample evidence showing a beneficial involvement of HO isozymes, especially HO-1 in neurodegenerative disorders, including Alzheimer's disease and Parkinson's disease. Early studies demonstrated that amyloid precursor proteins bind to HO, inhibit the HO activity, and decrease bilirubin formation in animal models [37]. The amyloid precursor protein-HO interactions are also associated with augmented oxidative neurotoxicity, an important event in Alzheimer's disease. Subsequently, exogenous administration of HO-1 protein has been shown to augment the phagocytosis and clearance of Aβ peptides in rat and mouse microglia. Overexpression of HO-1 in neuronal cells also leads to decreased levels of tau protein [38]. These in vivo and in vitro observations suggest that HO-1 acts as a protective molecule in the pathophysiological process of Alzheimer's disease.

Adenovirus-mediated HO-1 gene transfer has been reported to protect dopaminergic neurons from 1-methyl-4-phenylpyridinium (MPP^+)-induced neurotoxicity, a commonly used experimental model of parkinsonism [39]. The inflammatory stress in substantia nigra induced by MPP^+ is diminished by HO-1 overexpression. In contrast, inhibition of HO enzymatic activity by zinc protoporphyrin-IX aggravates MPP^+-elicited parkinsonism in mice. In addition, overexpression of HO-1 in cultured dopaminergic neurons also renders these cells resistant to MPP^+-induced neurocytotoxicity [39]. Moreover, HO-1 is found to protect mouse brain from glutamatergic excitotoxicity [40], an important mechanism underlying neurodegenerative disorders, including Parkinson's disease. Together, these in vitro and in vivo data support a beneficial effect of HO-1 in experimental parkinsonism.

3.1.5. Role in Pulmonary Diseases

Substantial evidence suggests a critical role for HO-1 in various pulmonary disorders involving oxidative stress and inflammation [41]. These include hyperoxic lung injury, lung ischemia-reperfusion injury, airway inflammation, and drug-induced pulmonary toxicity.

3.1.5.1. HYPEROXIC LUNG INJURY

Overexpression of HO-1 in lung tissue via intratracheal adenovirus-mediated gene transfer markedly attenuates hyperoxia-induced pulmonary injury, as evidenced by decreased lung inflammation and apoptosis as well as improved survival [42]. In line with the in vivo observations, overexpression of HO-1 in lung epithelial cells protects these cells from oxidative injury. However, targeted disruption of HO-1 in mice is reported to render these animals more resistant to hyperoxia-induced lung oxidative stress and mortality as compared with wild-type animals [43]. Deletion of HO-1 gene is also associated with decreased lung reactive iron and iron-associated proteins. It remains unclear why HO-1 overexpression and gene knockout studies yield opposite conclusions on the role of HO-1 in hyperoxic lung injury. Evidence from in vivo models implicates a potential toxic threshold for HO-1 overexpression related to iron accumulation. For instance, hyperoxia sensitivity is observed at high levels of HO-1 overexpression, whereas hyperoxia resistance is seen at moderate levels of transient overexpression of HO-1 in experimental animals [41].

3.1.5.2. LUNG ISCHEMIA-REPERFUSION INJURY

Lung ischemia-reperfusion injury is the inciting event in acute lung failure following transplantation, surgery, and shock. It is reported that HO-1-null mice are much more suscept-

ible to lung ischemia-reperfusion injury as compared with wild-type littermates. HO-1-null mice exhibit lethal ischemic lung injury, but are rescued from death by inhaled carbon monoxide [44]. Intranasal administration of siRNA targeting HO-1 results in selective knockdown of lung HO-1 and exacerbation of ischemia-reperfusion-induced lung cell apoptosis in mice [45]. Together, these findings demonstrate a beneficial effect of HO-1 in lung ischemia-reperfusion injury.

3.1.5.3. Airway Inflammation

The increasing recognition of the anti-inflammatory activity of HO-1 has led to extensive studies on this enzyme in protecting against airway inflammation. Induction of HO-1 by hemin markedly attenuates allergen-induced airway inflammation, oxidative stress, and hyperreactivity in guinea pigs and mice [46, 47]. Conversely, inhibition of HO activity by metalloporphyrins aggravates the above pathophysiological responses in animal models of airway inflammation. Carbon monoxide, one of the products of HO-catalyzed degradation of heme (Scetion 2.1), is also shown to attenuate airway inflammation and hyperreactivity in experimental animals.

3.1.5.4. Drug-induced Pulmonary Toxicity

Lung fibrosis represents one of the most serious adverse effects of drugs, such as bleomycin. Adenovirus-mediated HO-1 gene transfer is shown to protect against bleomycin-induced pulmonary fibrosis in a mouse model. The protection is associated with decreased epithelial cell apoptosis and increased interferon-γ production, and is independent of Fas-Fas ligand pathway [48]. Paradoxically, treatment of mice with the HO inhibitor, zinc-deuteroporphyrin IX-2,4-bisethylene glycol is reported to attenuate bleomycin-induced lung fibrosis and production of transforming growth factor-β [49]. This observation apparently conflicts with that of the aforementioned HO-1 gene transfer study. As stated above in Section 3.1.1, pharmacological inhibitors of HO may also exert other potential actions, which can confound the effects due to HO inhibition.

3.1.6. Role in Hepatic and Gastrointestinal Diseases

HO-1 plays a role in protecting against various liver and gastrointestinal diseases in animal models. Among them are liver ischemia-reperfusion injury, liver injury from systemic inflammatory response syndrome, drug/xenobiotic-induced hepatotoxicity, nonalcoholic fatty liver disease, and colitis.

3.1.6.1. Liver Injury from Ischemia-Reperfusion and Systemic Inflammatory Response Syndrome

Either gene transfer or chemical induction of HO-1 protects liver from ischemia-reperfusion injury, as evidenced by prolonged animal survival, attenuated oxidative and inflammatory stress, and decreased apoptosis of hepatocytes [50, 51]. On the other hand, suppression of HO enzyme activity leads to potentiation of liver ischemia-reperfusion injury and oxidative and inflammatory stress in experimental animals. Adenovirus-mediated gene transfer of HO-1 also attenuates hepatocellular injury and liver dysfunction caused by systemic inflammatory response syndrome, supporting a potent anti-inflammatory activity of HO-1 in liver injury [52].

3.1.6.2. Hepatotoxicity and Oxidative Stress

The antioxidative and anti-inflammatory activities of HO-1 are also implicated in hepatoprotection against drug/xenobiotic-induced toxicity [53-55]. Overexpression of HO-1 in liver either by gene transfer or chemical induction results in attenuation of hepatotoxicity induced by acetaminophen, carbon tetrachloride, alcohol, and heavy metals. It is noted that hepatotoxicity induced by the above insults involves oxidative and inflammatory stress mechanisms. These findings support an important function of HO-1 in protecting hepatocytes from oxidative and inflammatory stress in experimental animals. Recently, hepatocyte-specific deletion of HO-1 is found to cause oxidative stress in the absence of exogenous stressors, indicating a critical role for HO-1 in maintaining the redox homeostasis of hepatocytes under basal physiological conditions [56].

3.1.6.3. Nonalcoholic Fatty Liver Disease and Inflammatory Bowel Disease

There is evidence for a protective effect of HO-1 in experimental steatohepatitis, a severe form of nonalcoholic fatty liver disease. It is further found that the protection by HO-1 results from decreased production of proinflammatory cytokines and modulation of fatty acid turnover [57]. Studies using various models of murine colitis also demonstrate a crucial role for HO-1 in attenuating colonic inflammation and mucosal injury. In this context, the products derived from HO-mediated catabolism of heme, including bilirubin, carbon monoxide, and biliverdin have all been shown to be protective against experimental murine colitis [58, 59]. Upregulation of HO-1 by pharmacological agents also contributes to the inhibition of experimental colitis in animal models. These findings point to the feasibility of developing therapeutic modalities for inflammatory bowel disease via targeting HO-1 in the gut [60-62].

3.1.7. Role in Renal Diseases

While the basal levels of HO-1 in renal tissue are relatively low, constitutive expression of HO-2 is detectable in various cell populations of kidney. There is increasing experimental evidence for an important cytoprotective role of HO isozymes, especially HO-1 in diverse forms of kidney diseases. These include renal ischemia-reperfusion injury and kidney transplantation, nephrotoxicity induced by drugs/xenobiotics, diabetic nephropathy, kidney injury induced by systemic inflammatory response syndrome, and obstructive kidney disease [63, 64]. Notably, increased renal expression of HO-1 is often observed under the above disease conditions, implicating a possible compensatory mechanism of HO-1 induction in protecting against the disease pathophysiology.

3.1.7.1. Renal Ischemia-Reperfusion Injury and Kidney Transplantation

Using genetic mouse models and gene transfer of HO-1, extensive studies have established a critical involvement of HO-1 in protecting against ischemia-reperfusion-induced renal cell death, inflammation, and oxidative stress [64]. Expression of HO-1 or administration of carbon monoxide and biliverdin has also been causally linked to prolonged xenograft survival in animal models of kidney transplantation [65-67].

3.1.7.2. Drug-induced Nephrotoxicity and Diabetic Nephropathy

Studies in animal models with either overexpression or suppression of HO-1 demonstrate that HO-1 is a critical cytoprotector against nephrotoxicity induced by cisplatin, an effective anticancer drug whose clinical use is limited by nephrotoxicity [68, 69]. There is also evi-

dence suggesting a protective activity of HO-1 in kidney injury induced by cyclosporine A and radiocontrast agents.

Both HO-1 and HO-2 have been implicated in diabetic nephropathy. It is shown that HO-2 deficiency (HO-$2^{-/-}$) in mice aggravates diabetes-mediated renal dysfunction and oxidative stress, as reflected by increased levels of plasma creatinine, acute tubular damage, microvascular pathology, and augmented superoxide production. Upregulation of HO-1 by administration of cobalt protoporphyrin prevents the above pathophysiological changes, whereas inhibition of HO activity by treatment with tin mesoporphyrin accentuates the renal injury in the HO-2-deficient diabetic mice [27]. These observations indicate an important function for both HO-1 and HO-2 in suppressing diabetic nephropathy and associated oxidative stress in experimental animals.

3.1.7.3. Renal Injury from Systemic Inflammatory Response Syndrome and Ureteral Obstruction

On the one hand, deficiency of HO-1 in mice impairs renal hemodynamics and aggravates systemic inflammatory responses to renal ischemia-reperfusion [70]. On the other hand, it is shown that as compared with wild-type counterparts, HO-1-deficient mice are much more susceptible to renal cell apoptosis, kidney dysfunction, and inflammation caused by systemic inflammatory response syndrome [71]. Both scenarios point to HO-1 as a potent anti-inflammatory defense in kidney injury.

Targeted disruption of HO-1 in mice is reported to promote epithelial-mesenchymal transition and renal fibrosis following unilateral ureteral obstruction [72]. HO-1 deficiency also results in increased formation of tubular transforming growth factor-β1 and augments inflammation in the above mouse model of obstructive kidney disease.

3.1.8. Role in Skin Diseases

Skin is constantly exposed to both endogenous and environmental oxidants and proinflammatory stimuli. Oxidative stress and inflammation are critical mechanisms underlying various human skin disorders, including dermatitis. Multiple studies show that overexpression of HO-1 via chemical induction attenuates contact dermatitis elicited by different sensitizers in animal models, whereas inhibition of HO-1 activity potentiates the dermatitis [73-75]. It is further found that HO-1 inhibits T cell-dependent skin inflammation and the function of antigen-presenting cells [76]. Consistent with a protective effect of HO-1 in skin inflammation and dermatitis, this enzyme is often found to be highly induced in the epidermis during dermatitis in experimental animals.

HO-1 is also remarkably elevated in epidermis after skin wounding in animal models. Inhibition of HO activity by tin protoporphyrin-IX results in retardation of cutaneous wound closure [77]. Healing is also delayed in HO-1-deficient mice, in which lack of HO-1 leads to complete suppression of reepithelialization and thereby the formation of extensive skin lesions, accompanied by impaired neovascularization. Transgenic overexpression of HO-1 selectively in keratinocytes improves the neovascularization and hastens the closure of wounds. It is further demonstrated that induction of HO-1 in wounded skin tissue is relatively weak in diabetic (db/db) mice. Angiogenesis and wound healing are also impaired in these diabetic animals. Local delivery of HO-1 transgene using adenoviral vectors promotes wound healing and increases the vascularization in these diabetic mice [77]. Together, these data strongly

support a critical function of HO-1 in skin wound healing and vascularization. The promoting activity of HO-1 in angiogenesis may also have implications for tumor growth and progression, as discussed in the next section.

3.1.9. Role in Cancer

Recently, increasing evidence suggests that HO-1 may play a role in cancer development via influencing multiple processes. These include cancer cell growth, angiogenesis, metastasis, resistance to drug therapy, and chemical carcinogenesis [6].

3.1.9.1. Cancer Cell Growth

HO-1 overexpression is frequently observed in various types of cancer, and its expression also correlates with the rate of cancer cell proliferation. In cell cultures, overexpression of HO-1 leads to increased cell proliferation and decreased apoptosis, whereas suppression of HO-1 reverses the proproliferative effect. High levels of HO-1 expression resulting either from chemical induction or genetic manipulation are shown to also promote the growth of various types of tumors in experimental animals, including pancreatic cancer, melanoma, angioma, hepatocellular carcinoma, and prostate cancer [78-82]. Conversely, inhibition of HO-1 decreases the tumor growth in the animal models.

3.1.9.2. Angiogenesis and Cancer Metastasis

Angiogenesis, formation of new blood vessels from pre-existing ones, plays a critical role in tumor growth and progression. There is substantial in vitro and in vivo evidence showing that HO-1 possesses potent proangiogenic properties, which may be an important mechanism underlying the tumor-promoting activities of this enzyme [6, 7]. The proangiogenic properties of HO-1 may also contribute to tumor progression and metastasis mediated by HO-1 expression, as angiogenesis is essential not only for tumor growth, but also for metastasis.

3.1.9.3. Cancer Drug Resistance

The ability of HO-1 to protect cells from various stressors, including cytotoxic compounds implicates that this enzyme may also be involved in tumor cell resistance to chemotherapy. Indeed, chemical inhibition or targeted knockdown of HO-1 increases the sensitivity of tumor cells to anticancer drugs, whereas upregulation of HO-1 protects these cells from anticancer drug-induced cytotoxicity. In line with the in vitro observations, studies in animal models of cancer demonstrate that inhibition of HO-1 improves the survival of cancer-bearing animals after treatment with anticancer drugs. Conversely, overexpression of HO-1 renders the tumor cells more resistant to anticancer drugs, and thereby decreases the effectiveness of chemotherapy [6]. As such, suppression of HO-1 has been suggested as a potential approach to sensitizing cancer cells to chemotherapy [6, 83].

3.1.9.4. Chemical Carcinogenesis

While a growing body of evidence indicates a role for HO-1 in promoting tumor growth and progression in a variety of animal models, the involvement of HO-1 in chemical carcinogenesis remains unclear. On the one hand, HO-1 acts as a cytoprotective protein in healthy tissues exposed to harmful stimuli, including chemical carcinogens, and may thus protect against chemical carcinogenesis. On the other hand, the antiapoptotic and proproliferative properties of HO-1 may facilitate tumor promotion and progression during multistage chemi-

cal carcinogenesis. The exact role of HO-1 in chemical carcinogenesis awaits further investigations using HO-1 gene knockout and overexpression animal models.

3.1.10. Role in Other Diseases and Conditions

As an important defense against oxidative and inflammatory stress, HO enzymes have also been implicated in protecting against various other disease processes. These include anemia [84], arthritis [85], viral and parasitic infections [86-88], sepsis [89], eye disorders [90], sickle cell disease [91], and reproductive disorders [92] .

3.2. Human Studies and Clinical Perspectives

Our understanding of the role of HO enzymes, particularly HO-1 in human health and disease, results from studies in three general areas. They are (1) case reports of HO-1 deficiency, (2) observational studies on HO-1 changes in tissue samples of patients with various disease conditions, and (3) HO-1 gene polymorphism studies in human populations in relation to changes in disease risk.

3.2.1. HO-1 Deficiency

The first human case of HO-1 deficiency due to deletion of exons 2 and 3 was reported by A. Yachie and coworkers in 1999 [93]. This patient exhibited marked growth retardation, severe hemolytic anemia, a low level of serum bilirubin, and tissue iron deposition among other clinical abnormalities. In addition, a lymphoblastoid cell line derived from the patient has been shown to be highly vulnerable to oxidative stress injury as compared with normal human cells. Notably, growth retardation, anemia, iron deposition, and vulnerability to oxidative stress injury found in this patient are all characteristics observed in mice lacking HO-1 ($HO\text{-}1^{-/-}$) [84].

3.2.2. HO-1 Activity in Diseased Tissues

HO-1 activity is often induced in tissue samples from patients with various disorders, including cardiovascular diseases, neurodegeneration, pulmonary diseases, and cancer. However, such observations do not allow us to discriminate whether HO-1 upregulation is a cause, a consequence, or an accompanying phenomenon of the disease pathophysiology. Nevertheless, such studies do provide important clues to the possible involvement of HO-1 in human disease conditions, and necessitate further research to assess the causal role of HO-1 in disease processes.

3.2.3. HO-1 Gene Polymorphisms in Non-cancer Diseases

The involvement of HO-1 in human health and disease has been investigated by analysis of clinical data and comparison of HO-1 allele frequency distribution in healthy people versus patients. Human HO-1 gene promoter is highly polymorphic, and several polymorphisms are shown to modulate the expression of HO-1. HO-1 gene polymorphisms are associated with a wide range of disease conditions involving almost all the major human organs or systems. Although some studies report lack of an association between HO-1 gene polymorphisms and risk of developing certain diseases, overwhelming evidence supports the notion that HO-1

may play an important role in protecting against human diseases that involve mechanisms of oxidative stress and inflammation [94-101]. Studies on HO-1 gene polymorphism may help identify human subpopulations susceptible to developing certain diseases, and may thus provide a rational basis for devising effective strategies for disease intervention in the high risk subgroups.

3.2.4. HO-1 Gene Polymorphisms in Cancer

Gene polymorphism studies have also provided important insights into the role of HO-1 in human cancer development. Multiple reports demonstrate that polymorphisms leading to lower HO-1 expression are linked to higher incidence of cancers, including lung adenocarcinoma and oral squamous cell carcinoma associated with use of tobacco products, as well as gastric adenocarcinoma [102-105]. Conversely, polymorphisms leading to increased HO-1 expression are associated with decreased risk of developing oral squamous cell carcinoma as well as a lower tumor recurrence and better relapse-free survival [104]. However, the role of HO-1 gene polymorphisms in carcinogenesis may depend on the types of cancer. For example, polymorphisms leading to increased HO-1 expression are found to be associated with increased risk of malignant melanoma [106]. This suggests that higher levels of HO-1 expression may facilitate the growth and progression of malignant melanoma, which is in line with the observations made in the mouse melanoma model. Polymorphisms leading to increased HO-1 expression are also associated with increased risk of gastrointestinal stromal tumor as well as higher tumor recurrence rates and shorter survival [107]. Hence, the exact function of HO-1 in human cancer development varies with cancer types. Further elucidation of the precise role of HO-1 in various types of human cancer will facilitate the development of effective strategies targeting HO-1 (either upregulation or downregulation of HO-1 depending on the types of cancer) in cancer therapy.

4. Conclusion and Future Directions

HO enzymes, particular HO-1 are among the most extensively studied antioxidative and anti-inflammatory enzymes in mammalian systems. These enzymes protect against various oxidative stress- and inflammation-associated disease processes in animal models. HO-1 may play a dual role (inhibitory or promoting) in cancer development depending on the types of cancer. In line with the experimental findings, studies in human subjects also point to an important involvement of HO-1 in many disease conditions. Future efforts should focus on further elucidation of the precise role of HO enzymes, including both HO-1 and HO-2 in cancer as well as other common human diseases, and addressing how to translate the current discoveries on HO enzymes into new therapeutic and/or preventive modalities to combat human diseases.

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Chapter 16

NAD(P)H:Quinone Oxidoreductase

Abstract

NAD(P)H:quinone oxidoreductase (NQO) refers to a family of flavoproteins that catalyze the two-electron reduction of quinones and derivatives. In mammas, there are two members, namely, NQO1 and NQO2. NQO1 uses NAD(P)H whereas NQO2 employs dihydronicotinamide riboside (NRH) as the electron donors. In addition to the well-recognized function in metabolizing quinones and preventing formation of reactive oxygen species, NQO enzymes also possess other important activities. These include anti-inflammatory effects, direct scavenging of superoxide, stabilization of p53 and other tumor suppressors, and acting as a melatonin-binding site to mediate the biological functions of this neurohormone. Studies in animal models have demonstrated an important role for NQO enzymes in protecting against various pathophysiological processes, including chemical toxicity, carcinogenesis, and cardiovascular injury. Functional gene polymorphisms are identified in both human NQO1 and NQO2 genes. Studies on the association between NQO gene polymorphisms and susceptibility to disease development suggest a beneficial involvement of NQO enzymes in a number of human disorders, especially cancer.

1. Overview

NAD(P)H:quinone oxidoreductase (NQO) refers to a family of flavoproteins that include two members, namely, NQO1 and NQO2 in mammals. NQO1 and NQO2 strand for NAD(P)H:quinone oxidoreductase 1 and NRH:quinone oxidoreductase 2, respectively. NQO2 uses dihydronicotinamide riboside (NRH) rather than NAD(P)H as the electron donor. Hence, using the general term NAD(P)H:quinone oxidoreductase to refer to NQO2 is not accurate. NQO1 was discovered in rat liver and named DT-diaphorase by L. Ernster and F. Navazio in 1958. NQO1 and NQO2 catalyze two-electron reduction of various quinones and derivatives. As described below, these two enzymes also possess other novel biological activities. NQO1 and NQO2 are cytosolic enzymes with a molecular mass of 31 and 25 kDa, respectively. They are widely distributed in mammalian tissues though the highest levels are usually found in liver. In humans, NQO1 and NQO2 are localized on chromosomes 16q22.1 and 6pter-q12, respectively.

2. Biochemistry and Molecular Regulation

2.1. Biochemistry

The biochemical properties of NQO enzymes are summarized as follows.

- Two-electron reduction and detoxification of quinone compounds and derivatives
- Maintenance of α-tocopherol and ubiquinone in their reduced and active forms
- Direct scavenging of superoxide
- Stabilization of the tumor suppressor p53 protein and other tumor suppressors
- As a gatekeeper of the 20S proteasome
- Binding to melatonin

2.1.1. Two-Electron Reduction of Quinones and Derivatives

Both NQO1 and NQO2 catalyze two-electron reduction of quinones to hydroquinones, which then undergo conjugation reactions, leading to their excretion from the body. This two-electron reduction reaction prevents the unwanted one-electron reduction of quinones by other enzymes, such as cytochrome P450 reductase. The one-electron reduction results in the formation of reactive semiquinone radicals, and the subsequent formation of reactive oxygen species (ROS) via redox cycling (Figure 16-1). Two-electron reduction of quinones by NQO enzymes thus prevents the formation of reactive semiquinone radicals and ROS. In addition to quinones, NQO enzymes also catalyze two-electron reduction of quinoneimines, nitroaromatics, and azo dyes.

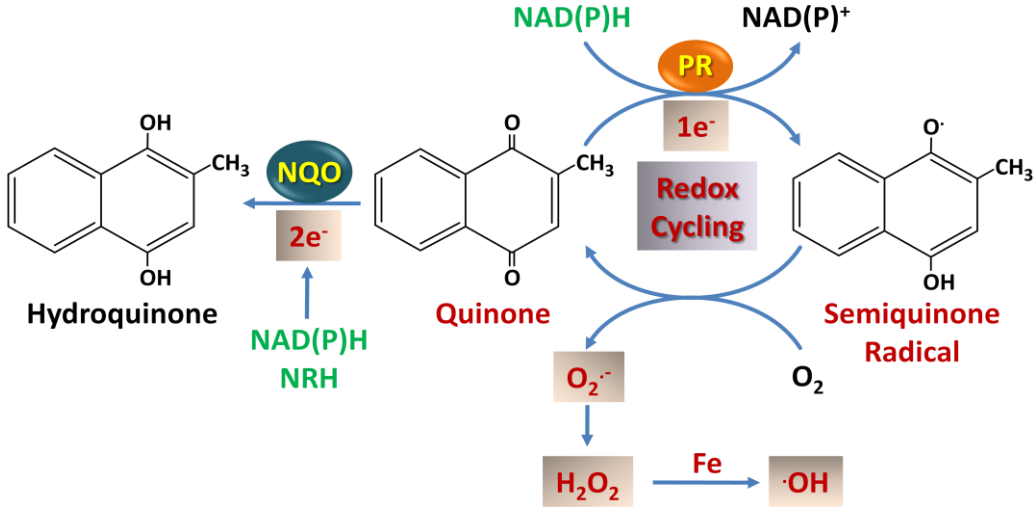

Figure 16-1. NAD(P)H:quinone oxidoreductase (NQO) enzyme-catalyzed two-electron reduction of quinones. As illustrated, NQO enzymes catalyze two-electron reduction of quinones to hydroquinones. This prevents quinones from undergoing one-electron reduction catalyzed by enzymes, such as cytochrome P450 reductase (PR). One-electron reduction of quinones leads to the formation of semiquinone radicals, which reduce oxygen to superoxide ($O_2^{\cdot-}$), leading to the formation of other reactive oxygen species, such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$).

2.1.2. Maintenance of Endogenous Antioxidants α-Tocopherol and Ubiquinone

NQO1 is thought to help maintain certain endogenous antioxidants in their reduced and active forms. Both ubiquinone (coenzyme Q) and α-tocopherol-quinone, two important lipid-soluble antioxidants are substrates for NQO1 in vitro. NQO1 catalyzes two-electron reduction of ubiquinone and α-tocopherol-quinone to their hydroquinone forms, which then protect against lipid peroxidation of membranes.

2.1.3. Scavenging of Superoxide

It is well-known that both NQO1 and NQO2 prevent ROS formation from quinone redox cycling. In addition, NQO1 also directly scavenges superoxide in an NAD(P)H-dependent manner [1]. This effect may be particularly significant in tissues, such as vasculature, where NQO1 is highly expressed [2].

2.1.4. Stabilization of p53 and Other Tumor Suppressors

The p53 tumor suppressor protein suppresses tumorigenesis by mediating either growth arrest or apoptosis in response to stresses, such as DNA damage. NQO1 is shown to play an important role in stabilizing p53 protein via processes including protein-protein interaction that prevents ubiquitin-independent degradation of p53 by 20S proteasome [3, 4]. Stabilization of p53 protein by NQO1 may contribute to the protective role of NQO1 in carcinogenesis (see Sections 3.1.4 and 3.2.2 for more discussion). In addition to p53, NQO1 also regulates the ubiquitin-independent 20S proteasomal degradation of p73α and p33, two other tumor suppressors [5, 6].

2.1.5. As a Gatekeeper of the 20S Proteasome

It has been demonstrated that in murine liver tissue majority of NQO1 is associated with the 20S proteasome, and NQO1 may function as a gatekeeper for protein degradation through the 20S proteasome. More recently, NQO1 is found to protect eukaryotic translation initiation factor (eIF) 4GI from proteasomal degradation, and as such may participate in the regulation of translation [7].

2.1.6. Binding to Melatonin

NQO2 has a melatonin-binding site, and as such may mediate some of the biological activities of melatonin, such as the antioxidant effects [8, 9]. However, the exact biological significance of the binding of melatonin to NQO2 remains to be elucidated.

2.2. Molecular Regulation

NQO1 is highly inducible by a wide variety of chemical inducers, including oxidants, electrophiles, and phenolic compounds. It is well-recognized that Keap1/Nrf2/ARE pathway plays a central role in regulating the gene expression of mammalian NQO1 [10, 11]. Here ARE refers to antioxidant response element. Indeed, there are multiple AREs in the promoter region of mammalian NQO1 gene. Mammalian NQO1 gene promoter also contains a functional xenobiotic response element (XRE), and arylhydrocarbon receptor (AhR) via binding

to XRE is crucially involved in the induction of NQO1 gene expression by xenobiotics, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). There is also evidence suggesting that Nrf2/ARE and AhR/XRE coordinate in the regulation of NQO1 expression in mammalian tissues [10].

NQO2 expression is also responsive to a variety of xenobiotics, and NQO2 gene expression is coordinately induced with NQO1 in response to xenobiotics and antioxidants. Nucleotide sequence analysis of mammalian NQO2 gene promoter reveals the presence of several cis-elements, including the binding sites for SP1, SP3, XRE, and ARE. It has been demonstrated that SP1 activates whereas SP3 represses the promoter activity of NQO2 gene [12]. A recent study shows that human NQO2 gene expression is also upregulated by antioxidants via an Nrf2-dependent mechanism [13].

3. Role in Health and Disease

3.1. Animal Studies

3.1.1. Experimental Approaches

Gene knockout mouse models have been created for NQO1 and NQO2. Mice deficient in either NQO1 or NQO2 are born and reproduced adeptly as the wild-type mice, indicating that neither gene is essential for murine embryonic development. In addition, NQO1 and NQO2-double-knockout mouse model has been generated, and the mice lacking both NQO1 and NQO2 are born and reproduced normally. Much of our knowledge on the in vivo biological functions of NQO enzymes results from studies using these knockout models.

In addition to the genetic models, several chemicals have been shown to potently inhibit NQO enzyme activity, and as such serve as useful tools for studying the biochemical activities of NQO enzymes in various cellular systems. For example, dicumarol at micromolar concentrations is a commonly used competitive inhibitor of NQO1 in biological systems. However, at concentrations that inhibit NQO1 activity, dicumarol may also interfere with other cellular enzymes, including the enzyme complexes of the mitochondrial electron transport chain [14]. Recently, a more selective and potent NQO1 inhibitor, namely, ES936 has been synthesized and characterized by D. Ross and coworkers [15]. ES936 is a quinone compound and a mechanism-based inhibitor of NQO1 in cellular systems, in which over 90% NQO1 activity can be inhibited by this chemical at 100 nM. More recently, a range of triazoloacridin-6-ones have been synthesized and evaluated for ability to inhibit NQO1 and NQO2, and one of them, NSC66041, is identified as the most potent inhibitor of NQO2 with an IC_{50} of 6 nM [16, 17]. Notably, NQO2 is also shown to be inhibited by novel compounds, including resveratrol [18, 19], the anti-leukemia drug imatinib [20], and the antimalarial quinolines [21, 22]. It is suggested that NQO2 may serve as a unique molecular target of these compounds to mediate their biological activities.

3.1.2. An Overview of the Biological Functions of NQO Enzymes in Animal Models

The biological functions of NQO enzymes are closely associated with their biochemical properties as described above in Section 2.1. Based on studies in cell cultures and animal

models, the best-recognized biological functions of NQO enzymes are (1) protection against quinone compound-induced toxicity and (2) protection against carcinogenesis. In addition, studies primarily in NQO knockout animals have suggested that NQO enzymes may also play an important role in certain other disease conditions, among which are cardiovascular disorders, lung injury, and autoimmunity. The sections below describe in detail these biological activities of NQO enzymes in experimental models.

3.1.3. Role in Chemical Toxicity

The involvement of both NQO1 and NOQ2 in quinone metabolism has been investigated in various in vitro and in vivo studies. Quinones exist as environmental toxic compounds. They are also formed endogenously, such as from metabolism of estrogens. NQO1 knockout ($NQO1^{-/-}$) mice are more susceptible to menadione-induced tissue injury compared with wild-type littermates [23]. In contrast, NQO2 knockout ($NQO2^{-/-}$) mice show decreased sensitivity to menadione toxicity, suggesting that NQO2 may metabolically activate menadione to cause tissue injury [24]. NQO1 deficiency in cultured cells also generally aggravates quinone-induced cytotoxicity, whereas overexpression of NQO1 usually protects the cells from quinone toxicity. Quinones are also reactive metabolites of benzene, and are responsible for hematotoxicity induced by this human leukemogen. Targeted disruption of NQO1 in mice markedly sensitizes the animals to benzene-induced hematotoxicity and bone marrow toxicity, as assessed by peripheral blood counts and bone marrow histopathology [25]. As described below in Section 3.2.2, deficiency of NQO1 in humans also increases the susceptibility to benzene-induced leukemias.

It should be noted that some quinone anticancer drugs, such as mitomycin C can be bioactivated by NQO1 under hypoxic conditions. This is due to the fact that two-electron reduction of these anticancer quinone drugs results in the formation of more reactive metabolites [26]. This unique mechanism of bioactivation may lead to enhanced and selective tumor killing because tumor cells usually express higher levels of NQO1 and have lower oxygen concentrations than do normal cells. On the other hand, NQO1-mediated bioactivation of mitomycin C may also contribute to the adverse effects of this anticancer drug. Indeed, $NQO1^{-/-}$ mice show a complete resistance to mitomycin C-induced bone marrow suppression. The heterozygous ($NQO1^{+/-}$) mice also exhibit partial resistance to mitomycin C-induced bone marrow toxicity compared with wild-type mice [27]. In cultured cells, NQO2 is also able to bioactivate mitomycin C as well as several other quinones [28]. However, targeted disruption of NQO2 in mice does not aggravate mitomycin C-induced bone marrow suppression and hematotoxicity [27]. Thus, NQO1 and NQO2 may exert differential effects on quinone metabolism and toxicity in mammalian systems.

3.1.4. Role in Carcinogenesis

Metabolism of environmental carcinogens leads to the formation of reactive quinones. Likewise, quinones are also metabolites of endogenous estrogens, which are implicated in estrogen carcinogenesis [29]. Both $NQO1^{-/-}$ and $NQO2^{-/-}$ mice show increased susceptibility to skin carcinogenesis induced by environmental carcinogens, including benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene [30-33]. $NQO1^{-/-}$ mice spontaneously develop myelogenous hyperplasia due to decreased apoptosis [34]. In addition, as compared with wild-type mice, $NQO1^{-/-}$ mice are much more susceptible to benzene-elicited genotoxicity and radiation-induced myeloproliferative disease [35]. Targeted disruption of NQO2 in mice also leads to

myeloid hyperplasia of bone marrow and increased neutrophils, basophils, eosinophils, and platelets in the peripheral blood. It is further shown that decreased apoptosis of bone marrow cells and circulating granulocytes contributes to myeloid hyperplasia and hyperactivity of bone marrow in $NQO2^{-/-}$ mice [24]. $NQO2^{-/-}$ mice are shown to be highly susceptible to radiation-induced B-cell lymphomas, suggesting that NQO2 may function as an endogenous factor in prevention against radiation-induced lymphomas [36].

Both NQO1 and NQO2 are able to reduce estrogen-derived quinone metabolites, thereby protecting cells from these quinone-induced genotoxicity [37, 38]. Estrogen-derived quinones have been implicated in breast carcinogenesis. As discussed below, gene polymorphism studies in humans suggest a protective role for both NQO1 and NQO2 in breast cancer development (Section 3.2). Induction of NQO1 by oltipraz, a chemoprotective agent is shown to attenuate chemically-induced colon carcinogenesis [39, 40]. Targeted disruption of NQO1 in mice also leads to increased oxidative DNA lesions in liver and kidney under basal conditions, indicating that NQO1 may be important in attenuating endogenous oxidative DNA damage in vivo [41].

3.1.5. Role in Cardiovascular Diseases and Related Conditions

NQO1 is a highly expressed antioxidant and anti-inflammatory enzyme in cardiovascular cells and tissues under basal conditions [2, 42, 43]. Its expression in cardiovascular system is also strongly inducible by various stimuli, including oxidants, reactive aldehydes, homocysteine, cigarette smoke, inflammatory cytokines, sheer force, as well as chemoprotective agents. Many of these factors are critically involved in cardiovascular disorders. Overexpression of NQO1 in cultured cardiovascular cells protects them not only from oxidative cytotoxicity, but also from cytokine-induced proinflammatory responses [44, 45]. A recent study reports that activation of NQO1 prevents arterial restenosis by suppressing vascular smooth muscle cell proliferation in an animal model of balloon injury of carotid artery [46]. Adenoviral vector-mediated overexpression of NQO1 in cultured vascular cells attenuates tumor necrosis factor-α-induced endothelial adhesion molecule expression and smooth muscle cell migration [44, 45]. These in vivo and in vitro data suggest that NQO1 functions as an important protector in atherogenesis, and induction of NQO1 in vasculature may thus represent an effective strategy for the intervention of vascular disorders.

NQO1 also plays a role in lipid metabolism and insulin resistance. As compared with wild-type mice, $NQO1^{-/-}$ mice exhibit higher ratios of NAD(P)H to $NAD(P)^+$, lower levels of abdominal adipose tissue, and higher levels of hepatic triglycerides. Insulin tolerance test demonstrates that $NQO1^{-/-}$ mice are insulin-resistant [47]. In rodent models of metabolic syndrome, β-lapachone-mediated pharmacological stimulation of NADH oxidation by NQO1 is shown to dramatically ameliorate obesity and related phenotypes, including glucose intolerance, dyslipidemia, and fatty liver [48]. β-Lapachone is a specific and high-affinity substrate of NQO1, and able to potently stimulate NADH oxidation by NQO1 in vitro and in vivo [49, 50]. These observations open the possibility of developing strategies targeting NQO1 for the treatment of metabolic syndrome.

3.1.6. Role in Pulmonary Diseases

As stated above, NQO1 and NQO2 double-knockout ($NQO1^{-/-}/NQO2^{-/-}$) mice are born and reproduced normally. However, the double-knockout mice show myelogenous hyperpla-

sia of bone marrow and development of bronchial-associated lymphoid tissue (BALT) infiltrated with neutrophils and macrophages [51]. The BALT is absent in wild-type and single-knockout mice. The double-knockout mice also develop systemic inflammation, as evidenced by increased serum levels of tumor necrosis factor-α, interleukin-1, and interleukin-6, as well as increased expression of inducible nitric oxide synthase and higher nitric oxide production in lung macrophages. $NQO1^{-/-}/NQO2^{-/-}$ mice upon exposure to hyperoxia demonstrate severe intra-alveolar edema, perivascular inflammation, and massive infiltration of neutrophils as compared with wild-type mice [51]. These findings suggest that NQO1 and NQO2 together function to suppress BALT development, lung inflammation, and hyperoxic lung injury in experimental animals.

Epidemiological studies have identified NQO1 as a host susceptible factor for ozone exposure. This has prompted experimental studies on the role of NQO1 in ozone toxicity in both animal models and cell cultures. Targeted disruption of NQO1 in mice renders them resistant to ozone-induced toxicity, as evidenced by decreased oxidative stress, attenuated airway inflammation, and diminished airway hyperresponsiveness [52]. In cultured primary human airway epithelial cells, inhibition of NQO1 by dicumarol attenuates ozone-induced F_2-isoprostane production and interleukin-8 gene expression [52]. F_2-Isoprostane is a highly reliable and specific biomarker of oxidative stress. These in vivo and in vitro data indicate that NQO1 may play a detrimental role in ozone-induced lung inflammation and oxidative stress. These results on ozone toxicity contradict the observations that NQO1 protects against hyperoxia-induced lung injury, in which oxidative stress and inflammation play a major role. It remains unknown how NQO1 functions as a susceptible factor in ozone toxicity, and if this is an ozone-specific phenomenon.

3.1.7. Role in Immunity and Autoimmunity

There is ample evidence for a beneficial function of NQO1 in inflammatory responses in both in vitro and in vivo systems. Studies in gene knockout mouse models demonstrate that both NQO1 and NQO2 participate in regulating humoral immunity and autoimmunity. Although targeted disruption of either NQO1 or NQO2 in mice leads to myeloid hyperplasia of bone marrow, these knockout mice show decreased lymphocytes in peripheral blood [53]. $NQO1^{-/-}$ and $NQO2^{-/-}$ mice also exhibit lower germinal center response, altered B cell homing, and impaired primary and secondary immune responses. Both $NQO1^{-/-}$ and $NQO2^{-/-}$ mice display increased susceptibility to autoimmune disease, as revealed by decreased apoptosis of thymocytes and predisposition to collagen-induced arthritis. As compared with wild-type mice, the autoimmunity-induced arthritis in $NQO1^{-/-}$ mice is more severe, has an earlier onset, and lasts longer. There is no significant difference between $NQO2^{-/-}$ and wild-type mice in the onset and severity of the arthritis. However, the arthritis lasts longer in $NQO2^{-/-}$ mice as compared with wild-type mice [53]. These observations implicate that NQO1 and NQO2 are endogenous factors in the regulation of immune response and autoimmunity in animal models. The detailed mechanisms by which NQO1 and NQO2 modulate immune response and autoimmunity await further investigations.

3.1.8. Role in Other Diseases and Conditions

NQO1 is expressed in the central nervous system, and may play a protective role in neurodegenerative disorders, particularly Parkinson's disease. Metabolism of dopamine to reac-

tive quinone metabolites with the concurrent production of ROS is believed to be responsible, at least partially, for dopaminergic neuron degeneration in Parkinson's disease. NQO1 is shown to catalyze two-electron reduction of dopamine-derived quinones, and protect cultured neurons from dopamine toxicity [54]. In contrast, overexpression of NQO2 in a dopaminergic neuronal cell line augments dopamine-mediated ROS formation [55]. Therefore, the exact role of NQO1 and NQO2 in neurodegeneration warrants further studies in animal models. In this regard, $NQO1^{-/-}$ and $NQO2^{-/-}$ mouse models will be important tools for understanding the role of NQO enzymes in experimental parkinsonism as well as other neurodegenerative disorders, such as Alzheimer's disease.

Melatonin is a neurohormone implicated in both biorhythm synchronization and neuroprotection from oxidative stress. Melatonin is also involved in cytoprotection against oxidative stress in various other tissues and disease conditions (see Chapter 19 for discussion of melatonin). The functions of melatonin are believed to be mediated by two G-protein-coupled-receptors (MT1 and MT2) and MT3, which corresponds to NQO2 [8, 9]. Indeed, all tissues from NQO2-null mice are depleted of the melatonin binding site MT3. However, it remains to be elucidated if the specific interaction between melatonin and NQO2 is responsible for the antioxidant activities of this neurohormone.

NQO1 may participate in regulating skin pigmentation process. NQO1 expression is diminished in multiple amelanotic melanoma cell lines. Treatment of both cultured normal human melanocytes and zebrafish with the NQO1 inhibitor ES936 or dicumarol markedly decreases melanogenesis. In contrast, adenoviral vector-mediated overexpression of NQO1 augments melanogenesis, concomitantly with an increased protein level of tyrosinase, the key enzyme involved in the biosynthesis of melanin [56]. These observations indicate that NQO1 may be a positive regulator of the skin pigmentation process via suppression of tyrosinase degradation.

3.2. Human Studies and Clinical Perspectives

3.2.1. Study Approaches and Gene Polymorphisms

The role of NQO enzymes, especially NQO1 in health and disease has been extensively studied in humans. The study approaches in human subjects primarily consist of (1) determination of the changes of NQO enzymes in tissue samples from patients with various disease conditions and (2) clinical and epidemiological studies on the association between NQO gene polymorphisms and disease risk. Both NQO1 and NQO2 genes are polymorphic. Two genetic polymorphisms have been identified in NQO1, which are designated as NQO1*2 and NQO1*3, respectively. The wild-type allele of NQO1 is designated as NQO1*1.

NQO1*2 is a single nucleotide polymorphism, a C to T change at position 609 of the NQO1 cDNA. This nucleotide substitution results in a proline to serine substitution at position 187 of the amino acid sequence of the protein. As such, NQO1*2 is frequently denoted as C609T or Pro187Ser. The mutant NQO1*2 protein is very unstable, and is rapidly ubiquitinated and degraded by the proteasome. Cells with one NQO1*1 allele and one NQO1*2 allele have approximately half of the NQO1 activity of NQO1*1/*1 cells (the wild-type cells), but NQO1*2/*2 cells have no NQO1 activity. Thus, both NQO1 protein and activity are virtually undetectable in tissue samples of individuals carrying the homozygous NQO1*2 genotype (i.e., NQO1*2/*2, also denoted as null genotype). Approximately 10% of the human popula-

tions have the homozygous NQO1*2 genotype, but the frequency varies in different ethical groups [10, 11, 57-59].

A second NQO1 polymorphism has been identified at base 465 of the NQO1 gene also involving a base change from cytosine to thymine, designated as NQO1*3 or C465T. This nucleotide change results in an arginine to tryptophan substitution at position 139 of the amino acid sequence of the protein. As such, this polymorphism is also denoted as Arg139Trp. The NQO1*3 genotype also results in a phenotype with lower NQO1 activity due to the decreased stability of the variant RNA and decreased activity of the resulting protein [58]. However, this polymorphism occurs at a much lower frequency than does NQO1*2. As such, most of the epidemiological studies on the association of NQO1 gene polymorphisms with diseases have dealt with NQO1*2. NQO1*2 polymorphism has been linked to a wide range of disease conditions, including cancer, cardiovascular diseases, diabetes, and neurological disorders. In addition, polymorphisms in NQO2 gene have been shown to affect the expression and activity of NQO2 protein, and be associated with altered risk of developing certain human diseases as well.

3.2.2. Role in Human Cancer

Knowledge on the critical role of NQO enzymes in protecting against experimental carcinogenesis and the p53 stabilizing activity of NQO1 has prompted studies on the involvement of NQO enzymes in human cancer development. Epidemiological studies have shown an increased frequency of NQO1*2 allele in patients with cancer, including leukemias, breast cancer, and colon cancer compared with healthy controls, suggesting a protective role for the normal NQO1 protein in cancer development. An association of the NQO1*2 polymorphism and cancer has been most frequently found in leukemias. Individuals with NQO1 deficiency (NQO1*2/*2) also exhibit an increased risk of developing leukemias following occupational exposure to benzene [60].

It is recently reported that NQO1*2 genotype is a strong prognostic and predicting factor in breast cancer [61]. The homozygous NQO1*2 strongly predicts poor survival in women with breast cancer, which is particularly evident after anthracycline-based adjuvant chemotherapy with epirubicin and in p53-aberrant tumors. Survival after metastasis is reduced among NQO1*2 homozygotes, further implicating NQO1 deficiency in breast cancer progression and treatment resistance. Consistently, response to epirubicin is impaired in NQO1*2-homozygous breast carcinoma cells in vitro [61]. In cultured human breast cancer cells, cDNA-mediated expression of the polymorphic variants (NQO1*2 and NQO1*3) of NQO1 is found to poorly reduce estrogen quinones, thereby leading to increased formation of estrogen quinone-DNA adducts [62]. Such DNA adducts are implicated in estrogen-induced breast carcinogenesis. Polymorphisms of NQO2 that lead to decreased NQO2 expression are also associated with increased susceptibility to breast cancer [63]. Wild-type NQO2 is shown to reduce estrogen quinones, leading to decreased DNA adduct formation [38]. Nevertheless, the precise role of NQO-mediated reduction of estrogen quinones in breast cancer development in human patients awaits further evaluation.

Multiple studies suggest that NQO1 plays a significant role in preventing the development of colorectal cancer, and individuals with an NQO1*2/*2 genotype are at an increased risk of developing this disease [64]. This conclusion is in line with results from the experimental studies showing that NQO1 may participate in preventing colorectal carcinogenesis in a rat model [39, 40].

Although extensive studies suggest a significant protective role for NQO enzymes, especially NQO1 in numerous types of cancer, there are also other epidemiological studies reporting no differences or mixed results in occurrence of NQO1*2 in various types of cancer, including renal cancer, lung cancer, gastric cancer, prostate cancer, gliomas, and lymphomas. The involvement of NQO1 and NQO2 gene polymorphisms in human cancer development can be potentially influenced by many factors. These include different ethical backgrounds, stage of cancer, concomitant alterations in other antioxidant genes or disease-modifying genes, dietary and environmental factors, as well as study design. Confirmation in experimental systems, including cell cultures and animal studies will further strengthen the conclusion regarding a positive association between NQO gene polymorphisms and risk of human disease development.

3.2.3. Role in Human Cardiovascular Diseases and Related Conditions

NQO1 is highly expressed and inducible in cardiovascular tissues and cells. As described above in Section 3.1.5, activation of NQO1 attenuates atherogenesis in experimental animals. A recent epidemiological study involving 834 patients with type 2 diabetes reports that patients with NQO1*2 polymorphism exhibit a higher prevalence of atherosclerotic plaques of carotid artery than those without this polymorphism [65]. Another study in patients undergoing standard coronary artery bypass grafting shows a greater inflammatory response, as revealed by increased serum interleukin-6 levels, in the subjects with NQO1*2 genotype as compared with the patients without this polymorphism [66]. Significantly higher levels of C-reactive protein, a biomarker for coronary heart disease, are reported to occur in coronary heart disease patients with lower NQO1 activity [67], suggesting a protective role for NQO1 in cardiovascular inflammation.

Studies in humans have revealed that NQO1 gene is also highly expressed in adipose tissue, and its mRNA expression is reduced during diet-induced weight loss [68]. The expression levels of NQO1 mRNA are found to positively correlate with adiposity, glucose tolerance, and markers of liver dysfunction, suggesting a possible involvement of NQO1 in the metabolic complications of human obesity. However, such studies provide no evidence as to whether NQO1 plays an etiological role in the disease pathophysiology or increased NQO1 mRNA expression is simply an accompanying phenomenon of obesity and its metabolic complications. Alternatively, the increased NQO1 mRNA expression could be an adaptive response to obesity and its metabolic complications, and might serve as a protective mechanism. In this context, NQO1 is inducible by inflammation, a critical event in metabolic syndrome. The involvement of NQO1*2 polymorphism in both type 1 and type 2 diabetes has been investigated in two epidemiological studies in a Chinese population and a Danish population, and no association between NQO1*2 genotype and diabetes (either type 1 or type 2) is demonstrated [69, 70]. The potential involvement of NQO1 in human diabetes and metabolic syndrome deserves further investigations, including studies in other ethical groups and geographical regions.

3.2.4. Role in Human Neurological Diseases

Oxidative stress and dopamine metabolism are implicated in Parkinson's disease. NQO enzymes are proposed to protect against Parkinson's disease via their ability to inhibit oxidative stress and augment the detoxification of dopamine quinones. In this context, NQO1 expression is found in both normal and Parkinsonian substantia nigra [71]. Furthermore, a

marked increase in the astroglial and neuronal expression of NQO1 is consistently observed in the Parkinsonian substantia nigra. However, studies to date on the association between NQO1*2 gene polymorphism and risk of developing Parkinson's disease have produced null results [72, 73]. In contrast, polymorphisms in NQO2 gene promoter are shown to be associated with altered susceptibility to Parkinson's disease [55, 72]. The promoter of NQO2 gene is found to be highly polymorphic. Several novel polymorphic alleles are reported, among which are 29-bp insertion (I-29 allele) and 29-bp deletion (D-29 allele). Although the D-29 allele was initially reported to be associated with decreased NQO2 expression, subsequent studies demonstrate the opposite, i.e., the D-29 allele in the promoter directs augmented NQO2 gene expression that results in greater enzymatic activity than wild-type allele [55]. Two studies report a significantly higher frequency of the D-29 allele in patients with Parkinson's disease than in control, suggesting that this NQO2 polymorphism and the resulting elevated NQO2 expression may be associated with increased susceptibility to Parkinson's disease. As described in Section 3.1.8, overexpression of NQO2 in a dopaminergic neuronal cell line leads to increased production of ROS when being exposed to exogenous dopamine, indicating a possible role for NQO2 in augmenting dopamine-mediated oxidative stress and neuronal injury. In contrast to the above two human studies, another population-based case-control study with 190 Parkinson's disease patients and 305 unrelated controls matched on age and sex demonstrates no association between the I/D-29 polymorphisms and development of Parkinson's disease [73].

NQO1 expression is found to co-localize with the Alzheimer's disease pathology, and the ratio of frontal to cerebellar NQO1 enzyme activity is significantly increased in patients with Alzheimer's disease as compared with that in control subjects [74], suggesting a possible involvement of NQO1 in this neurodegenerative disorder. A recent study in a Chinese population consisting of 311 Alzheimer's disease patients and 330 controls shows a higher frequency of NQO1*2 allele and a lower frequency of wild-type allele (NQO1*1) in the Alzheimer's patients compared with controls [75]. These observations implicate a possible protective effect of NQO1 in the development of Alzheimer's disease. However, a similar study also in a Chinese population reveals no association between NQO1*2 genotype and late-onset Alzheimer's disease [76].

Multiple studies suggest an involvement of NQO2 in other neurological/psychiatric disorders. For example, a study in a Japanese population of 102 patients with schizophrenia and 234 controls reports that the D-29 polymorphism of NQO2 gene promoter is associated with an increased susceptibility to developing schizophrenia [77]. In the same study, D-29 genotype is found to be associated with a lower NQO2 mRNA in white blood cells. Functional polymorphisms of NQO2 gene are also associated with methamphetamine psychosis [78] as well as the pathogenesis of alcoholism and alcohol withdrawal symptoms [79], indicating a possible role for NQO2 in modulating drug abuse-related mental and behavioral changes. A more recent study using a cohort of 722 community-dwelling older individuals aged 50 years and over demonstrates that a functional NQO2 polymorphism is associated with cognitive decline.

3.2.5. Role in Other Human Diseases

NQO gene polymorphisms are also associated with a number of other human disease conditions. These include asthma [80, 81], acute lung injury [82], inflammatory bowel disease [83], urolithiasis [84], and clozapine-induced agranulocytosis [85]. However, further studies

are needed to better characterize the involvement of NQO gene polymorphisms in modulating the risk of developing the above human diseases.

4. Conclusion and Future Directions

NQO enzymes are multifunctional proteins that play a beneficial role in diverse pathophysiological processes involving oxidative stress and inflammation. This notion has been supported by extensive studies in animal models as well as in human subjects. Future studies should focus on how to translate the enormous amounts of basic and clinical knowledge about NQO enzymes into novel preventive and therapeutic modalities for the intervention of human diseases in which NQO enzymes play a causative role. In this context, further investigations of the role of NQO gene polymorphisms in disease processes in well-designed clinical and epidemiological studies will also contribute to the development of sensitive and selective NQO-based genetic biomarkers of disease susceptibility and progression. Use of these biomarkers will help identify susceptible subjects to whom NQO-based interventional modalities may be selectively applied.

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Chapter 17

Paraoxonase

Abstract

Paraoxonase (PON) refers to a family of three enzymes, namely, PON1, PON2, and PON3. Mammalian PON1 and PON3 are found in circulation bound to high-density lipoprotein, whereas PON2 is an intracellular protein. PON1 was first discovered as an enzyme to hydrolyze paraoxon, an activity that both PON2 and PON3 lack. All three PON enzymes are able to degrade oxidized lipids and protect against oxidative stress. PON enzymes also function to suppress inflammation. Animal studies demonstrate a critical role for PONs, especially PON1 in protecting against diverse disease processes involving oxidative stress and inflammation, such as atherosclerosis, diabetes, and metabolic syndrome. In line with the findings in experimental models, substantial evidence from clinical and epidemiological studies also suggests a potential beneficial role for PONs in a variety of human diseases.

1. Overview

Paraoxonase (PON) refers to a family of enzymes that includes three members in mammals, namely, PON1, PON2, and PON3. PON1 was first identified by W.N. Aldridge in 1953 as an enzyme to hydrolyze paraoxon [1, 2]. PON was thus named for its ability to metabolize paraoxon, the primary metabolite of the organophosphate insecticide parathion. When two closely related enzymes were identified in the mid 1990s, the original PON was referred to as PON1, while the two new enzymes were named PON2 and PON3. In contrast to PON1, neither PON2 nor PON3 exhibits any significant ability to hydrolyze paraoxon or other organophosphate pesticides.

PON1 (43 kDa) is synthesized and secreted by liver, and is primarily associated with high-density lipoprotein (HDL) in plasma. PON1 catalyzes the breakdown of oxidized lipids and inhibits oxidation of HDL as well as low-density lipoprotein (LDL). PON2 is an intracellular protein that is widely distributed in mammalian tissues. Similar to PON1, PON2 with a molecular mass of 44 kDa also acts as an antioxidant protecting against oxidative stress. PON3 (40 kDa) is primarily expressed in liver, and associated with HDL in plasma. Like other members of the paraoxonase family, PON3 has significant antioxidant activity in protect-

ing both HDL and LDL from oxidation. In humans, PON1, PON2, and PON3 are localized next to each other on chromosome 7q21.3-22.1.

2. Biochemistry and Molecular Regulation

2.1. Biochemistry

Since its discovery, mammalian PON1 has been extensively studied with respect to its role in metabolizing organophosphate pesticides, lipid hydroperoxides, and a number of other substrates. In contrast, studies on PON2 and PON3 have been less extensive. Nevertheless, these two new isozymes have been recently found to possess unique biochemical functions. This section focuses on discussing the biochemistry of PON1 along with a description of recent findings on the biochemical properties of PON2 and PON3.

2.1.1. Biochemistry of PON1

The biochemical properties of PON1 are summarized as follows.

- Hydrolysis of organophosphates
- Degradation of oxidized lipids
- Lactonase activity

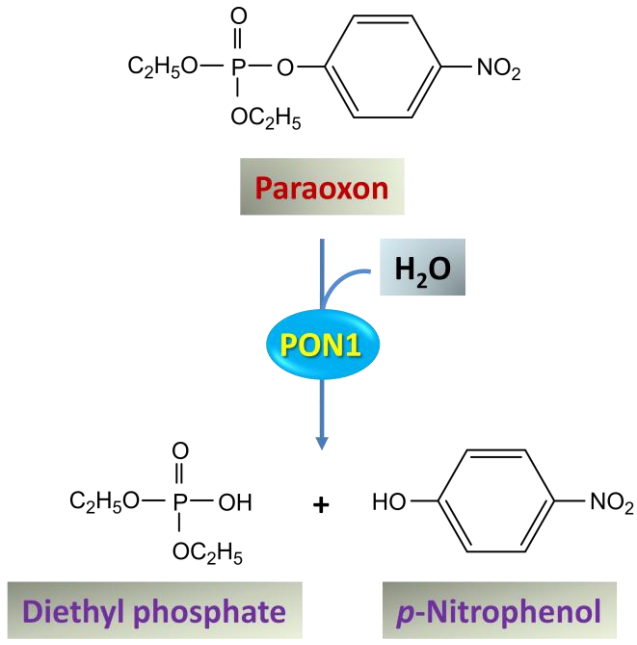

Figure 17-1. Hydrolysis of paraoxon by paraoxonase 1 (PON1). As illustrated, PON1-catalyzed hydrolysis of paraoxon forms diethyl phosphate and *p*-nitrophenol. See text (Section 2.1.1) for detailed description.

PON1 is an excellent example of a multitasking protein, displaying at least two important biochemical functions: (1) hydrolysis of organophosphates (pesticides and nerve agents) and (2) inhibition of lipoprotein oxidation by catalyzing degradation of oxidized lipids. PON1 effectively hydrolyzes a variety of organophosphate pesticides and nerve agents, including paraoxon, diazoxon, chlorpyrifos-oxon, sarin, and soman [3]. Hydrolysis of these organophosphates by PON1 leads to their detoxification. Figure 17-1 illustrates the chemical reaction of PON1-catalyzed hydrolysis of paraoxon.

Since paraoxon and other organophosphate toxicants are not present in the human body under normal conditions, other molecules would need to be the physiological substrates of PON1. Indeed, extensive studies have demonstrated that PON1 protects LDL and HDL from lipid peroxidation by catalyzing the degradation of oxidized lipids contained in the oxidized lipoproteins [4]. Notably, cysteine 284 is required for the enzyme's ability to hydrolyze oxidized lipids, but not for its activity against organophosphates. This suggests that differential mechanisms of hydrolysis may operate towards organophosphates and oxidized lipids [5]. It has been shown that PON1 can be inactivated by S-glutathionylation [6], a redox signaling mechanism characterized by formation of a mixed disulfide bridge between a protein sulfhydryl group and the oxidized form of glutathione (GSSG) (see Chapter 9 for discussion of protein glutathionylation).

In addition to hydrolyzing organophosphates, PON1 also hydrolyzes aromatic esters, preferably those of acetic acid. Indeed, phenyl acetate is one of the most commonly used substrates for assaying the enzymatic activity of PON1 in serum. More recently, PON1 has been reported to catalyze the hydrolysis of a variety of aromatic and aliphatic lactones as well as the reverse reaction, lactonization, of γ- and δ-hydroxycarboxylic acids [7]. PON1 is suggested to act as a lactonase or lactonizing enzyme. PON1 as well as PON2 and PON3 metabolize very efficiently 5-hydroxy-eicosatetraenoic acid 1,5-lactone and 4-hydroxydocosahexaenoic acid, which are products of both enzymatic and nonenzymatic oxidation of arachidonic acid and docosahexaenoic acid, respectively. These compounds may represent the PONs' endogenous substrates. PON1 may also possess other biochemical activities, such as metabolizing estrogen esters as well as certain drugs.

2.1.2. Biochemistry of PON2 and PON3

As noted above, PON2 and PON3 lack the ability to degrade organophosphates. However, PON2 and PON3 effectively hydrolyze lactones, and as such also act as lactonases. Of particular note is the ability of PONs, especially PON2 to hydrolyze and thereby inactivate acrylhomoserine lactones, which are quorum sensing signals of pathogenic bacteria [8] (see Section 3.1.6 below for discussion of quorum sensing). PON3 has been shown to hydrolyze bulky drug substrates, such as lovastatin and spironolactone in vitro. However, the in vivo significance of PON3 in the metabolism of these important drugs and thereby in modulating their pharmacological effects remains to be further elucidated.

Less is known regarding the detailed activities of PON2 and PON3 in metabolizing oxidized lipids. Similar to PON1, purified PON2 and PON3 are shown to inhibit lipid peroxidation of LDL in vitro. Since PON2 is an intracellular protein and not present in plasma, it is suggested to act as a protector against cellular oxidative stress. Indeed, intracellular overexpression of PON2 attenuates oxidative stress in various types of vascular cells and inhibits cell-mediated oxidation of LDL [9, 10].

2.2. Molecular Regulation

Among the three members of the mammalian PON family, PON1 has been most extensively studied with regard to the regulation of gene expression. Multiple regulatory mechanisms and signaling pathways have been implicated in the modulation of PON1 gene expression in mammalian systems. PON1 expression is inducible by various agents or biomolecules, including dietary phenolic compounds [11-13], aspirin [14], statin drugs [15], alcohol [16], glucose [17], and interleukin-6 [18]. On the other hand, several compounds, such as bile acids, interleukin-1β, and tumor necrosis factor-α are found to suppress PON1 gene expression [18, 19] .

One study in a cultured human liver cell line demonstrates a critical role for SP1 and protein kinase C in the positive regulation of PON1 gene transcription [20]. The transcription factor SP1 as well as sterol regulatory element-binding protein-2 are involved in statin-induced activation of PON1 gene expression in human liver and kidney cell lines [15, 21]. SP1 is also implicated in glucose-induced PON1 gene expression in cultured liver cells [17]. On the other hand, induction of PON1 gene expression by dietary polyphenols, such as resveratrol and quercetin occurs via an arylhydrocarbon receptor (AhR)-dependent mechanism [12, 22]. Nucleotide sequence analysis reveals a xenobiotic response element (XRE)-like sequence within the PON1 promoter, and this XRE-like sequence mediates the induction of PON1 gene transcription by phenolic compounds. A more recent study reports a role for peroxisome proliferator-activated receptor-gamma (PPARγ) pathway in regulating increased expression of PON1 gene by polyphenols in hepatocytes [13].

PON2 is also inducible by polyphenols. In macrophages, induction of PON2 by polyphenols is shown to occur via PPARγ- and AP-1-dependent mechanisms [23]. PON2 gene expression is also upregulated by unesterified cholesterol and dexamethasone. Induction of PON2 expression by unesterified cholesterol occurs through activation of the phosphatidylinositol 3-kinase (PI3K) pathway [24]. A putative glucocorticoid response element in the promoter region of PON2 is suggested to mediate the activation of PON2 gene expression by glucocorticoid drugs [25]. The in vivo biological relevance of these two regulatory mechanisms of PON2 expression remains to be determined. Currently, the molecular regulation of PON3 gene expression is largely unknown.

3. Role in Health and Disease

3.1. Animal Studies

3.1.1. Experimental Approaches

Both transgenic overexpression and gene knockout mouse models have been created to investigate the biological functions of PON isozymes in health and disease. In addition, recombinant PON proteins have been made available for studying the protective role of PONs in disease conditions in experimental animals. Mice with homozygous knockout of PON1 or PON2 are born and reproduced adeptly, indicating neither of these two PONs is essential for murine embryonic development. To date, PON3-null mouse model has not been reported in the literature. Viral vector-mediated gene transfer and siRNA-mediated gene knockdown

have also been utilized to elucidate the biological functions of PONs in various experimental models.

Selective chemical inhibitors of PON enzymes would be useful tools for studying the biological activities of PONs. In this context, 2-hydroxyquinoline was claimed to be a selective inhibitor of PON1 [26]; however its usefulness as a specific inhibitor of PON1 in biological systems remains to be determined. Recently, a high throughput PON1 assay has been developed for discovery of small molecule modulators of PON1 activity. With this assay, an effective inhibitor of PON1, designated as BAS03551158, has been identified, which is 10-fold more potent than 2-hydroxyquinoline in inhibiting purified PON1 [27]. But, this compound is less potent in inhibiting serum PON1 than 2-hydroxyquinoline. There are also studies showing selective inhibition of PON1 by negatively charged lipids, such as lysophospholipids [28]. However, the value of these PON1 inhibitors in biological systems remains unclear.

3.1.2. An Overview of the Biological Activities of PONs in Animal Models

As stated earlier, PONs hydrolyze oxidized lipids, and protect against lipid peroxidation as well as cellular oxidative stress. In addition, these enzymes possess other novel biological functions, including anti-inflammation and stimulation of macrophage cholesterol efflux [29, 30]. These findings implicate that PONs may potentially affect a variety of disease processes involving oxidative stress and inflammation, such as atherosclerosis, diabetes and metabolic syndrome, hepatic injury, and infectious diseases. Furthermore, PON1 efficiently hydrolyzes various organophosphates, including pesticides and nerve agents [3], implicating that this PON enzyme may also act as an important defense against organophosphate poisoning. The sections below discuss the role of PONs in a number of pathophysiological conditions in animal models.

3.1.3. Role in Cardiovascular Diseases

The best characterized biological activity of PONs in experimental animals is the suppression of atherogenesis. There is substantial evidence for an important role of all three PON enzymes in protecting against experimental atherosclerosis, vascular inflammation, and oxidative stress [31, 32]. In this regard, targeted disruption of PON1 markedly augments high-fat diet-induced vascular inflammation, oxidative stress, and atherosclerosis. Conversely, either transgenic overexpression of PON1 or viral vector-mediated PON1 gene transfer greatly attenuates the above detrimental effects caused by high-fat diet [33-36]. Furthermore, HDL isolated from the PON1-deficient animals is unable to protect LDL from lipid peroxidation, suggesting that PON1 mediates the antioxidant activities of HDL [33]. Recently, PON1 gene transfer is found to also attenuate vascular oxidative stress and improve vasomotor function in ApoE-deficient mice with pre-existing atherosclerosis, pointing to a therapeutic activity of PON1 in atherogenesis [37].

The protective effects of both PON2 and PON3 in experimental atherosclerosis have been recently demonstrated. Despite lower levels of very low-density lipoprotein (VLDL) and LDL cholesterol, mice deficient in PON2 develop significantly larger atherosclerotic lesions compared with their wild-type counterparts [38]. Three potential mechanisms may account for the enhanced atherosclerotic lesions in PON2-deficient mice. They are: (1) enhanced inflammatory properties of LDL, (2) diminished anti-atherogenic capacity of HDL, and (3) a heightened state of oxidative stress coupled with an exacerbated inflammatory response from PON2-deficient macrophages [38]. In line with these findings, adenovirus-mediated expression of

PON2 protects against the development of atherosclerosis in ApoE-deficient mice [39]. Similarly, adenovirus-mediated gene transfer of human PON3 augments HDL antioxidant effects against lipid peroxidation of LDL, and inhibits the progression of atherosclerosis in ApoE-deficient mice [40]. Transgenic overexpression of human PON3 also attenuates atherosclerosis lesion and vascular inflammation in both wild-type mice fed atherogenic diet and in LDL receptor-deficient mice [41]. Notably, a recent study demonstrates that transgenic overexpression of a human PON gene cluster containing PON1, PON2, and PON3 not only represses atherosclerosis, but also promotes atherosclerotic plaque stability in ApoE-deficient mice [42]. In summary, extensive studies in animal models support a critical role for PONs in protecting against atherosclerosis. However, it remains unknown if PONs also have a beneficial role in other forms of cardiovascular disorders.

3.1.4. Role in Diabetes and Metabolic Syndrome

Diabetes is characterized by increased inflammation and oxidative stress, and decreased PON1 activity. Transgenic overexpression of PON1 attenuates diabetes-induced macrophage oxidative stress, represses diabetes development, and decreases mortality in mice treated with streptozotocin. Conversely, mice lacking PON1 are more susceptible to streptozotocin-induced diabetes and oxidative stress [43]. Streptozotocin is a commonly used chemical for inducing type 1 diabetes in experimental animals. It has been suggested that pharmacological and nutritional intervention directed toward increasing PON1 activity may be beneficial in arresting diabetes development and its cardiovascular complications [43]. Recently, the potential role of PON2 in diabetes has been studied in cellular systems under hyperglycemic conditions. It is shown that PON2 decreases high glucose-induced macrophage triglyceride accumulation and oxidative stress by inhibiting NAD(P)H oxidase and diacylglycerol acyltransferase-1 [44]. Hypertriglyceridemia is an independent factor for atherosclerosis, and is also associated with the development of diabetes. As such, accumulation of triglycerides by macrophages under diabetic conditions may promote foam cell formation and contribute to diabetic vascular complications. Thus, augmentation of macrophage PON2 expression may attenuate macrophage atherogenicity, foam cell formation, and the resulting vascular complications in diabetes.

There is also evidence supporting a beneficial role for PONs in metabolic syndrome and associated cardiovascular complications. For instance, transgenic overexpression of human PON3 in mice is shown to decrease adiposity and lower circulating leptin levels, implicating a critical role for PON3 in protecting against obesity [41]. In mice with combined leptin and LDL receptor deficiency, a model of metabolic syndrome, adenovirus-mediated overexpression of human PON1 significantly reduces the total atherosclerotic plaque volume, the volume of plaque macrophages, and the amount of plaque-associated oxidized LDL [45].

3.1.5. Role in Liver Diseases

Liver is the major source of PON synthesis, and PON enzymes may play a protective role in liver injury involving oxidative stress and inflammation [46, 47]. Early studies suggest an association between decreased PON1 activity and development of chronic liver disease induced by carbon tetrachloride [48]. Adenovirus-mediated overexpression of either human PON1 or PON3 in mice attenuates carbon tetrachloride-elicited acute hepatic cell death, lipid peroxidation, and inflammation, indicating a causal role for PON1 and PON3 in inhibiting toxic liver injury [49]. The effects of PONs in protecting against other forms of liver disord-

ers, including alcoholic liver injury, acetaminophen overdose-induced hepatotoxicity, and nonalcoholic fatty liver disease deserve investigations with the use of genetically manipulated animal models.

3.1.6. Role in Infectious Diseases

PONs are able to hydrolyze and thereby inactivate acrylhomoserine lactones, which are quorum sensing signals of pathogenic bacteria [8]. Several lines of evidence suggest a role for PONs in innate immunity and in protection against infections by pathogenic microorganisms, including *Pseudomonas aeruginosa* and *Trypanosoma congolense*.

3.1.6.1. PSEUDOMONAS AERUGINOSA INFECTION

P. aeruginosa is an opportunistic bacterium which causes serious infections in immune compromised individuals, such as AIDS patients. Using small signaling molecules called acrylhomoserine lactones, populations of *P. aeruginosa* can coordinate phenotypic changes, including biofilm formation and virulence factor secretion, a process known as quorum sensing. Interference with quorum sensing has been identified as a potential strategy for the intervention of *P. aeruginosa* infection. Human PON enzymes, especially PON2 are able to effectively hydrolyze and inactivate acrylhomoserine lactones, and as such may play an important role in protecting against *P. aeruginosa* infection. Indeed, PON2 is shown to inactivate the quorum sensing molecule *N*-3-oxododecanoyl homoserine lactone in mouse tracheal epithelia [50]. The lysates of tracheal epithelia from PON2, but not PON1 or PON3 knockout mice have impaired *N*-3-oxododecanoyl homoserine lactone inactivation compared with wild-type mice. Using a quorum sensing reporter strain of *P. aeruginosa*, it is further found that quorum sensing is augmented in PON2-deficient tracheal epithelia as compared with those of wild-type, suggesting that PON2 may play a critical role in airway defense against *P. aeruginosa* infection [50].

An in vitro study demonstrates that expression of any of the three PON isozymes in *P. aeruginosa* leads to inhibition of biofilm formation and decreased resistance to antibiotics gentamicin and ceftazidima [51]. In an in vitro biofilm model, serum PON1 is also shown to prevent *P. aeruginosa* quorum sensing and biofilm formation by inactivating *N*-3-oxododecanoyl homoserine lactone [52]. These in vitro observations suggest a protective function of PON1 in *P. aeruginosa* infection. However, PON1 knockout mice are found to be paradoxically more resistant to *P. aeruginosa* infection as compared with wild-type littermates [52]. The PON1 knockout mice show increased levels of PON2 and PON3 mRNA in epithelial tissues, suggesting a possible compensatory mechanism. A recent study demonstrates that transgenic overexpression of human PON1 in *Drosophila melanogaster* dramatically decreases the lethality caused by *P. aeruginosa* infection. The protection is dependent on the lactonase activity of PON1 and the specific inactivation of the *N*-3-oxododecanoyl homoserine lactone, which is critical for the virulence of *P. aeruginosa* in *Drosophila melanogaster* [53]. Taken together, these novel findings indicate that PON1 can interfere with quorum sensing in vivo, and may play an important role in innate immune response to quorum sensing-dependent pathogens.

3.1.6.2. TRYPANOSOMA CONGOLENSE INFECTION

PONs may also play a role in protecting against parasitic infections. In vitro studies have suggested that a fraction of human HDL, termed trypanosome lysis factor, can suppress try-

panosome infection. The trypanosome factor fraction consists of PON1 and apolipoprotein A-II. Both PON1 transgenic overexpression and gene knockout mice have been used to investigate the involvement of PON1 in protection against trypanosome infection. When challenged with the same dosage of trypanosomes, mice overexpressing PON1 live significantly longer than wild-type mice, and mice deficient in PON1 live significantly shorter. In contrast, mice overexpressing another HDL-associated protein, apolipoprotein A-II have the same survival as wild-type mice following trypanosome infection [54]. The study also shows that HDL particles containing PON1 and those depleted of PON1 do not differ in their ability to lyse the trypanosome parasites in vitro, suggesting that the in vivo protection by PON1 may be indirect. Hence, the exact mechanism underlying the protective effects of PON1 in trypanosome infection remains to be elucidated.

3.1.7. Role in Organophosphate Poisoning

PON1 effectively hydrolyzes organophosphate pesticides and nerve agents, and as such plays an important role in protecting against organophosphate poisoning. Targeted disruption of PON1 in mice markedly sensitizes the animals to organophosphate-induced toxicity [33]. Administration of either rabbit or human PON1 purified from serum to rats and mice also protects them from organophosphate toxicity. Because human PON1 exhibits limited stability, engineered recombinant PON1 variants with higher activity and increased stability have been developed. Recently, it has been reported that injection of such a recombinant human PON1 variant, designated as $rHuPON1_{k192}$, protects the $PON1^{-/-}$ mice against up to three median lethal doses of the organophosphate diazoxon [55]. $rHuPON1_{k192}$ exhibits greater hydrolysis of diazoxon, chlorpyrifos-oxon, and paraoxon compared with $PON1_{R192}$, the more efficient one of the two natural human PON1 alloforms (see Section 3.2.2 and Table 17-1). After injection, $rHuPON1_{k192}$ is found to be nontoxic in mice, persist in serum for at least two days, and protective against diazoxon both prophylactically and therapeutically [55]. These observations point to the potential value for using the engineered recombinant PON1 for: (1) protection of military personnel or civilians from organophosphate chemical warfare agents and (2) protection of agricultural workers and by-standers from accidental poisoning by organophosphate pesticides. Such engineered recombinant human PON1 variants may also be of value for the intervention of chronic cardiovascular disorders like atherosclerosis considering that this enzyme is important in protecting against atherosclerotic vascular diseases in experimental animals (Section 3.1.3).

3.2. Human Studies and Clinical Perspectives

3.2.1. Study Approaches and General Considerations

The potential involvement of PONs in human diseases has been extensively investigated over the last decade. Numerous studies evaluate changes in the levels or activities of PON enzymes in sera of patients with various disease conditions. Many others involve the epidemiological assessment of the association of PON gene polymorphisms with risk or incidence of particular diseases. There are also studies aiming to link gene polymorphisms to changes of PON activities, and further to risk of disease development. These studies in human subjects have provided important information on the involvement of PONs in disease processes; however, they do not establish a casual role for PONs in disease pathophysiology. Nevertheless,

the data obtained from both experimental animal models and human studies have yielded valuable insights into the functions of PON enzymes in human health and disease. The sections below begin with a description of common PON gene polymorphisms, followed by a summary of the major clinical and epidemiological findings on the role of PONs in prevalent human diseases.

3.2.2. PON Gene Polymorphisms

Among the three members of human PONs, PON1 gene polymorphisms and their influence on enzyme activity have received the most extensive studies. PON1 gene has two well-studied polymorphisms in the coding region, which have been associated with various human diseases. Substitution of glutamine (Gln or Q) by arginine (Arg or R) at position 192, and methionine (Met or M) by leucine (Leu or L) at position 55 of the amino acid sequence of the protein independently influence the PON1 activity, and constitute an important molecular basis for interindividual variability. These two coding region polymorphisms of PON1 are designated as Gln192Arg (or Q192R) and Met55Leu (or M55L), respectively.

The Q192R polymorphism exhibits a striking substrate specific difference in the hydrolytic activity of the enzyme. The 192R alloenzyme shows greater activity toward paraoxon hydrolysis than the 192Q alloenzyme, whereas the 192Q alloenzyme provides greater protection against the accumulation of lipid peroxides in LDL than the R alloenzyme in vitro [56, 57]. However, it should be noted that the in vitro effects of the PON alloenzymes on LDL oxidation may not be translated into the corresponding in vivo activities in human subjects (see Section 3.2.3 for further discussion).

The M55L polymorphism also affects PON1 activity/concentration though less than does the Q192R polymorphism. Individuals carrying a leucine at position 55 (L alloenzyme) have a higher PON1 concentration than those with a methionine (M alloenzyme) at this position. There are also five known polymorphisms located within the PON1 promoter region that have been shown to affect PON1 expression. However, their association with human diseases has not been clearly demonstrated [32, 58]. Table 17-1 summarizes the impact of Q192R and M55L polymorphisms on serum PON1 activity/concentration.

Table 17-1. PON1 gene polymorphisms and effects on serum PON1 activity/concentration

Polymorphism	Effects on Serum PON1 Activity/Concentration
Q192R	RR - high serum PON1 activity QQ - low serum PON1 activity
M55L	LL - high serum PON1 concentration MM - low serum PON1 concentration

Note: See text (Section 3.2.2) for detailed description of the Q192R and M55L polymorphisms of PON1 gene and their effects on PON1 activity/concentration.

A number of polymorphisms have been identified in PON2 gene. Among them substitution of cysteine by serine at position 311 of the amino acid sequence of the protein, designated as Cys311Ser (or C311S) has received most attention. The C311S polymorphism has been found to affect PON2 activity (with the C variant showing impaired lactonase activity) [59], and be associated with the development of certain human diseases.

Much less is known about the gene polymorphisms of human PON3 and their association with diseases. A study in an Italian population has identified five point mutations in PON3 gene, with three being silent mutations and two being missense mutations. The two missense mutations give rise to amino acid substitutions at position 311 (S to T) and 324 (G to D) [60]. Currently, the effects of the genetic variations on PON3 activity and the linkage between these variations and diseases remain unknown though one study shows an association of PON3 gene variation with PON1 activity [61]. This suggests a potential cross-effect among PON isozymes.

3.2.3. Role in Human Cardiovascular Diseases

A beneficial role for PON enzymes in atherosclerosis is strongly supported by transgenic overexpression and gene knockout studies in experimental animals (Section 3.1.3). There is also ample evidence supporting a protective effect of PONs, especially PON1 in human coronary heart disease. Many epidemiological studies suggest a correlation between low serum PON1 activity and coronary heart disease. Numerous gene polymorphism studies demonstrate an association between functional polymorphisms of PON1, such as Q192R and M55L, and the development of coronary heart disease though some others report conflicting results [32, 62]. In general, serum PON1 activity is a better predictor of risk for coronary heart disease than PON1 genotype. Therefore, it becomes necessary that when performing case-control studies, the observed differences in enzyme activities are matched by genotypes. In this context, a recent study demonstrates that PON1 genotype exhibits a significant dose-dependent association with decreased levels of serum PON activity (QQ192<QR192<RR192) and with increased levels of systemic oxidative stress (QQ192>QR192>RR192) [63]. Hence, among the three genotypes, QQ192 is associated with the lowest levels of PON1 activity and the highest levels of systemic oxidative stress, whereas RR192 exhibits the opposite. Compared with individuals possessing either PON1 RR192 or QR192 genotype, individuals with the QQ192 genotype have an increased risk of all-cause mortality and major adverse cardiac events. The incidence of major adverse cardiac events is significantly lower in individuals in the highest PON1 activity quartile compared with those in the lowest activity quartile [63]. These observations are consistent with findings in murine models and provide evidence for a cardiovascular protective function of PON1 in humans.

A more recent study shows that among symptomatic male coronary heart disease patients, carriership of the PON1 Q192 or M55 alleles is associated with an increase of the 10-year risk of myocardial infarction and mortality from ischemic heart disease. [64]. Both Q192 and M55 isotypes are associated with lower PON1 activity/concentration (Table 17-1). The data thus support the notion that PON1 activity may predict the adverse coronary events, and that PON1 may be a potential target of secondary prevention of coronary heart disease. In line with a protective role of PON1 in cardiovascular diseases as well as other aging-related disorders (see sections below), a recent meta-analysis of studies involving a total of 5962 subjects reveals that individuals carrying PON1 RR192 and QR192 genotypes are favored in reaching extreme ages, implicating that PON1 may be a longevity gene [65].

Gene polymorphisms of PON2 are also associated with an increased risk of cardiovascular diseases. As noted earlier in Section 3.2.2, the C311S polymorphism of PON2 affects PON2 activity, with the C isoform showing impaired lactonase activity [59]. A recent study in an Italian population reports that patients with at least one PON2 C allele represent a category of subjects at a higher risk for the development of acute myocardial infarction with a

worse prognosis [66]. The genotype with the C allele of the PON2 gene is also shown to be a risk factor for large vessel disease-associated stroke in a Polish population, further supporting a vasoprotective function of PON2 in humans [67]. These observations, however, contradict an early study showing S allele carriers of the PON2 C311S polymorphism at a greater risk of developing coronary heart disease than C allele carriers [68].

3.2.4. Role in Human Diabetes and Metabolic Syndrome

There are a number of clinical studies showing a significant decrease in serum PON1 activity in diabetic patients as compared with control subjects. The decrease in serum PON1 activity is also associated with the development of diabetic complications. A more recent study demonstrates that lower serum PON1 activity is related to higher plasma C-reactive protein and obesity [69]. These observations indicate a potential beneficial role for PON1 in diabetes and metabolic syndrome, which is also in line with findings in animal models (Section 3.1.4). However, epidemiological studies on the association of gene polymorphisms of PON1 with diabetes have produced inconsistent results. Some studies demonstrate that the PON1 genotypes causing decreased PON1 activity (e.g., PON1-QQ192 and PON1-MM55) are associated with poorer diabetes control and complications [70]. In contrast, other studies report that the PON1-192R allele causing increased PON activity can be an independent cardiovascular risk factor in type 2 diabetic patients [71].

As a further line of supportive evidence for a beneficial effect of PON1 in diabetes, the T allele of PON1 promoter polymorphism (-107C>T) causing reduced expression of PON1 is reported to be associated with lower serum levels of the enzyme and an increased risk for coronary heart disease in type 2 diabetic patients [72]. Moreover, the lower expressor genotype of PON1 (-107TT) is also associated with higher blood glucose concentration in non-diabetic subjects, suggesting that this polymorphism may predispose the individuals to insulin resistance [73]. Thus, a majority of the clinical and epidemiological findings support the view that PON1 may act as a potential protector in diabetes and its complications, as well as in metabolic syndrome.

3.2.5. Role in Human Neurological Diseases

Clinical and epidemiological studies have implicated PONs in various neurological disorders, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, schizophrenia, and anxiety. A decrease in the serum levels of PON1 activity is often observed in patients with Alzheimer's disease compared with control subjects. Some studies report that the R allele of PON1 Q192R polymorphism is associated with an increased serum PON1 activity and a decreased risk of developing Alzheimer's disease [74, 75]. Among the Alzheimer's patients, individuals who responded to cholinesterase inhibitors (drugs used for the treatment of Alzheimer's disease) had a significantly higher frequency in the R allele compared to non-responders, suggesting that PON1 may facilitate response to drug therapy [76]. The MM genotype of PON1 L55M polymorphism (causing decreased serum levels of PON1) is also associated with an increased risk of Alzheimer's disease, and correlated with the pathophysiological changes underlying the disease [77]. Taken together, these observations indicate that PON1 may be protective against Alzheimer's disease. However, there are also studies showing no association between PON1 gene polymorphisms and susceptibility to Alzheimer's disease [78]. In addition to PON1, a study in a Chinese population shows that the C allele of the C311S polymorphism of PON2 is associated with Alzhei-

mer's disease [79]. As noted earlier in Section 3.2.2, the C allele displays a decreased lactonase activity of PON2.

A lower PON1 activity is frequently observed in the sera of patients with Parkinson's disease as compared with control subjects. PON1 gene polymorphisms are implicated as risk factors for Parkinson's disease, but case-control studies have generated controversial results. A meta-analysis of the studies relating PON1 M55L and PON1 Q192R polymorphisms to risk of developing Parkinson's disease reveals that the MM55 genotype of PON1 (showing decreased serum levels of PON1) is associated with an increased risk of developing Parkinson's disease compared with the LL55 genotype. However, there is no association between the PON1 Q192R polymorphism and susceptibility to Parkinson's disease [80].

Differences in PON1 polymorphism frequencies and enzyme activity/levels have been investigated in several other neurological disorders. Polymorphisms in PON gene cluster are associated with amyotrophic lateral sclerosis [81]. Decreased serum levels/activity of PON1 are observed in patients with multiple sclerosis [82], schizophrenia [83], or anxiety disorder [84], supporting the notion that oxidative stress-mediated damage may contribute to the pathogenesis of these neurological diseases. Notably, PON1 expression is upregulated in the fetal liver of Down's syndrome patients, which may provide a clue as to why these patients have a lower incidence of atherosclerotic vascular disease than the general population [85].

3.2.6. Role in Human Liver Diseases

The fact that liver is the major synthesis organ for PON1 has prompted clinical investigations into the potential role of this protein in various human liver disorders involving oxidative stress and inflammation. Early studies demonstrated a significant decrease in serum PON1 activity in patients with liver cirrhosis, which has been confirmed by subsequent studies in patients with varying degrees of chronic liver diseases, including chronic hepatitis, chronic alcoholic liver injury, and nonalcoholic fatty liver disease [86-88]. Indeed, serum PON1 activity can reflect liver function, and as such has been proposed as a biomarker for monitoring severity and progression of chronic liver diseases [89]. The association of PON1 gene polymorphisms with chronic hepatitis C virus (HCV) infection has been investigated in one study. It shows that patients with HCV-related chronic hepatitis have a higher frequency of the RR isoform of the Q192R polymorphism than healthy subjects [87]. The plasma concentrations of peroxides are also higher in patients with the hepatitis than control subjects. As noted earlier, the PON1 RR192 genotype is associated with an increased serum activity of the enzyme (Table 17-1). Thus, the significance of above gene polymorphism study remains unclear. Further studies on the association of PON gene polymorphisms with chronic liver diseases will provide insights into the potential impact of PON genotypes on susceptibility to chronic liver diseases. Such studies may lead to identification of PON-associated risk factors for developing chronic liver diseases.

3.2.7. Role in Human Cancer

Oxidative stress and inflammation are critical events underlying carcinogenesis. By suppressing oxidative stress and inflammation, PON enzymes may protect against cancer development. However, to date little is known about the role of PONs in carcinogenesis in animal models. In contrast, there have been many studies in human subjects, suggesting a potential protective effect of PON1 in the development of different types of human cancer. A lower serum PON1 activity has been consistently observed in patients with various types of cancer

compared with control subjects. Gene polymorphisms of PON1 that cause decreased serum PON1 activity are associated with increased risk of developing malignancies, including lung cancer [90], breast cancer [91], ovarian epithelial carcinoma [92], prostate cancer [93], and brain cancer [94]. The causative role of PON1 as well as the other two members of PON family in cancer development warrants further investigations in both human subjects and experimental animals.

3.2.8. Role in Other Human Diseases

PON1 has also been implicated in many other human disorders based on observations from clinical and epidemiological studies. These include viral infections (e.g., HIV infection, hepatitis B infection) [95], *Helicobacter pylori* infection [96], idiopathic chronic pancreatitis [97], progressive IgA nephropathy [98], age-related macular degeneration [99], erectile dysfunction [100], and organophosphate poisoning [101]. The exact role of PON1 as well as other PON isozymes in the pathophysiology of the above disease conditions awaits further confirmation by additional well-designed studies.

4. Conclusion and Future Directions

PONs are multitasking antioxidant enzymes that have a beneficial role in a variety of oxidative stress- and inflammation-associated pathophysiological processes in experimental animals. Increasing evidence from studies in human subjects also suggests PONs as protective factors in many diseases, including atherosclerotic coronary heart disease, diabetes, neurodegeneration, and cancer. Currently, there is a great need to translate the important discoveries on PON isozymes into effective interventional strategies to prevent and treat human diseases that involve a PON-dependent mechanism. In this regard, future studies should focus on developing effective pharmacological inducers of PONs and evaluating their effectiveness in upregulating PON enzymes and in protecting against oxidative and inflammatory tissue degeneration underlying common human disorders.

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Chapter 18

Metal Ion Sequestering Proteins

Abstract

Metal ions such as iron and copper are essential elements, yet dysregulation of their homeostasis results in tissue injury due partly to their pro-oxidant properties. Mammals have evolved a number of mechanisms to maintain metal ion homeostasis, among which are ferritin and metallothionein (MT) that possess the ability to sequester metal ions in addition to other biological activities. In mammals, ferritin is a 24-subunit intracellular protein whose principal role within cells is the storage of iron in a nontoxic, but bioavailable form. Studies in experimental models demonstrate that ferritin plays a role in protecting against pathophysiological conditions involving oxidative stress, such as myocardial ischemia-reperfusion injury and neurodegeneration. A number of ferritin genetic disorders have been reported in human subjects with iron-facilitated oxidative stress being a contributing factor to the disease pathogenesis. MT refers to a superfamily of small molecular mass proteins with high cysteine content and the capacity to bind various heavy metals, including copper, zinc, cadmium, and mercury. MTs regulate copper and zinc homeostasis. They are also important defenses against heavy metal toxicity as well as oxidative and inflammatory stress. Extensive studies in animal models demonstrate an important protective function for MTs in a number of disease conditions, such as cardiovascular disorders, diabetes, neurodegeneration, and cancer. Studies in human subjects also support a potential involvement of MTs in certain disease processes, including atherosclerosis and diabetes.

1. Introduction

Metal ions, including copper and iron ions are essential elements in mammals. For example, these metal ions form the active centers of a variety of important enzymes in mammalian cells or tissues. However, these metals, particularly copper and iron when exist as free ions can also cause detrimental effects. Three mechanisms are involved in the detrimental effects mediated by copper and iron ions. They are summarized as following.

- Participation in Fenton/Fenton-type reactions to produce the extremely reactive hydroxyl radical (Section 2.3.3 of Chapter 1)

- Catalysis of auto-oxidation of biomolecules (e.g., ascorbate, catecholamines) to form reactive species, such as free radicals and reactive quinones
- Involvement in decomposition of lipid hydroperoxides to form peroxyl and alkoxyl radicals (Section 2.3.6 of Chapter 1)

Due to the adverse effects of free metal ions, mammals have evolved diverse mechanisms for sequestration of these essential but also potentially toxic metal ions. This chapter focuses on discussing two important metal ion-sequestering proteins in mammalian systems: ferritin and metallothionein.

2. Ferritin

2.1. General Aspects, Biochemistry, and Molecular Regulation

2.1.1. General Aspects and Biochemistry

Ferritin was first discovered in 1973 by V. Laufberger, who isolated a new protein from horse spleen that contained up to 23% by dry weight of iron. In mammals, ferritin is a 24-subunit intracellular protein whose principal role within cells is the storage of iron in a non-toxic, but bioavailable form [1]. The assembled ferritin molecule, often referred to as a nanocage, can store up to 4,500 atoms of iron. Iron in the ferritin nanocage is insoluble and most likely redox inactive. In mammals, there are two ferritin genes that encode for the heavy (H) and light (L) subunits of ferritin, with a molecular mass of 19 and 21 kDa, respectively. Although the two subunits share approximately 55% of their sequences as well as their multihelical three-dimensional structures, they are functionally distinct. The heavy subunit is primarily responsible for the ferrioxidase activity of the ferritin complex, whereas the light subunit facilitates the storage of iron into the ferritin core. The efficient storage of iron requires the coordinated actions of both subunits.

Ferritin is primarily localized in cytosol, existing as a heteropolymers of both heavy and light subunits. It is also present in nuclei and plasma [2, 3]. Nuclear ferritin is found to be the same as cytosolic ferritin, and not the product of a separate gene. Similarly, plasma ferritin is believed to be also derived from cytosolic ferritin though plasma ferritin is composed mainly of the light subunits and is relatively iron-poor. Recently, a third mammalian nuclear gene has been identified to code for the precursor of the mitochondrial ferritin (FtMt). The mature mitochondrial ferritin subunit possessing ferrioxidase activity assembles into homopolymers [4]. In humans, the genes for ferritin heavy subunit, light subunit, and mitochondrial ferritin are localized on chromosomes 11q12, 19q13.33, and 5q21.3, respectively.

2.1.2. Molecular Regulation

The expression of ferritin subunits is under both transcriptional and translational regulation. The translational regulation of ferritin subunits has been well-characterized. The mRNAs of heavy and light subunits contain iron regulatory element (IRE), which binds with high affinity the repressor, named iron response proteins (IRPs including IRP1 and IRP2). The binding activity of IRPs to IRE is modulated by cellular availability of iron. When cellular iron is abundant, IRPs lose their binding activity to IRE, and thereby leading to increased

translation of ferritin subunits. On the other hand, under cellular iron deficiency, the binding activity of IRPs to IRE is increased, thus resulting in decreased translation of ferritin subunits [1]. Redox status may also affect the translation of ferritin subunits. Reactive oxygen species (ROS) cause ferritin induction by inactivating IRP1 through reversible oxidation of critical cysteine residues [5]. As discussed below, ROS can also cause ferritin induction via activating transcription of the ferritin subunit genes.

At the transcriptional level, several factors can enhance the expression of ferritin subunits. These factors include inflammatory cytokines, oxidants, and heme. A number of transcription factors have been shown to mediate the transcription of ferritin subunit genes. For example, Nrf2 and Jun D activate transcription of human ferritin heavy subunit gene via an antioxidant response element (ARE)-driven mechanism [6, 7]. This Nrf2/ARE pathway is shown to mediate the induction of ferritin heavy subunit by phenolic compounds, dithiolethiones, and oxidants [7]. Nrf2 may also control the transcriptional expression of ferritin light subunit gene [7]. NF-κB is demonstrated to mediate the transcriptional activation of ferritin heavy subunit by tumor necrosis factor-α [8]. It is reported that p53 downregulates the promoter activity of ferritin heavy subunit gene [9]. On the other hand, ferritin is shown to interact with p53 and increase p53 protein level and transcriptional activity during oxidative stress [10]. This suggests that ferritin cooperates with p53 to cope with oxidative stress.

2.2. Role in Health and Disease

2.2.1. General Biological Activities

Ferritin has a central role in the regulation of cellular iron homeostasis. Sequestration of iron ions by ferritin is an important mechanism for controlling iron-mediated ROS formation and oxidative damage in mammalian systems (Figure 18-1). As noted earlier, ferritin is mostly cytosolic but also found in mitochondria and nuclei. There is substantial evidence that mitochondrial ferritin may protect this organelle from iron toxicity and oxidative damage [11]. Similarly, nuclear ferritin may bind and protect nuclear DNA from oxidative injury. In addition, nuclear ferritin has been suggested to participate in the transcriptional regulation of β-globin gene [2]. As noted earlier in Section 2.1.1, ferritin also exists in extracellular compartments, such as plasma. Ferritin in plasma has low iron content and a distinct subunit composition. Recently, plasma ferritin is shown to regulate blood vessel formation, a novel role beyond iron storage [12].

Iron can be released from ferritin by several reducing agents, including superoxide in vitro. As described in Section 2.3.1 of Chapter 1, superoxide is also reactive to iron-sulfur clusters in enzymes, leading to release of iron from the enzymes. The released iron further reacts with hydrogen peroxide, generating the highly reactive hydroxyl radical. Indeed, superoxide and iron have been suggested as partners in crime in oxidative tissue injury [13].

2.2.2. Animal Studies

2.2.2.1. Experimental Approaches

Animal models of both targeted disruption and transgenic overexpression of ferritin heavy subunit (FTH) have been reported in the literature. Homozygous knockout of the heavy

subunit results in early embryonic lethality, indicating an essential role for FTH in murine development [14].

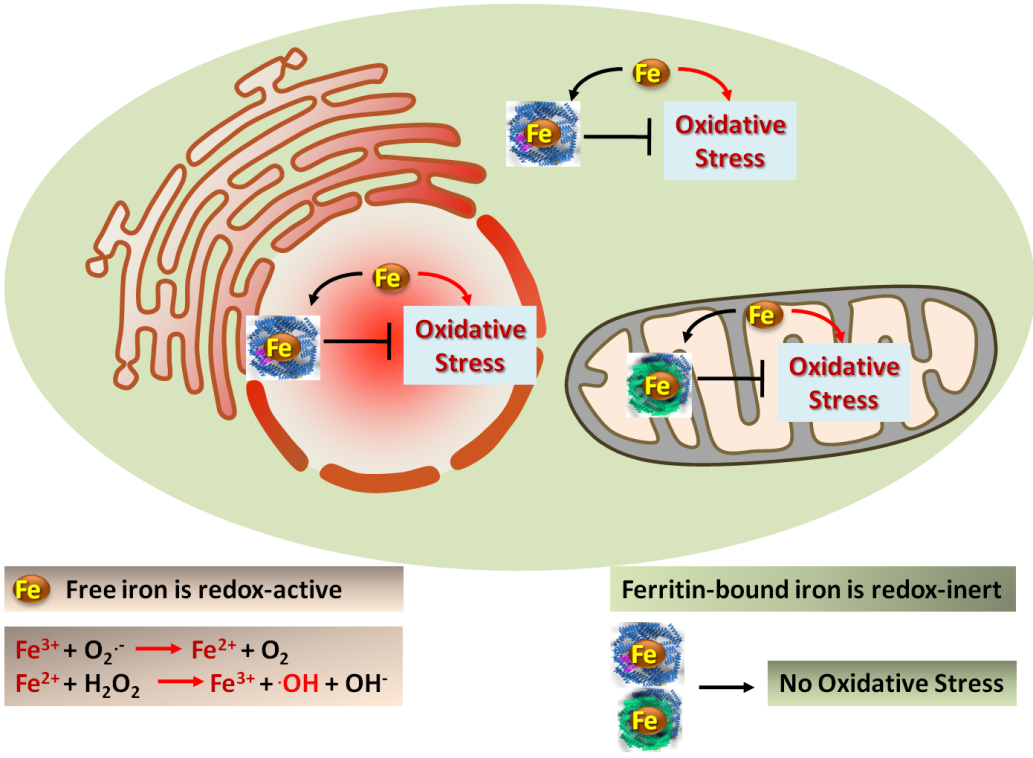

Figure 18-1. Protection against iron-mediated oxidative stress by ferritin in cytosol, mitochondria, and nuclei. As shown, free iron is redox-active and participates in Fenton reaction, generating hydroxyl radical. In contrast, iron sequestered by ferritin is redox-inert.

In contrast, the heterozygous knockout ($FTH^{+/-}$) mice are born and reproduced normally [15]. Due to the embryonic lethality of the global homozygous knockout of FTH, conditional FTH knockout mice have recently been characterized with respect to phenotypic changes [16]. Generation and phenotypic characterization of these genetically-manipulated animal models have been key strategies for understanding the biological functions of ferritin in mammals.

2.2.2.2. Role in Cardiovascular Diseases and Related Conditions

Evidence indicates a protective role for ferritin in cardiovascular disorders, such as ischemic heart injury. Induction of myocardial ferritin is observed during cardiac ischemia [17]. It has been suggested that cardiac protection by ischemic preconditioning is mediated by ferritin. Cardiac ischemic preconditioning increases de novo synthesis of ferritin in cardiac tissue of mice, and the elevated ferritin may serve as a sink for redox-active iron, thus protecting myocardium from iron-mediated oxidative damage associated with ischemia-reperfusion injury [18]. In cultured cardiomyocytes, downregulation of ferritin heavy subunit by siRNA

increases labile iron pool, oxidative stress, and cell death, supporting a protective role for ferritin in iron-mediated oxidative cardiac injury [19]. As discussed in Chapter 15, heme oxygenase-1 is a critical protector against oxidative pathophysiology underlying various disease conditions, including cardiovascular disorders. Induction of heme oxygenase-1 is often associated with augmented synthesis of ferritin, which has been suggested to be partially responsible for the beneficial effects of heme oxygenase-1. In this regard, adenovirus-mediated overexpression of ferritin heavy subunit is shown to protect cardiovascular cells from apoptosis elicited by various insults, including ROS [20].

A potential role for ferritin in metabolic syndrome has been proposed based on its interaction with adiponectin. Adiponectin is an adipocyte-derived protein that acts to reduce insulin resistance in liver and muscle and also inhibits atherosclerosis. In primary murine skeletal cells, adiponectin is found to upregulate ferritin heavy subunit via an NF-κB-dependent mechanism. The adiponectin-mediated antioxidative stress activity is shown to rely on the induction of ferritin. Hence, upregulation of ferritin may partially explain how adiponectin protects against oxidative stress and insulin resistance in skeletal muscle [21]. In this context, oxidative stress is an important event underlying insulin resistance and other manifestations of metabolic syndrome.

2.2.2.3. Role in Neurodegenerative Diseases

As noted earlier, homozygous deletion of ferritin heavy subunit causes embryonic lethality, whereas mice with heterozygous deletion are born and reproduced normally. As compared with wild-type mice, the heterozygous mice have less than half the levels of ferritin heavy subunit and exhibit an increase in the indices of oxidative stress and apoptosis in the brain, implying a beneficial role for ferritin in oxidative neurodegeneration [22]. Transgenic overexpression of ferritin heavy subunit in dopaminergic neurons of the substantia nigra results in increased levels of ferritin in the neurons. This affords protection against neurodegeneration and oxidative stress elicited by two parkinsonism-inducing toxicants, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and paraquat, in young mice [23, 24]. Similarly, pharmacological iron chelation also protects against experimental parkinsonism [23, 24]. These observations suggest a causative role for iron-mediated oxidative stress in the pathogenesis of experimental parkinsonism and the feasibility of developing strategies targeting ferritin for the intervention of this neurodegenerative disorder. However, a recent study shows that chronic expression of ferritin heavy subunit in dopaminergic midbrain neurons of older mice results in an age-related expansion of the labile iron pool and subsequent oxidative neurodegeneration [25], pointing to a complex role of ferritin in iron metabolism and neurodegeneration associated with aging.

Genetic manipulation of ferritin light subunit has been employed to study the molecular involvement of ferritin in neurodegenerative disorders. Insertional mutations in exon 4 of the ferritin light subunit gene are associated with hereditary ferritinopathy (also called neuroferritinopathy), an autosomal dominant neurodegenerative disease characterized by progressive impairment of motor and cognitive functions [1] (Section 2.2.3.1). Transgenic overexpression of one of the mutant human ferritin light subunit cDNAs in mice leads to abnormal iron metabolism likely due to the compromised ability of the mutant ferritin to sequester iron. The transgenic mice exhibit increased lipid peroxidation and oxidative protein damage in the brain tissue [26].

Mitochondrial ferritin, a newly discovered ferritin, is expressed in relatively high levels

in high energy-consuming cells, such as neurons. A recent study demonstrates that overexpression of mitochondrial ferritin inhibits 6-hydroxydopamine-induced dopaminergic neuron damage and oxidative stress [27].

2.2.2.4. Role in Hepatic Diseases

As noted above in Section 2.2.2.1, a conditional deletion of ferritin heavy subunit in adult mice has recently been reported. This approach deletes ferritin heavy subunit almost completely in the liver, bone marrow, spleen, and thymus, but less prominently in other tissues. The mice are viable and survive up to two years without visible abnormalities. However, mice fed on a high iron diet prior to ferritin heavy subunit deletion suffer from severe liver damage, as evidenced by markedly increased release of liver enzymes into circulation, hepatic cell death, macrosteatosis, hemorrhage, and infiltration of inflammatory cells [16]. Cell depleted of ferritin heavy subunit show increased sensitivity to iron salt-induced death in cultures, and the cell injury can be prevented by overexpression of a wild-type ferritin heavy subunit but not by a mutant subunit lacking ferrioxidase activity. The cell death is preceded by an increase in cytoplasmic free iron, ROS, and mitochondrial dysfunction [16]. These results indicate that the iron-sequestering function of ferritin plays a major role in preventing iron-mediated cell and tissue damage.

Iron-elicited oxidative stress is involved in tissue ischemia-reperfusion injury. A study demonstrates that adenovirus-mediated overexpression of ferritin heavy subunit gene protects rat livers from ischemia-reperfusion injury and prevents hepatocellular damage upon transplantation into syngeneic recipients [28]. The protective effects of overexpression of ferritin heavy subunit are associated with the inhibition of endothelial cell and hepatocyte apoptosis in vivo. In vitro studies further show that overexpression of the ferritin heavy subunit in endothelial cells suppresses apoptosis induced by a variety of stimuli [28]. These findings demonstrate that ferritin acts as a critical protector in liver degeneration associated with oxidative stress.

2.2.2.5. Role in Cancer

It has been proposed that ferritin by blocking iron-mediated oxidative stress may play a dual role in cancer development. On the one hand, ferritin may prevent cancer development by inhibiting iron-mediated oxidative DNA damage and multistage carcinogenesis. Indeed, overexpression of ferritin heavy subunit decreases intracellular labile iron pool and oxidative stress, and attenuates transforming growth factor-induced hepatic epithelial-mesenchymal transition, a process known to be critical in carcinogenesis [29]. On the other hand, by protecting against oxidative damage and apoptosis, ferritin may promote the survival of the already formed cancer cells. In this regard, serum ferritin levels are often found to be increased in cancer patients, suggesting a possible involvement of ferritin in facilitating cancer development (see Section 2.2.3.2 below). Indeed, serum ferritin has recently been shown to promote angiogenesis in a tumor angiogenesis model [12]. Overexpression of ferritin light subunit is also associated with metastatic phenotype of human melanoma cells. Conversely, downregulation of ferritin light subunit in melanoma cells strongly inhibits their proliferation and invasiveness in both in vitro and in vivo models [30]. Downregulation of ferritin light subunit also sensitizes the melanoma cells to oxidative stress and apoptosis [30]. These data suggest that ferritin may facilitate melanoma progression by promoting cell growth and invasiveness as well as by inhibiting apoptosis in the experimental models. Hence, whether ferri-

tin plays an inhibitory or promoting role in cancer development may depend on the stage of carcinogenesis.

2.2.3. Human Studies and Clinical Perspectives

The involvement of ferritin in human health and disease can be summarized into the following two scenarios.

- Genetic ferritin disorders
- Changes of serum ferritin as a marker or risk factor of disease conditions

2.2.3.1. Genetic Ferritin Disorders

Genetic variations have been reported for both ferritin heavy and light subunit genes. A single point mutation (A49U) in the IRE motif of H-ferritin mRNA was identified in four of seven members of a Japanese family affected by dominantly inherited iron overload [31]. This mutation is responsible for tissue iron deposition and is a novel cause of hereditary iron overload. A more extensive study of 660 human subjects with abnormalities in serum ferritin and iron status identified only two additional novel mutations in the 5'-untranslated region of ferritin heavy subunit, which however did not seem to contribute to the abnormalities in serum ferritin and iron status [32]. This suggests that ferritin heavy subunit gene is not easily subject to mutations.

In contrast to genetic variations in ferritin heavy subunit, mutations of ferritin light subunit gene are more frequent and often lead to disorders. For example, heterozygous mutations in the 5'-untranslated region of the mRNA cause hereditary hyperferritinemia cataract syndrome (HHCS), whereas the mutations that modify the C-terminal part of the protein sequence cause hereditary ferritinopathies with neurodegeneration. Individuals with HHCS have mutations that alter the structure and functionality of the iron regulatory element (IRE), and reduce its binding affinity to the iron regulatory proteins (IRPs), thereby leading to increased expression of light subunit. Excess of ferritin light subunit in the lens causes aggregation into microcrystals, resulting in bilateral nuclear cataract [1]. Patients with hereditary ferritinopathies (also known as neuroferritinopathies) exhibit movement abnormalities, heterozygous extrapyramidal Parkinson-type symptoms, and spherical inclusions containing ferritin and iron in brain and other organs [1]. Studies in experimental models suggest that these mutations cause dysfunction of ferritin, leading to increased labile iron pool and oxidative stress as well as protein aggregation [26].

2.2.3.2. Serum Ferritin in Disease Conditions

Increased levels of serum ferritin, known as hyperferritinemia have been frequently observed in patients with various pathophysiological conditions, including iron overload, acute inflammation and infection, chronic inflammatory disorders, autoimmunity, and malignancies [3]. Under certain circumstances, serum ferritin can serve as a useful marker for the above disorders; however, interpretation of the results should be done with caution due to the multiplicity of conditions affecting serum ferritin levels. On the other hand, certain pathophysiological conditions, such as iron deficiency anemia have been associated with decreased levels of serum ferritin. Indeed, serum ferritin is routinely ordered in the evaluation of anemia in human patients [26].

Extensive studies suggest that changes in serum ferritin may be a risk factor for certain human diseases. For example, higher serum ferritin concentrations in postmenopausal women are associated with an increased risk of ischemic stroke [33]. Several recent studies also demonstrate that hyperferritinemia is associated with insulin resistance and fatty liver [34, 35]. Elevated maternal serum ferritin is also related to a higher risk of gestational diabetes and intra-uterine growth retardation [36]. As mentioned in Section 2.2.2.2, studies in experimental models suggest a protective role for ferritin in ischemic heart disease. However, epidemiological studies on ferritin/iron status and cardiovascular diseases have yielded conflicting results, with two recent larger studies failing to show a linear association between serum ferritin/iron status and risk of ischemic heart disease [37, 38].

Taken together, although serum ferritin can be used as a biomarker for certain pathophysiological conditions, including iron overload and iron-deficient anemia, the value of serum ferritin as a biomarker or risk factor in other prevalent diseases, such as diabetes and cardiovascular disorders remains to be further determined. In addition, it should be noted that the association of hyperferritinemia with increased risk of certain diseases does not establish a causal role for ferritin in disease development. Instead, changes in serum ferritin most likely reflect the body's iron status or responses to pathophysiological conditions. Thus, future studies should focus on identification of the causal involvement (either protective or detrimental) of ferritin in human diseases. In this regard, continued genetic variation studies may lead to discovery of new mutations in ferritin subunit genes as well as in mitochondrial ferritin gene. Characterization of the novel mutations and the associated phenotypic changes will provide important insights into the molecular pathways through which ferritin contributes to human health and disease.

3. Metallothionein

3.1. General Aspects, Biochemistry, and Molecular Regulation

3.1.1. General Aspects and Biochemistry

Metallothionein (MT) refers to a superfamily of small molecular mass proteins (6-7 kDa) with high cysteine content and the capacity to bind various heavy metals, including copper, zinc, cadmium, and mercury. MT was first isolated from horse kidney and characterized by M. Margoshes and B.L. Vallee in 1957. In mammals, there are four types of MT proteins, namely, MT-1, MT-2, MT-3 and MT-4, among which MT-1 and MT-2 have been most extensively investigated. MT-1 and MT-2 are widely distributed in mammalian tissues. MT-3 is found mainly in brain, kidney and reproductive organs, whereas MT-4 is localized primarily in squamous epithelia [39].

In humans, the MT genes are tightly clustered in the q13 region of chromosome 16, consisting of seven functional MT-1 genes (MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, and MT-1X) and a single gene encoding each of the other three MT isozymes, namely, MT-2 (MT-2A gene), MT-3, and MT-4. Mice have a much simpler gene structure with only one functional gene for each of the four isozymes, namely MT-1, MT-2, MT-3, and MT-4, all located on chromosome 8 [39]. It should be noted that majority of MT basic research has been performed in rodents, predominantly mice.

3.1.2. Molecular Regulation

The genes of MT-1 and MT-2 are usually expressed coordinately in mammalian tissues, whereas MT-3 and MT-4 exhibit a much more restricted tissue expression. Regulation of MT-1 and M-2 expression has received much more extensive studies than that of MT-3 and MT-4, and occurs primarily at the transcriptional level. Both MT-1 and MT-2 are highly inducible in response to a wide range of stimuli, including heavy metal ions, glucocorticoids, cytokines, inflammation, electrophiles, and oxidants. A number of transcriptional regulatory pathways have been identified for the upregulation of MT-1 and MT-2 gene expression by various stimuli. For example, metal ion-induced expression is mediated by metal-response element-binding transcription factor-1 (MTF-1) via a metal response element (MRE)-driven mechanism. Similarly, glucocorticoid-induced expression of MT-1 and MT-2 genes is mediated by a glucocorticoid response element (GRE)-driven mechanism [40]. In addition to MRE and GRE, the promoter region of MT-1 and MT-2 genes also contains the binding sites for a number of other transcription factors, such as SP1, Nrf1, and Nrf2. Although both Nrf1 and Nrf2 are able to bind to the antioxidant response elements (AREs) of the MT-1 and MT-2 genes, only Nrf1 activates the transcription [41]. As discussed in Chapter 2, Nrf2/ARE pathway plays a central role in the upregulation of antioxidant gene expression by metals, oxidants, electrophiles, and inflammatory cytokines. Therefore, the role of Nrf2/ARE in the regulation of MT-1 and MT-2 gene expression under the above conditions merits further investigations. Currently, the molecular regulation of MT-3 and MT-4 gene expression remains largely unknown.

3.2. Role in Health and Disease

3.2.1. General Biological Activities

The biological functions of MT proteins are summarized below.

- Storage of the heavy metals in a nontoxic form
- Regulation of cellular copper and zinc metabolism
- Antioxidant and anti-inflammatory activities

As illustrated in Figure 18-2, the antioxidant activities of MT proteins stem from two main mechanisms. They are: (1) direct ROS-scavenging ability due to the high cysteine content of the MT proteins and (2) chelation of copper ions, thus preventing the formation of hydroxyl radical and other reactive species from copper ion-mediated redox reactions. Since oxidative stress and inflammation are intimately related processes, the antioxidant activities of MT proteins may also explain their anti-inflammatory functions under various pathophysiological conditions.

3.2.2. Animal Studies

3.2.2.1. Experimental Approaches

Much of our current knowledge on MTs results from studies in murine models, especially gene knockout and transgenic overexpression mouse lines. Due to the coordinated expression

of MT-1 and MT-2, simultaneous knockout of both genes (MT-1/MT-2-null) in mice has been described, and the mice are born and reproduced normally. MT-3 knockout mouse model has also been reported in the literature, and the mice develop neurological/behavioral disorders [42, 43] (Section 3.2.2.4).

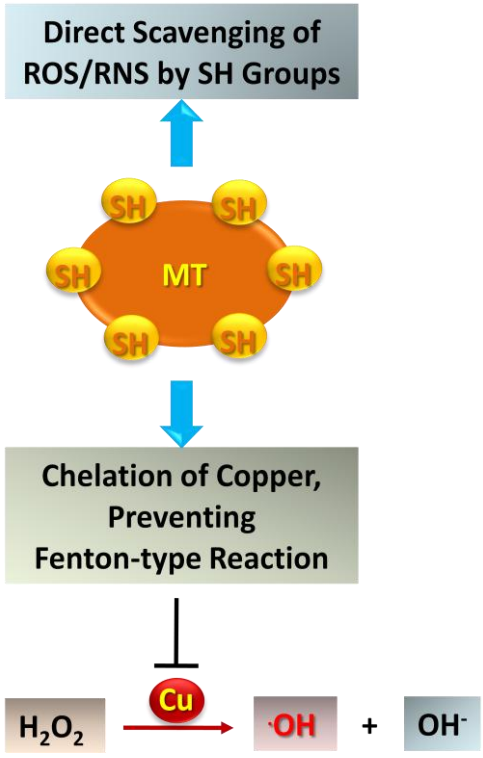

Figure 18-2. Mechanisms underlying metallothionein (MT)-mediated antioxidative activities. As illustrated, MT proteins can directly scavenge reactive oxygen and nitrogen species (ROS/RNS) due to the high content of cysteine residues. MT proteins by binding to copper ion also prevent copper from participating in Fenton-type reaction to generate hydroxyl radical.

3.2.2.2. Role in Cardiovascular Diseases

Studies in genetically manipulated animal models have demonstrated a protective role for MT-1/MT-2 in various forms of cardiovascular disorders. These include ischemia-reperfusion injury [44], alcoholic cardiomyopathy [45], doxorubicin-induced cardiotoxicity [46], as well as aging-, high-fat diet-, or sepsis-induced cardiac contractile dysfunction [47-49]. Overexpression of MT-1/MT-2 attenuates the oxidative damage and inflammatory responses in myocardium under the above pathophysiological conditions, pointing to an involvement of the antioxidant activities of MT proteins in disease protection. Recently, it has been shown that MT-1/MT-2 can also enhance angiogenesis and arteriogenesis by upregulating endothelial growth factor expression and modulating smooth muscle cell and macrophage function [50]. These novel activities may result in collateral flow recovery and contribute to the cardioprotective effects of MT proteins in ischemic heart disease.

3.2.2.3. Role in Diabetes and Metabolic Syndrome

Mounting evidence supports a beneficial role of MT proteins in diabetes and metabolic syndrome in experimental models. Overexpression of MT-1/MT-2 in the pancreatic β-cells renders these cells more resistant to oxidative damage [51]. Transgenic overexpression of the MT proteins in mice results in attenuation of streptozotocin-induced diabetes [52]. MT overexpression also protects against diabetic complications, such as diabetic cardiomyopathy in experimental animals [53]. Insulin resistance-induced cardiac dysfunction in mice is alleviated by transgenic MT overexpression in myocardium [54], suggesting a beneficial effect of MT proteins in metabolic syndrome-associated complications. MT proteins may also function to suppress the development of metabolic syndrome. In this regard, an early study demonstrated that targeted disruption of MT-1/MT-2 genes in mice results in moderate obesity and hyperleptinemia [55]. More recently, MT-1/MT-2-null mice have been shown to be more susceptible to high-fat diet-induced obesity as compared with wild-type counterparts [56]. Together, these findings indicate a protective function of MT-1/MT-2 in the genesis of metabolic syndrome.

3.2.2.4. Role in Neurological Diseases

One of the best characterized functions of MT-1/MT-2 is the protection against various forms of neurological disorders, including cerebral ischemia-reperfusion injury [57, 58], toxin-induced parkinsonism [59, 60], excitotoxicity [61], autoimmune encephalomyelitis [62], and traumatic brain or spinal cord injury [63, 64]. Overall, the beneficial effects of MT-1/MT-2 observed in these experimental models are consistent with the protective functions of the MT proteins in oxidative stress and inflammation. Indeed, MT-1/MT-2 proteins have been proposed as multipurpose neuroprotectants in brain pathogenesis [65]. Recently, studies in MT-3-null and -overexpressing mice also demonstrate an important role for this brain-selective MT isoform in attenuating cerebral ischemic injury and kainic acid-induced seizure [42, 66]. Moreover, MT-3-null mice develop abnormalities in psychological behaviors, suggesting a possible involvement of MT-3 in higher levels of brain function [43].

3.2.2.5. Role in Pulmonary Diseases

Multiple studies demonstrate a protective role for MT proteins in acute lung injury and inflammation induced by toxicants, such as nickel and ozone [67, 68]. The lung injury and inflammation elicited by lipopolysaccharide (given via either intratracheal instillation or systemic administration) are much more severe in MT-1/MT-2-null mice than that in wild-type animals [69, 70], implicating a potent anti-inflammatory function of MT-1/MT-2. In line with this notion, targeted disruption of MT-1/MT-2 genes in mice also sensitizes the animals to ovalbumin-induced airway inflammation and oxidative stress, as evidenced by increased proinflammatory cytokine production, infiltration of inflammatory cells, and oxidative DNA damage [71]. Ovalbumin-induced airway inflammation is a commonly used experimental model of asthma.

3.2.2.6. Role in Hepatic and Gastrointestinal Diseases

Liver is a major target organ of chemical toxicity. MT proteins play a central part in protecting against hepatic injury induced by heavy metals, such as cadmium [72]. MT proteins also alleviate liver injury caused by other chemicals, including carbon tetrachloride [73], alcohol [74], acetaminophen [75], cisplatin [76], and lipopolysaccharide [77]. There is also evi-

dence indicating an involvement of MT proteins in liver cell regeneration [78]. Moreover, adenovirus-mediated human MT-2A gene transfer is shown to reverse liver fibrosis and promote hepatocyte regeneration in a mouse model of carbon tetrachloride-induced irreversible liver damage [79].

MT proteins possess a beneficial activity in certain gastrointestinal disorders. Increased expression of MT proteins is observed in experimental animals with inflammatory bowel disease or *Helicobacter pylori* infection [80, 81]. A recent study demonstrates that targeted disruption of MT-1/MT-2 genes in mice markedly aggravates *H. pylori*-induced gastric inflammation and ulceration as compared with wild-type mice [82]. However, it remains unknown if selective overexpression or induction of MT proteins in gastrointestinal tissues is also protective against gut inflammatory disorders.

3.2.2.7. Role in Renal Diseases

Studies implicate MT-1/MT-2 as important protective molecules in nephrotoxicity induced by toxicants and drugs, including cadmium, arsenicals, and cisplatin. For instance, targeted disruption of MT-1/MT-2 in mice greatly sensitizes the animals to cadmium-induced nephrotoxicity following chronic oral exposure [72]. Long-term exposure to arsenicals leads to renal injury in wild-type mice; however, the renal toxicity is dramatically aggravated in MT-1/MT-2-null mice, as manifested by increased tubular cell vaculolization, inflammatory cell infiltration, and interstitial fibrosis [83].

Cisplatin is a widely used anticancer drug whose clinical application is limited by nephrotoxicity. Targeted disruption of MT-1/MT-2 in mice exacerbates cisplatin-induced renal injury. Pre-induction of renal MT proteins by bismuth nitrate or zinc sulfate protects the wild-type mice from cisplatin-induced nephrotoxicity. However, the same treatment in MT-1/MT-2-null mice does not induce MT proteins and fails to suppress cisplatin-elicited nephrotoxicity [84]. These results indicate that MT proteins are an important endogenous defense against cisplatin-associated renal injury and point to the possibility to attenuate this anticancer drug-induced nephrotoxicity by upregulating renal MT proteins.

3.2.2.8. Role in Skin Diseases

The proangiogenesis properties of MTs along with their role in cell regeneration implicate that these metal-binding proteins may be involved in skin wound healing. MT-1/MT-2 are upregulated in skin tissue following topical application of zinc and copper. These proteins are also elevated in the wound margins, particularly in regions of high mitotic activity. In addition, MT expression in the wound margins is accompanied by accumulation of zinc, a metal involved in wound healing [85, 86]. Induction of MTs in the wound margins may reflect their role in promoting cell proliferation and reepithelialization. Indeed, either chemical or physical injury-induced epidermal cell proliferation is attenuated in mice with targeted disruption of MT-1/MT-2 [87].

MT-1/MT-2-null mice also exhibit reduced tolerance to ultraviolet B (UVB)-induced skin injury, as revealed by increased numbers of sunburn cells and apoptotic cells compared with wild-type littermates [88]. These experimental data suggest that MT-1/MT-2 have a photoprotective activity in vivo. The in vivo protective effect of MTs on UV irradiation-induced skin injury is also consistent with the observations that MT proteins in skin are highly inducible by UVB, and that induction of MTs in cultured keratinocytes attenuates UVB-elicited cell injury and oxidative stress.

3.2.2.9. Role in Cancer

As described in prior sections, MTs exhibit antioxidant and anti-inflammatory activities, and they also possess antiapoptotic, proliferative, and proangiogenic properties. On the one hand, the antioxidant and anti-inflammatory activities of MTs implicate a protective role for these proteins in carcinogenesis as oxidative stress and inflammation are critical events underlying multistage carcinogenesis. On the other hand, the antiapoptotic, proliferative, and proangiogenic properties of MTs would suggest a potential tumor promoting activity. The protection by MTs against anticancer drug-induced cytotoxicity may also implicate them in anticancer drug resistance. However, direct evidence for a causal tumor promoting activity of MTs in experimental animal models has been largely lacking though multiple studies have shown an increased expression of MT proteins in malignancies [89].

In contrast, extensive studies using genetically manipulated mouse models demonstrate a critical role for MTs in protecting against multistage carcinogenesis induced by various chemical carcinogens as well as γ-irradiation. For example, MT-1/MT-2-null mice are found to be hypersensitive to lead-induced kidney carcinogenesis and cisplatin-induced hepatocellular carcinoma as compared with wild-type counterparts [90, 91]. MT-1/MT-2 deficiency in mice also markedly sensitizes the animals to multistage skin carcinogenesis caused by 7,12-dimethylbezn(a)anthracene and 12-*O*-tetradecanoylphorbol-13-acetate [92]. In addition to chemical carcinogenesis, targeted disruption of MT-1/MT-2 in mice also renders the animals more susceptible to γ-irradiation-induced thymic lymphoma and oxidative DNA damage as compared with wild-type littermates [93].

3.2.2.10. Role in Other Diseases and Conditions

Studies using mice with genetically manipulated MT genes indicate that these metal-binding antioxidant proteins may be protective in many other pathophysiological conditions. These include chemically-induced retinal neuron damage [94], selective degeneration of central photoreceptors caused by hyperbaric oxygen [95], X-irradiation-induced bone marrow depression [96], and multiple organ damage following lipopolysaccharide-elicited systemic inflammation [70].

3.2.3. Human Studies and Clinical Perspectives

The involvement of MTs in protection against various pathophysiological conditions as revealed in experimental animals suggests that these proteins may also have a beneficial role in human health and disease. Indeed, studies show that levels of MTs are altered in some human disorders, especially malignancies [89]. MT expression is found to be upregulated in certain human malignancies, such as breast and lung cancer, and downregulated in others, including hepatocellular carcinoma. It has been recently reported that MT-1G acts as a potential tumor suppressor gene in human hepatocellular carcinoma [97].

Genetic variation studies in human populations have identified multiple polymorphisms in both promoter and coding regions of the MT genes. Among them several alleles are shown to be associated with increased risk of developing certain disorders, including atherosclerosis [98, 99], diabetes and diabetic complications [100, 101], and heavy metal toxicity [102, 103]. In addition, a recent study in an Italian population demonstrates that a polymorphism in MT-1A gene coding region is associated with longevity [104]. While these studies indicate an association between genetic variations of MT genes and susceptibility to developing certain

pathophysiological conditions, they do not establish a causal involvement of MT proteins in the disease processes. As such, it becomes necessary that when performing case-control studies, MT protein levels are detected, and the observed differences in MT protein levels are matched by the genotypes. In addition, the association revealed by epidemiological studies should be further investigated in experimental models, including cell cultures and genetically manipulated animals. Such experimental studies will help establish a causative relationship between genetic variations of MT genes, changes of MT proteins, and susceptibility to disease development.

4. Conclusion and Future Directions

Both ferritin and MTs are critically involved in regulating essential metal ion homeostasis. They also possess antioxidant and anti-inflammatory activities as well as other novel functions, such as regulation of blood vessel formation. These multifunctional proteins play an important part in protecting against various disease processes involving oxidative stress and inflammation in animal models. Although being not as compelling as the findings in animal studies, observations from clinical and epidemiological studies do suggest a possible involvement of these metal-sequestering proteins in human health and disease. Continued basic and clinical research is warranted to further elucidate the causative role (either beneficial or detrimental) for both ferritin and MTs in disease pathophysiology, and develop strategies selectively targeting these proteins to combat human disorders involving oxidative and inflammatory stress.

5. References

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Chapter 19

Other Non-Protein Antioxidants Synthesized by Cells

Abstract

Previous chapters have discussed the endogenous protein antioxidants as well as the tripeptide glutathione synthesized in mammalian cells. There are also a variety of non-protein antioxidants produced by mammalian cells, including bilirubin, coenzyme Q, estrogens, α-lipoic acid, melatonin, pyruvate, and uric acid. This chapter describes each of these common non-protein compounds with respect to their antioxidant activities and role in health and disease.

1. Introduction

The most important non-protein antioxidant synthesized by cells is perhaps the tripeptide, glutathione, which is described in chapter 5. In addition to glutathione, a number of other non-protein compounds with antioxidant properties are also synthesized by mammalian cells. These include bilirubin, coenzyme Q, estrogens, α-lipoic acid, melatonin, pyruvate, and uric acid. This chapter discusses the biochemical properties and role in health and disease for each of the above non-protein antioxidants.

2. Bilirubin

2.1. General Aspects and Biochemistry

2.1.1. Bilirubin and Heme Oxygenases

Bilirubin (structure shown in Figure 19-1) is the end product of heme metabolism in mammals. Heme oxygenases catalyze the cleavage of the heme ring to release ferrous ion, carbon monoxide, and biliverdin. The hydrophilic biliverdin is rapidly reduced by biliverdin reductase to hydrophobic bilirubin. As discussed in Chapter 15, heme oxygenases possess

important antioxidative and anti-inflammatory activities. It is now well-recognized that the biological activities of heme oxygenases are primarily mediated by the enzymatic reaction products, including bilirubin, biliverdin, and carbon monoxide. Among these products, bilirubin has received the most extensive studies with respect to the antioxidative and anti-inflammatory functions [1].

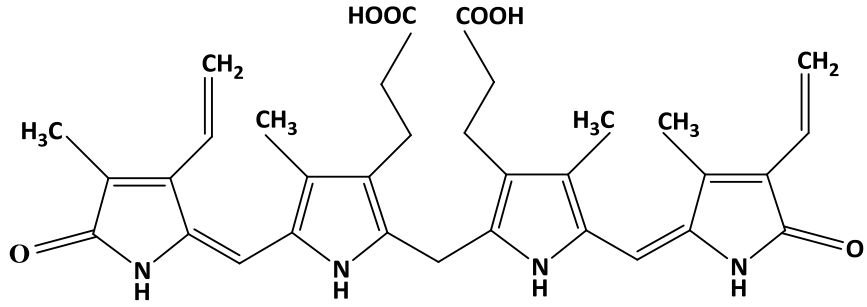

Figure 19-1. Structure of bilirubin. See text (Section 2) for description of bilirubin.

2.1.2. Physiological Concentrations and Metabolism

In adult humans, approximately 250-350 mg bilirubin are produced daily primarily via breakdown of hemoglobin. The normal human plasma concentrations of unconjugated bilirubin are between 5 and 15 μM, and over 99% is bound to albumin. In contrast, tissue concentrations of unconjugated bilirubin are much lower and estimated to be approximately 20-50 nM. Unconjugated, albumin-bound bilirubin is transported to liver, where it dissociates from albumin and enters hepatocytes. Inside hepatocytes, bilirubin undergoes glucuronidation to form water-soluble monoglucuronide and diglucuronide metabolites, which are then excreted into bile and eliminated from the body [1].

2.1.3. Biochemical Properties and Functions

Bilirubin is a potent scavenger of reactive oxygen and nitrogen species (ROS/RNS), including peroxyl radical and peroxynitrite [2, 3]. At physiological tissue concentrations (~20-50 nM), bilirubin is shown to provide cytoprotection against oxidative damage in various biological systems. The potent antioxidant effects of bilirubin at physiologically relevant concentrations are believed to stem from the biliverdin-bilirubin cycle, a concept first proposed by S.H. Snyder and coworkers [4, 5] (Figure 19-2). When bilirubin acts as an antioxidant, it is oxidized to biliverdin, which is rapidly reduced by biliverdin reductase to bilirubin. This cycle ensures the continuous availability of bilirubin for effective detoxification of ROS/RNS. Moreover, bilirubin and glutathione are found to have complementary antioxidant roles, highlighting the coordinated actions of these two important non-protein antioxidants in defense against oxidative stress [6]. In addition to bilirubin, the precursor biliverdin also possesses antioxidant activities in both cellular systems and experimental animals [1]. Due to its water solubility, biliverdin may scavenge ROS/RNS formed mainly in aqueous environment. In contrast, the ROS/RNS-scavenging activities of the lipophilic bilirubin are largely limited to lipid environment, such as lipoproteins and cell membranes.

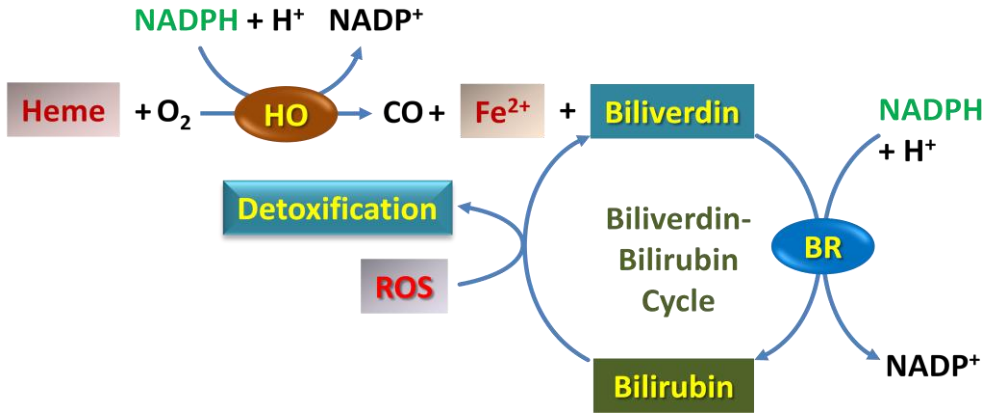

Figure 19-2. Biliverdin-bilirubin redox cycle in the detoxification of reactive oxygen species (ROS). As illustrated, bilirubin scavenges ROS, and is oxidized to biliverdin. Biliverdin reductase (BR) regenerates bilirubin from biliverdin to ensure the continuous detoxification of ROS. HO, heme oxygenases; CO, carbon monoxide.

2.2. Role in Health and Disease

It is well-recognized that heme oxygenases play an important protective role in various pathophysiological conditions (Section 3 of Chapter 15). In view of the potent antioxidant activity of bilirubin, it is believed that the beneficial effects of heme oxygenases are mediated, at least partially, by bilirubin. This notion is supported by the observations that administration of exogenous bilirubin can replicate the protective effects exerted by induction of heme oxygenases in different animal models of human diseases.

2.2.1. Animal Studies

2.2.1.1. Experimental Approaches

Direct administration of bilirubin to animals has been frequently used to study its biological activities in health and disease. It is known that large doses of bilirubin cause adverse effects, such as neurotoxicity. As such, doses of bilirubin are chosen to achieve physiologically relevant plasma concentrations in experimental animals to study the potential protection in disease conditions. Physiologically relevant concentrations of bilirubin have been employed in cell cultures to understand the cytoprotective effects and the underlying mechanisms. In addition to administration of exogenous bilirubin, another important approach to studying the biological activities of bilirubin is the use of Gunn rat model. Gunn rat is deficient in the bilirubin-conjugating enzyme UDP-glucuronosyltransferase 1A1 (UGT1A1), and as such develops hyperbilirubinemia. This rat model has been used to assess the beneficial role of bilirubin in various disease conditions, including cardiovascular disorders. As UGT1A1 is the primary enzyme involved in bilirubin metabolism, another approach to increasing plasma bilirubin in animals is through the use of UGT1A1 inhibitors, such as indinavir [7].

2.2.1.2. Role in Cardiovascular Diseases

Administration of bilirubin to animals protects against certain forms of cardiovascular disorders, including myocardial ischemia-reperfusion injury and atherogenesis [8-10]. Studies using animal models of hyperbilirubinemia demonstrate that elevated plasma bilirubin can attenuate angiotensin II- or deoxycorticosterone acetate salt-induced hypertension [11, 12]. In cultured cardiovascular cells, bilirubin at physiologically relevant concentrations inhibits cell proliferation and proinflammatory responses, as well as oxidative stress-induced cytotoxicity [13, 14]. Similar to bilirubin, exogenously administered biliverdin also protects against atherosclerosis in experimental animals and inhibits vascular smooth muscle cell proliferation in cultures [10, 15].

2.2.1.3. Role in Diabetes and Pancreatic Islet Transplantation

Exogenously administered bilirubin or biliverdin protects the pancreatic β-cells or islets from oxidative and inflammatory stress. Biliverdin also prevents endothelial sloughing and oxidative stress in a rat model of type 1 diabetes elicited by streptozotocin [16]. Bilirubin is shown to induce tolerance to islet allografts and prolong the survival of the allografts via mechanisms including antioxidant activities and modulation of T regulatory cells [17, 18].

2.2.1.4. Role in Neurological Diseases

At pathophysiologically high concentrations, bilirubin is known to cause neurotoxicity [19]. In contrast, substantial evidence supports an important role for physiologically relevant concentrations of bilirubin in neuroprotection. In cultured neuronal cells, bilirubin at physiologically relevant concentrations inhibits oxidative stress injury [20]. Administration of bilirubin to SJL/J mice has been demonstrated to also suppress autoimmune encephalomyelitis, a neurological disorder involving an oxidative stress mechanism [21].

2.2.1.5. Role in Pulmonary Diseases

An early study demonstrated that neonatal Gunn rats are more resistance to hyperoxia-induced lung oxidative stress and tissue injury, suggesting bilirubin as a protective molecule in pulmonary diseases [22]. Subsequent studies have shown that either bilirubin or biliverdin administration alleviates endotoxin-induced acute lung injury [23], and that bilirubin treatment also ameliorates bleomycin-induced pulmonary fibrosis and inflammation in experimental animals [24]. In a murine asthma model, administration of bilirubin inhibits VCAM-1-mediated airway inflammation likely through suppression of cellular ROS production [25]. VCAM-1 stands for vascular cell adhesion molecule-1.

2.2.1.6. Role in Hepatic and Gastrointestinal Diseases

Multiple studies demonstrate that exogenously administered bilirubin or biliverdin exhibits beneficial effects in several liver pathophysiological conditions, including hemin-induced hepatic oxidative stress [26], liver ischemia-reperfusion injury [27], and liver transplantation [28]. Bilirubin or biliverdin is also found to protect against intestinal ischemia-reperfusion injury and colitis in animal models [29, 30].

2.2.1.7. Role in Renal Diseases

Similar to what observed in liver ischemia-reperfusion injury, administration of bilirubin or biliverdin attenuates renal ischemia-reperfusion injury [31, 32]. A recent study using Gunn

rats shows that hyperbilirubinemia provides marked preservation of renal function and histology in a cisplatin nephrotoxicity model [33]. Notably, exogenous bilirubin does not interfere with the antineoplastic activity of cisplatin in vitro [33], pointing to the feasibility of using bilirubin to enhance the therapeutic index of cisplatin.

2.2.1.8. Role in Cancer

Bilirubin and biliverdin act as potent scavengers of peroxyl radical and other reactive species commonly involved in multistage carcinogenesis. These two pigments are also found to inhibit the mutagenic effects of various classes of mutagens in cultured cells [34]. In addition, bilirubin is shown to suppress tumor cell growth in both in vitro cultures and in vivo cancer xenografts [35, 36].

2.2.1.9. Role in Sepsis

As noted earlier in Section 2.2.1.5, exogenously administered bilirubin protects against endotoxin-induced acute lung injury. Exogenous bilirubin also reduces mortality and counteracts hypotension elicited by endotoxin through mechanisms involving a decreased induction of nitric oxide synthase 2 (NOS2) secondary to inhibition of NAD(P)H oxidase [37]. Notably, a single bolus injection of bilirubin is shown to improve the overall conditions in a mouse model of endotoxemia [38], indicating a potential value of employing bilirubin-based strategies in the clinical intervention of sepsis.

2.2.2. Human Studies and Clinical Perspectives

2.2.2.1. Genetic Variations Leading to Hyperbilirubinemia in Humans

Genetic variations have been demonstrated to cause elevated plasma bilirubin in humans. The most extensively studied genetic disorder of hyperbilirubinemia is Gilbert's syndrome [39]. This common syndrome is caused by decreased hepatic levels of the enzyme UDP-glucuronosyltransferase (mainly UGT1A1) due to gene mutations. As this enzyme is responsible for glucuronidation of bilirubin in the liver, reduced activity of this enzyme leads to an accumulation of unconjugated bilirubin in the circulation. Gilbert's syndrome is found in 5-10% of the population. People with Gilbert's syndrome have mild, chronic unconjugated hyperbilirubinemia in the absence of liver disease or overt hemolysis. Another genetic variation causing hyperbilirubinemia is a gene polymorphism designated as UGT1A1*28 [39]. Individuals with UGT1A1*28 allele show decreased liver UGT1A1 activity and increased plasma bilirubin levels.

2.2.2.2. Epidemiological Studies

The potent antioxidative and anti-inflammatory functions of bilirubin as reported in experimental models have prompted numerous studies on its potential beneficial effects in human diseases. Meta-analysis of a number of epidemiological studies has demonstrated a correlation between increased plasma bilirubin levels and decreased risk of developing coronary heart disease [39, 40]. In addition, multiple epidemiological studies have also suggested an association between elevated plasma bilirubin levels and reduced risk of developing diabetes and its complications [41, 42]. Moreover, there have been several studies indicating a possible association between hyperbilirubinemia and decreased risk of other diseases, including asthma, inflammatory disorders, and certain types of cancer. These observations in human sub-

jects are in line with the findings in animal models, pointing to a potential protective role for bilirubin in human health and disease.

2.3. Summary

Bilirubin and biliverdin possess potent antioxidative and anti-inflammatory activities. These properties contribute to the beneficial effects of these two pigments in various pathophysiological conditions in animal models. Consistent with the findings in experimental models, epidemiological studies also suggest a protective role for bilirubin in human diseases, including coronary heart disease, diabetes, and certain types of cancer.

3. Coenzyme Q

3.1. General Aspects and Biochemistry

3.1.1. Definition

Coenzyme Q (2,3-dimethoxy-5-methyl-6-multiprenyl-1,4-benzoquinone), also known as ubiquinone is a lipid-soluble compound comprised of a redox-active quinoid nucleus and a hydrophobic side chain containing a number of monounsaturated trans-isoprenoid units. The predominant form of coenzyme Q synthesized by mammalian cells is coenzyme Q_{10} (CoQ_{10}), with 10 isoprenoid units in the side chain (Figure 19-3).

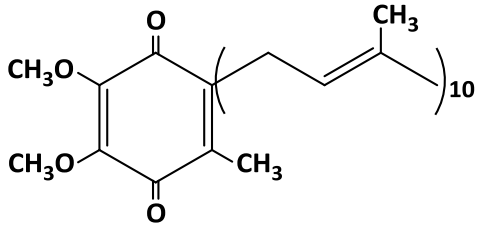

Figure 19-3. Structure of coenzyme Q_{10}. See text (Section 3) for description of coenzyme Q_{10}.

3.1.2. Discovery and Biosynthesis

CoQ_{10} was first isolated from beef heart mitochondria by F.L. Crane and coworkers in 1957 [43]. It is now known that CoQ_{10} biosynthesis occurs mainly in mitochondria. As noted above, CoQ_{10} is composed of a benzoquinone and a decaprenyl side chain. The quinone ring is derived from tyrosine or phenylalanine, whereas the isoprenoid side chain is produced by addition of isopentenyl diphosphate molecules (derived from the mevalonate pathway) to farnesyl diphosphate in multiple steps catalyzed by decaprenyl diphosphate synthase. Decaprenyl diphosphate and para-hydroxybenzoate (PHB) are condensed in a reaction catalyzed by PHB-polyprenyl transferase (COQ2), and the benzoate ring is then modified by at least six enzymes (COQ3-COQ8), which catalyze methylation, decarboxylation, and hydroxylation reactions to eventually form CoQ_{10} [44].

The molecular regulation of mammalian CoQ_{10} biosynthesis remains largely unknown. An early study demonstrated that peroxisome proliferator-activated receptor is involved in regulating CoQ_{10} biosynthesis [45]. A more recent study shows a critical role for NF-κB in the transcriptional activation of COQ7 gene and the subsequent induction of CoQ_{10} synthesis [46]. On the other hand, mutations have been identified in some of the genes encoding for enzymes involved in CoQ_{10} biosynthesis and have been linked to CoQ_{10} deficiencies in humans [47] (see Section 3.2.2 below).

3.1.3. Localization and Distribution

CoQ_{10} is an essential electron carrier of the mitochondrial electron transport chain. It is not only present in mitochondrial membrane, but also in other cellular membranes as well as in lipoproteins. The concentrations of CoQ_{10} in human plasma range from 0.40 to 2.0 μM [48]. The amounts of CoQ_{10} vary in different tissues, with highest levels found in heart, kidney, and liver.

3.1.4. Exogenous CoQ_{10}

CoQ_{10} is also found naturally in dietary sources, especially meats, and the daily intake of CoQ_{10} from food is estimated to be 3-5 mg. Dietary supplementation is another exogenous source of CoQ_{10}. Because of its insolubility in water, a variety of formulations have been developed to solubilize CoQ_{10} and enhance its absorption. In addition, CoQ_{10} analogs with improved pharmacokinetic profiles have been developed as promising drugs for disease intervention. Among them, idebenone and MitoQ have been investigated in clinical trials for both safety and efficacy [48]. MitoQ is a mitochondria-targeted antioxidant analogue of idebenone. It possesses a terminal positively charged triphenylphosphonium group, which is responsible for targeting the compound to mitochondria as the inner membrane of mitochondria is negatively charged.

3.1.5. Biochemical Properties and Functions

As mentioned above, CoQ_{10} is an essential electron carrier of the mitochondrial electron transport chain and thus indispensible for mitochondrial ATP production. In addition, CoQ_{10} also possesses other important biochemical activities, including (1) scavenging of ROS/RNS, (2) anti-inflammation, and (3) regulation of mitochondrial uncoupling proteins.

The two-electron reduced form of CoQ_{10} ($CoQ_{10}H_2$), also known as ubiquinol is a much more potent scavenger of ROS/RNS than the oxidized form of CoQ_{10}. NAD(P)H:quinone oxidoreductase 1 (NQO1) is shown to catalyze the two-electron reduction of CoQ_{10} to $CoQ_{10}H_2$ (Section 2.1.2 of Chapter 16). In particular, $CoQ_{10}H_2$ effectively scavenges peroxyl radical and inhibits lipid peroxidation (Reaction 1). $CoQ_{10}H_2$ is also able to regenerate α-tocopherol (α-TOH) from its radical form (α-TO$^{\cdot}$) in cellular membranes or lipoproteins (Reaction 2). The resulting coenzyme Q radical ($CoQ_{10}H^{\cdot}$) is relatively stable, and can be reduced back to $CoQ_{10}H_2$ by reducing agents, such as ascorbate (Section 2.2.2 of Chapter 20) and dihydrolipoic acid (Section 5).

$$CoQ_{10}H_2 + LOO^{\cdot} \longrightarrow CoQ_{10}H^{\cdot} + LOOH \quad (1)$$

$$CoQ_{10}H_2 + \alpha\text{-}TO^{\cdot} \longrightarrow CoQ_{10}H^{\cdot} + \alpha\text{-}TOH \quad (2)$$

CoQ_{10} exerts multiple anti-inflammatory effects by suppressing the expression of pro-inflammatory cytokines and adhesion molecules, and by modulating the activities of inflammatory cells [49]. The antioxidant activities of CoQ_{10} also contribute to its anti-inflammatory effects as oxidative stress and inflammation are intimately related processes. In addition to the well-recognized antioxidative and anti-inflammatory properties, CoQ_{10} is also involved in the regulation of uncoupling proteins. Mitochondrial uncoupling proteins play an important role in coordinating mitochondrial oxidative phosphorylation and heat production, as well as formation of ROS. There is evidence showing CoQ_{10} is an obligatory cofactor for uncoupling protein function [50]. The enhancement of uncoupling protein function by CoQ_{10} may lead to decreased formation of ROS from mitochondrial electron transport chain as uncoupling proteins act to decrease mitochondrial ROS production.

3.2. Role in Health and Disease

3.2.1. Animal Studies

The beneficial effects of both CoQ_{10} and its analogs (e.g., idebenone, MitoQ) have been extensively studied in various experimental systems, including cell cultures and animal models. At physiologically relevant concentrations, CoQ_{10} and analogs afford cytoprotection against ROS/RNS-mediated damage. They also exhibit marked anti-inflammatory activities in cell cultures. In experimental animals, administration of CoQ_{10} and its analogs results in increased levels of these antioxidant compounds in plasma and tissues, including the central nervous system. Numerous studies demonstrate that CoQ_{10} and its analogs protect against various pathophysiological processes involving oxidative and inflammatory stress mechanisms. These include cardiovascular disorders (e.g., hypertension, atherosclerosis, myocardial ischemia-reperfusion injury, heart failure, cardiotoxicity), diabetes and metabolic syndrome, neurodegenerative disorders (e.g., Parkinson's disease, Alzheimer's disease, Huntington's disease), sepsis, skin disorders, male infertility, and cancer. The beneficial effects of CoQ_{10} and its analogs on the above pathophysiological conditions are believed to result from their antioxidative and anti-inflammatory activities as well as their critical involvement in mitochondrial energetics.

3.2.2. Human Studies and Clinical Perspectives

Current information on the role of CoQ_{10} in human health and disease results primarily from studies on CoQ_{10} deficiencies as well as clinical trials on CoQ_{10} supplementation. In addition, the CoQ_{10} analogs idebenone and MitoQ have also been investigated in clinical trials for the intervention of human diseases.

3.2.2.1. HUMAN CoQ_{10} DEFICIENCIES

Deficiencies of CoQ_{10} are classified into primary and secondary deficiencies, and have been associated with four major clinical phenotypes [51]: (1) encephalomyopathy characterized by a triad of recurrent myoglobinuria, brain involvement, and ragged-red fibers, (2) infantile multisystemic disease typically with prominent nephropathy and encephalopathy, (3) cerebellar ataxia with marked cerebellar atrophy, and (4) pure myopathy with lipid storage myopathy and mitochondrial respiratory chain dysfunction.

Primary CoQ_{10} deficiencies due to mutations in CoQ_{10} biosynthetic genes have been

identified in patients with the infantile multisystemic and cerebellar ataxic phenotypes. On the other hand, secondary CoQ_{10} deficiencies due to mutations in genes not directly related to ubiquinone biosynthesis have been recognized in patients with cerebellar ataxia and pure myopathy. In addition, CoQ_{10} deficiencies have been reported in patients with other disorders, including Leigh syndrome encephalopathy, growth retardation, deafness, and cardiofaciocutaneous syndrome. In many patients with CoQ_{10} deficiencies, the causative genetic defects are unknown; therefore, it is likely that mutations in additional genes will be identified as causes of CoQ_{10} deficiencies. Regardless of the underlying molecular mechanisms, clinical manifestations of CoQ_{10} deficiencies are believed to be attributed to the compromised functions of CoQ_{10} in energy production as well as in protecting against oxidative and inflammatory stress. Numerous studies demonstrate that CoQ_{10} supplementation improves the clinical conditions in patients with CoQ_{10} deficiencies, further supporting a causal role of CoQ_{10} deficiencies in disease pathophysiology [48].

3.2.2.2. Clinical Studies

In line with the findings in experimental models, supplementation of CoQ_{10} has been shown to provide protection against cardiovascular disorders, diabetes, neurodegenerative diseases, and certain types of cancer in numerous clinical trials [48, 52]. In addition, clinical studies also demonstrate excellent safety profiles for oral CoQ_{10} supplementation. Although a large majority of clinical trials have provided compelling evidence for a beneficial role of CoQ_{10} supplementation in the intervention of common human diseases, inconsistent results are also reported. It is worthy of mentioning that most of the clinical trials so far on CoQ_{10} are relatively small-scaled with potential design limitations, which may explain the inconsistency in conclusions. In this context, several large-scale double-blind, placebo-controlled trials are currently underway to further determine the efficacy of CoQ_{10} supplementation in the intervention of common human diseases [48].

3.3. Summary

In addition to its essential role in mitochondrial ATP production, CoQ_{10} is also a potent antioxidant and anti-inflammatory compound. A growing body of evidence from studies in experimental animals supports CoQ_{10} as an important protector in various disease conditions involving oxidative stress and inflammation. Similarly, the beneficial effects of CoQ_{10} supplementation in human diseases have been suggested by numerous clinical studies. Ongoing and future large-scale double-blind, placebo-controlled trials will further define the exact role of this multifunctional compound in human health and disease.

4. Estrogens

4.1. General Aspects and Biochemistry

The estrogen hormones include estradiol, estrone, and estriol. Physiological levels of estrogens are within the nanomolar range. The beneficial effects of estrogens in human health

and disease have been increasingly recognized. However, the underlying molecular mechanisms remain to be elucidated. At non-physiologically relevant micromolar concentrations, estrogens are able to scavenge ROS/RNS and inhibit lipid peroxidation in cell-free systems. Estrogens at micromolar concentrations also induce cellular antioxidant proteins, including superoxide dismutase, glutathione peroxidase, and thioredoxin [53-55]. Induction of antioxidant enzymes by estrogens and estrogen derivatives is shown to be mediated by pathways including estrogen receptor activation as well as Nrf2 signaling [56, 57]. Consistent with these in vitro findings, administration of pharmacological doses of estrogens has been demonstrated to upregulate various tissue antioxidants in experimental animals. In humans, increased estrogen levels also lead to enhanced superoxide dismutase (ECSOD and MnSOD) expression in circulating monocytes [53]. In addition, estrogens are shown to suppress inflammatory responses in cultured cells and animal models [58]. These studies suggest potential antioxidative and anti-inflammatory activities of estrogens at pharmacological doses in vitro and in vivo. However, currently there is no clear evidence showing that estrogens at physiologically relevant concentrations possess any significant in vivo antioxidative and anti-inflammatory functions.

4.2. Role in Health and Disease

Consistent with their antioxidative and anti-inflammatory activities, estrogens given to animals at pharmacological doses are found to protect against pathophysiological conditions involving oxidative stress and inflammation. These include cardiovascular diseases, neurological disorders, and others.

4.2.1. Role in Cardiovascular Diseases

Administration of estradiol to animals attenuates hypertension and vascular oxidative stress, and improves endothelial function in spontaneous hypertensive rats [59]. Long-term treatment of ovarectomized $ApoE^{-/-}$ mice with oral estradiol (6 μg daily for 12 weeks) inhibits the development atherosclerosis [60]. In addition, the estradiol treatment also leads to decreased vascular oxidative stress and induction of vascular Cu,ZnSOD and MnSOD in these mice, suggesting that the antioxidative actions in the vessel wall may contribute to the atheroprotective effects of estrogens. Pharmacological doses of estrogens have also been shown to protect against myocardial ischemia-reperfusion injury in animal models, which is, at least partially, dependent on the antioxidative and anti-inflammatory activities of estrogens [61]. These findings in animal models are consistent with the cardiovascular protective effects of estrogens in humans. However, it should be borne in mind that the beneficial actions of estrogens in human health and disease are likely attributed to the multiple molecular and biochemical activities of these hormonal compounds, rather than their specific antioxidative and anti-inflammatory properties only.

4.2.2. Role in Neurological Diseases and Other Disease Conditions

Extensive studies indicate the neuroprotective effects of estrogens and estrogen derivatives at pharmacological doses in animal models of human neurological disorders, especially cerebral ischemic injury [62, 63]. The neuroprotective actions of estrogens are believed to

largely result from their antioxidant activities. Indeed, estrogens are shown to not only inhibit ROS formation from NAD(P)H oxidase, but also induce antioxidant enzymes in brain tissue of experimental animals [63, 64].

Estrogens also exert beneficial effects in other disease conditions. For instance, treatment with estrogens upregulates pancreatic antioxidants and attenuates experimental diabetes and diabetic complications in animal models [65, 66]. Administration of pharmacological doses of estrogens is also found to protect against oxidative multiorgan damage in experimental animals with chronic renal failure [67], attenuate liver ischemia-reperfusion injury and oxidative stress [68], and ameliorate toxicant-induced hepatic fibrosis [69, 70].

4.3. Summary

Estrogens at micromolar concentrations possess antioxidant activities, as evidenced by scavenging ROS/RNS and inducing endogenous cellular antioxidant enzymes. Administration of pharmacological doses of estrogens has been shown to attenuate oxidative and inflammatory pathophysiological conditions in various animal models. These experimental findings are in line with the established beneficial effects of estrogens in humans.

5. α-Lipoic Acid

5.1. General Aspects and Biochemistry

α-Lipoic Acid (LA; 1,2-dithiolane-3-pentanoic acid) is a naturally occurring sulfur-containing compound synthesized enzymatically in mitochondria from octanoic acid [71]. LA is a necessary cofactor for mitochondrial α-ketoacid dehydrogenase, and thus plays a critical role in energy metabolism of mammalian cells. In addition to its cellular synthesis, LA is also absorbed intact from dietary sources and distributes to various tissues. Micromolar concentrations of LA in plasma can be achieved following administration of LA in both experimental animals and humans.

LA and its reduced form dihydrolipoic acid (structures shown in Figure 19-4) at micromolar concentrations have been shown to scavenge various ROS/RNS, chelate redox-active metal ions, and restore cellular glutathione levels. LA at micromolar concentrations has also been found to induce endogenous cellular antioxidant enzymes and suppress inflammatory responses [72-74].

5.2. Role in Health and Disease

LA exhibits cytoprotective activities against cell injury elicited by ROS/RNS in vitro. Administration of LA to experimental animals provides protection against many pathophysiological processes involving oxidative stress and dysregulated inflammation. These include cardiovascular disorders, diabetes and metabolic syndrome, neurodegeneration, sepsis, and some others [73].

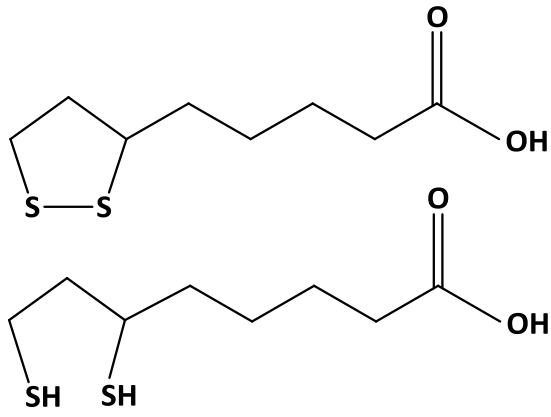

Figure 19-4. Structures of α-lipoic acid (top) and dihydrolipoic acid (bottom). See text (Section 5) for description of α-lipoic acid.

In humans, LA is used to prevent diabetic polyneuropathies [75]. Clinical trials also demonstrate an efficacy of LA supplementation in attenuating vascular dysfunction and high blood pressure in patients with metabolic syndrome [76, 77].

5.3. Summary

LA is a naturally occurring sulfur compound synthesized in mitochondria, and is also derived from dietary sources. LA at micromolar concentrations scavenges ROS/RNS, chelates redox-active metal ions, and upregulates cellular antioxidant enzymes. LA is also able to suppress inflammatory responses. Administration of LA to animals affords protection against a wide range of disease processes involving oxidative stress and inflammation. Studies in human subjects also show effectiveness of LA supplementation in the intervention of such common diseases as diabetes and metabolic syndrome.

6. Melatonin

6.1. General Aspects and Biochemistry

Melatonin (*N*-acetyl-5-methoxytryptamine) is synthesized in pineal gland of mammals, and the best known function of this molecule is to regulate circadian rhythms. It is also produced by many extrapineal tissues, including retina, ciliary body, lens, Harderian gland, brain, thymus, airway epithelium, bone marrow, gut, ovary, testes, placenta, lymphocytes, and skin. Although the physiological concentrations of melatonin are extremely low in plasma (usually less than 1 nM), the intracellular levels of melatonin in certain tissues, such as skin are estimated to be in micromolar range or even higher [78].

The highest intracellular concentrations of melatonin are found in mitochondria. In this context, melatonin is shown to protect mitochondria from oxidative damage by preventing cardiolipin oxidation. Melatonin is a potent scavenger of ROS/RNS in vitro. Melatonin at micromolar concentrations also induces certain endogenous cellular antioxidant enzymes and inhibits proinflammatory responses. In addition to the antioxidative and anti-inflammatory actions, melatonin participates in other cellular processes, including cell signal transduction, growth regulation, and angiogenesis [79-81].

6.2. Role in Health and Disease

As noted above, melatonin exhibits potent antioxidative and anti-inflammatory activities. At micromolar concentrations, melatonin has been shown to protect cells or tissues from ROS/RNS-mediated damage. Administration of melatonin to experimental animals has been demonstrated to provide protection against various disease conditions, including cardiovascular diseases, diabetes, neurodegeneration, cancer, skin disorders, sepsis, and many other inflammatory conditions [78, 79, 81]. The above beneficial effects of melatonin have also been suggested by extensive clinical studies [78, 79, 81]. The clinical studies also demonstrate excellent safety profiles for oral melatonin supplementation over a wide range of doses. The protective effects of melatonin on oxidative stress and inflammation together with its excellent safety profiles and other beneficial activities make this neurohormone an attractive compound for continued clinical research.

6.3. Summary

A growing body of evidence shows that melatonin is capable of attenuating oxidative and inflammatory stress. Administration of pharmacological doses of melatonin has been shown to be protective in a variety of animal models of human diseases involving oxidative stress and inflammation. Consistently, numerous clinical studies also suggest a beneficial role for melatonin supplementation in the intervention of many common human disease conditions.

7. Pyruvate

7.1. General Aspects and Biochemistry

Pyruvic acid ($CH_3COCOOH$) is a three-carbon α-keto acid that is present in cells and extracellular fluids as its conjugate anion, pyruvate. Pyruvic acid is the final product of glycolysis and the starting substrate for tricarboxylic acid cycle.

Pyruvic acid reacts nonenzymatically with hydrogen peroxide and protects cells from hydrogen peroxide-induced injury. Pyruvic acid also scavenges other ROS/RNS, such as hydroxyl radical and peroxynitrite. Due to the unstable nature of pyruvic acid in solution, a relatively stable derivative, namely, ethyl pyruvate has been developed and studied for its protective effects on ROS/RNS-induced damage. Ethyl pyruvate also effectively scavenges

ROS/RNS. In addition, ethyl pyruvate potently suppresses inflammatory responses via, at least partially, inhibiting NF-κB-mediated expression of proinflammatory cytokines [82, 83].

7.2. Role in Health and Disease

In line with its antioxidative and anti-inflammatory activities, treatment with ethyl pyruvate (or pyruvate in certain studies) has been shown to improve survival and ameliorate organ dysfunction in a variety of preclinical animal models of acute illnesses. These include severe sepsis, hemorrhagic shock, stroke, spinal cord ischemia-reperfusion injury, myocardial ischemia-reperfusion injury, ethanol-induced liver injury, liver ischemia-reperfusion injury, acute pancreatitis, bacterial peritonitis, acute burn injury, and whole-body radiation-induced injury [82, 83]. On the other hand, more prolonged treatment with ethyl pyruvate has been found to ameliorate inflammatory bowel disease and slow the growth rate of malignant tumors in animal models [84-86]. Prompted by these promising preclinical findings, ethyl pyruvate was investigated in a phase 2 clinical trial for the intervention of certain acute conditions in high-risk patients undergoing cardiac surgery with cardiopulmonary bypass [87]. Although ethyl pyruvate was found to be safe, it failed to improve the clinical outcome.

7.3. Summary

Pyruvate reacts nonenzymatically with hydrogen peroxide. The more stable pyruvate derivative, ethyl pyruvate also scavenges ROS/RNS and possesses potent anti-inflammatory activities. Ethyl pyruvate has been shown to be protective against disease pathophysiology in various animal models of human diseases; however, its effectiveness in disease intervention in clinical trials is unclear and warrants further investigations.

8. Uric Acid

8.1. General Aspects and Biochemistry

Uric acid is the end product of purine metabolism in humans. The final two reactions in the metabolism pathway that lead to the formation of uric acid are the conversion of hypoxanthine to xanthine, and xanthine to uric acid. These two reactions are catalyzed by xanthine oxidoreductase. As illustrated in Figure 19-5, in most animal species, uric acid is metabolized to allantoin by urate oxidase. Humans lack urate oxidase, and as such the plasma levels of uric acid in humans are much higher (200-400 μM) than those in other mammals.

Uric acid is also present intracellularly and in other body fluids, but usually at lower levels than in plasma. At physiological pH, almost all uric acid is ionized to urate. Uric acid at physiologically relevant concentrations is able to scavenge various ROS/RNS, such as peroxyl radical, hydroxyl radical, singlet oxygen, peroxynitrite, and ozone [88-90]. Uric acid also chelates redox-active metal ions, including copper and iron. These findings suggest that uric acid may be a physiologically relevant antioxidant compound in vivo.

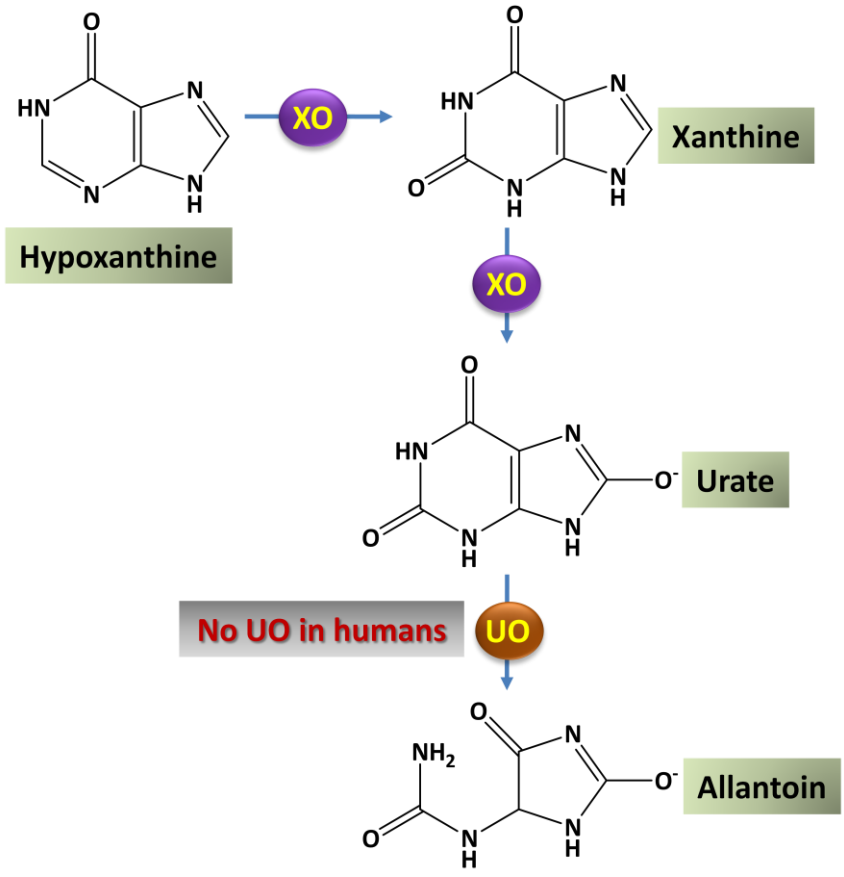

Figure 19-5. Formation of uric acid from purine metabolism. As illustrated, xanthine oxidoreductase (XO) catalyzes the formation of xanthine from hypoxanthine. XO further converts xanthine to uric acid. Uric acid is then metabolized by urate oxidase (UO) to allantoin. Humans lack UO, and as such plasma levels of urate in humans are high and usually in the range of 300 to 400 μM. Hyperuricemia, a condition with abnormally elevated levels of plasma urate, is a major risk factor for the development of gout in humans. (courtesy of Jason Z. Li)

8.2. Role in Health and Disease

8.2.1. Animal Studies

In experimental animals, uric acid is shown to protect against allergic encephalomyelitis and multiple sclerosis likely through its scavenging of peroxynitrite, a reactive species implicated in the pathogenesis of experimental encephalomyelitis and multiple sclerosis [91, 92]. Administration of uric acid at the onset of spinal cord injury in a mouse model attenuates the pathological changes, including oxidative damage and inflammatory cell infiltration in the spinal cord [93]. Uric acid also provides protection in other animal models of human diseases involving oxidative stress and inflammation, such as experimental parkinsonism [94], brain injury induced by acute ischemia [95, 96], and liver injury resulting from hemorrhagic shock [97] or obesity [98]. These findings are consistent with the antioxidant activities of urate.

8.2.2. Human Studies and Clinical Perspectives

In human healthy volunteers, administration of uric acid is shown to increase the serum free radical-scavenging capacity [99] and reduce exercise-induced oxidative stress [100]. Multiple epidemiological studies suggest that high plasma urate concentrations are associated with decreased risk of developing Parkinson's disease [101, 102] as well as other neurological disorders, including Huntington's disease and mild cognitive impairment [103, 104]. Furthermore, a recent clinical study demonstrates that decreased levels of plasma uric acid in acute ischemic stroke are correlated with increased stroke severity, unfavorable disease evolution, and poor long-term clinical outcome [105]. A pilot interventional study reported that in acute stroke patients treated with recombinant tissue plasminogen activator (rt-PA), co-administration of uric acid could reduce lipid peroxidation and prevent a fall of uric acid in blood that occurred early after stroke onset [106]. These clinical and epidemiological observations are in line with the findings in animal studies, pointing to a potential protective role of uric acid in human neurological disorders. However, there have been studies reporting inconsistent or contradictory results on the effects of uric acid in ischemic stroke in human subjects [107, 108], justifying the need for large-scale double-blind, placebo-controlled trials to further determine the efficacy of uric acid in stroke intervention.

On the one hand, there is substantial evidence supporting the antioxidant activities of uric acid in both in vitro systems and animal models, as well as in certain human disorders. On the other hand, epidemiological studies suggest that high levels of plasma uric acid are strongly associated with, and in many cases predict development of hypertension, diabetes, metabolic syndrome, kidney diseases, and cardiovascular events [109-111]. Hence, the effects of uric acid in human diseases vary with pathological conditions, and the potential value of uric acid in disease intervention warrants further clinical studies.

8.3. Summary

At physiologically relevant concentrations, uric acid scavenges ROS/RNS and protects against oxidative cell injury. Administration of uric acid to animals attenuates the pathophysiological processes of various diseases, especially neurological disorders. Epidemiological studies also suggest an inverse association between plasma uric acid levels and risk of developing Parkinson's disease. However, some epidemiological studies also demonstrate a positive correlation between plasma uric acid levels and risk of developing many other diseases, including cardiovascular disorders and diabetes. The causal involvement of uric acid in human diseases and the underlying mechanisms deserve further exploration so as to better define the beneficial and detrimental effects of uric acid in health and disease.

9. Conclusion and Future Directions

Bilirubin, CoQ_{10}, estrogens, LA, melatonin, pyruvate, and uric acid are endogenous non-protein compounds synthesized by mammalian cells. A growing body of experimental evidence supports that these compounds at physiologically or pharmacologically relevant concentrations possess antioxidative and anti-inflammatory activities. Some of them, such as

estrogens, LA, and melatonin are also able to upregulate endogenous cellular antioxidant enzymes. Both animal studies and clinical trials have provided substantial evidence for the beneficial effects of these antioxidant compounds in various disease conditions though other studies have reported inconsistent results. It should be noted that the beneficial effects of these compounds may also result from their non-antioxidant functions. Future studies should focus on characterization of the dose-response relationship when using these antioxidant compounds in disease intervention. It should be kept in mind that although being synthesized by cells, these antioxidant compounds may cause adverse effects when they are supplemented at pharmacological doses.

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SECTION III
ANTIOXIDANTS DERIVED FROM THE DIET

The human diet contains a number of compounds that have antioxidant properties. Among them are vitamin C, vitamin E, carotenoids, and phenolic compounds. These compounds are not made by human cells though vitamin C is synthesized in many mammalian species. It is important to note that these diet-derived non-protein compounds also possess other biological activities in addition to their antioxidant effects. Thus, the role of these compounds in health and disease may results from either the antioxidant or the non-antioxidant activities, or both. The biochemical properties and role in health and disease for each of the above compounds are discussed in the subsequent Chapters 20-23.

Chapter 20

Vitamin C

Abstract

Vitamin C is a water-soluble antioxidant synthesized in many animal species, but not in humans. Humans obtain vitamin C from dietary sources as well as via vitamin supplementation. Vitamin C possesses important biological functions, including serving as a cofactor for many enzymes, acting as an antioxidant, and participating in regulating cell growth, apoptosis, and signaling. Extensive research in animal models has conclusively established diverse beneficial functions of this antioxidant vitamin in a wide variety of disease conditions involving oxidative stress and inflammation. In line with the findings in experimental animals, observational epidemiological studies demonstrate an association between high dietary vitamin C intake and decreased risk of human diseases, including cardiovascular disorders and cancer. However, large-scale interventional trials in nonselected populations have failed to conclude a consistent beneficial role for vitamin C supplementation in cardiovascular diseases and cancer. In contrast, small-scale clinical trials in selected subpopulations of patients with significant oxidative stress have provided evidence supporting a potential beneficial effect of vitamin C in the intervention of certain disease conditions.

1. Overview

1.1. Definition and Dietary Sources

Vitamin C, also known as ascorbic acid or ascorbate, is a water-soluble molecule synthesized endogenously in animals except humans, monkeys, guinea pigs, and several other animal species [1]. Humans lost this capability as a result of a series of inactivating mutations of the gene encoding gulonolactone oxidase (GULO), a key enzyme in vitamin C biosynthesis. Humans normally acquire vitamin C from various dietary sources through a substrate-saturable transport mechanism. Dietary sources of vitamin C are mainly vegetables and fruits, including brussels sprouts, broccoli, bell peppers, lettuce, tomatoes, citrus fruits, strawberries, papayas, and mangoes. Because vitamin C is vulnerable to heat, raw fruits and vegetables usually have a higher nutrient density than their cooked counterparts. Another source is vitamin C supplement. Vitamin C is also frequently added to juice.

1.2. Pharmacokinetics

Oral vitamin C intake produces plasma concentrations that are tightly controlled; once its oral intake exceeds 200 mg daily, it is difficult to further increase its plasma concentration. The maximal plasma concentration attainable by oral intake of vitamin C has been estimated to be approximately 200 μM though the physiological plasma concentrations in healthy humans range from 60 to 100 μM. In contrast, intravenous injection of large doses of vitamin C produces millimolar concentrations of plasma vitamin C [2].

Under physiological conditions, intracellular levels of vitamin C can be in the millimolar range. This is due to selective intracellular accumulation via vitamin C transport system present in the plasma membrane [3].

1.3. Deficiency

Vitamin C deficiency in diet causes the dread disease scurvy. Other manifestations of vitamin C deficiency may include anemia, poor wound healing, rough skin, blotchy bruises, and frequent infections.

2. Biochemical Properties and Functions

The biochemical properties and functions of vitamin C can be summarized into the following three categories.

- As a cofactor for various enzymes
- As an antioxidant or a potential pro-oxidant
- Other novel activities

2.1. As a Cofactor for Various Enzymes

Vitamin C serves as a cofactor for at least eight enzymes in mammals. The most notable ones are proline hydroxylase and lysine hydroxylase, which are involved in collagen synthesis. The other enzymes for which vitamin C acts as a cofactor are involved in carnitine synthesis, catecholamine synthesis, peptide amidation, and tyrosine metabolism [4]. Due to its critical role in collagen synthesis, deficiency of vitamin C compromises the integrity of blood vessels, leading to scorbutic gums and pinpoint hemorrhage.

2.2. As an Antioxidant or a Potential Pro-oxidant

2.2.1. Redox Chemistry

The redox chemical properties of vitamin C are responsible for its potential antioxidant or pro-oxidant activities. Ascorbic acid ($AscH_2$) has two ionizable hydroxyl groups. At physio-

logical pH, ascorbic acid exists predominantly as a monoanion, i.e., ascorbate monoanion ($AscH^-$). $AscH^-$ acts as a reducing agent and is converted to ascorbate radical ($Asc^{\cdot-}$, also known as semidehydroascorbate) after donation of one electron. After losing another electron, $Asc^{\cdot-}$ is converted to dehydroascorbate (DHA). $Asc^{\cdot-}$ can be reduced back to $AscH^-$. DHA can also be reduced by either one electron to $Asc^{\cdot-}$ or by two electrons to $AscH^-$ (Figure 20-1). Due to its high instability, DHA, if not rapidly reduced, undergoes hydrolytic ring opening to 2,3-diketogulonic acid.

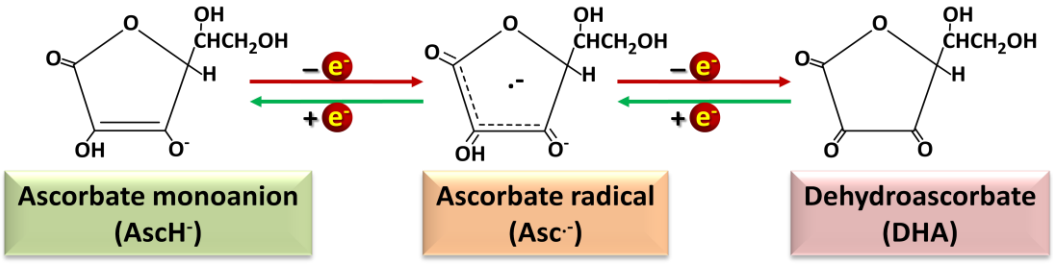

Figure 20-1. Vitamin C structure and related redox reactions. See text (Section 2.2.1) for detailed description.

2.2.2. Antioxidant Functions

In many in vitro systems, vitamin C has been found to scavenge various reactive oxygen and nitrogen species (ROS/RNS), and protect cells from oxidative damage. Vitamin C is able to regenerate α-tocopherol and coenzyme Q from α-tocopherol radical and coenzyme Q radical, respectively, and thereby plays a role in maintaining the antioxidant activities of α-tocopherol and coenzyme Q. It has recently been demonstrated that vitamin C is also able to reduce 1-Cys peroxiredoxin [5]. As discussed in Section 2.1 of Chapter 11, peroxiredoxin is critical for the detoxification of hydrogen peroxide, organic hydroperoxides, and possibly peroxynitrite. Early studies also showed that vitamin C and glutathione cooperate as an efficient antioxidant system in mammals [6]. In addition to the above activities involved in detoxifying ROS/RNS, vitamin C is found to inhibit NAD(P)H oxidase, thus decreasing the formation of ROS from this important cellular source [7].

2.2.3. Potential Pro-oxidant Activities

Under certain conditions, such as in the presence of redox-active metal ions, vitamin C may behave as a pro-oxidant, giving rise to ROS, damaging DNA, and causing protein glycation. Vitamin C is shown to induce decomposition of lipid hydroperoxides to genotoxins even in the absence of redox-active metal ions [8]. In a humanized mouse model, vitamin C is found to mediate chemical aging of lens crystallins via the Maillard reaction [9]. In experimental animals, administration of pharmacological doses of vitamin C causes formation of vitamin C radical and ROS in extracellular fluid and decreases growth of aggressive tumor xenografts [10] (see Section 3.1.10 below for more discussion of the role of vitamin C in cancer). Hence, the pro-oxidant activities of vitamin C may exert either detrimental or beneficial effects depending on the physiological and pathophysiological conditions.

2.3. Other Novel Activities

Vitamin C reduces ferric ion to ferrous ion and thus facilitates iron absorption in the duodenum. Recent studies suggest an important role for vitamin C in nucleic acid and histone demethylation, as well as proteoglycan deglycanation. Due to its crucial involvement in hydroxylation reactions, vitamin C participates in downregulating hypoxia-inducible transcription factor-1α (HIF-1α). In this regard, proline hydroxylation targets HIF-1α for ubiquitin-mediated degradation [11]. Moreover, vitamin C is shown to regulate angiogenesis [12, 13]. These novel mechanisms also contribute to the biological functions of vitamin C in health and disease.

3. Role in Health and Disease

3.1. Animal Studies

3.1.1. Experimental Approaches

A number of approaches have been employed to study the biological activities of vitamin C in experimental animals. These include direct administration of vitamin C either orally or parenterally. As noted earlier in Section 1.2, intravenous injection of large doses of vitamin C produces millimolar plasma concentrations, causing pharmacological effects such as antitumor activity. Due to limited oral bioavailability of vitamin C, lipophilic vitamin C derivatives (e.g., ascorbyl stearate) with improved bioavailability have been developed as potential protectors in animal models of human diseases.

As aforementioned, humans are unable to synthesize vitamin C endogenously due to inactivating mutations of the gene encoding gulonolactone oxidase (GULO). Targeted disruption of GULO gene in mice leads to creation of mutant mice of vitamin C deficiency [14]. This vitamin C deficient GULO knockout mouse model has been used to study the biological functions of vitamin C under experimental conditions. For example, vitamin C deficiency in GULO knockout mice is shown to cause aortic wall damage and augment the instability of atherosclerotic plaque likely due to the compromised synthesis of collagen and enhanced oxidative stress [14, 15].

3.1.2. Role in Cardiovascular Diseases

One of the most extensively investigated biological activities of vitamin C in experimental animals is cardiovascular protection. Numerous studies have conclusively established that vitamin C protects against many forms of cardiovascular disorders in animal models, including hypertension, atherosclerosis, and ischemic heart disease [16-18]. Mechanistic studies have suggested that the beneficial effects of vitamin C in cardiovascular pathophysiology may result from the following mechanisms.

- Protection against ROS/RNS-mediated oxidative modifications of low-density lipoprotein (LDL) [16]
- Maintenance of vascular nitric oxide bioavailability and amelioration of endothelial dysfunction [17]

- Stabilization of atherosclerotic lesion due to production of collagen and inhibition of oxidative stress and inflammation [17].

3.1.3. Role in Diabetes and Metabolic Syndrome

There is evidence supporting the protective effects of vitamin C in diabetes and metabolic syndrome in experimental animals. For example, vitamin C supplementation is found to decrease insulin glycation and improve glucose homeostasis in obese hyperglycemic mice [19]. Administration of vitamin C alone or in combination with vitamin E also attenuates oxidative stress and pancreatic β-cell injury, as well as protects against diabetic complications in various animal models [20-22].

3.1.4. Role in Neurological Diseases

The highest concentrations of vitamin C are found in brain and in neuroendocrine tissues (e.g., adrenal gland). There is increasing experimental evidence supporting that vitamin C is a vital antioxidant in brain. For instance, vitamin C deficiency in GULO knockout mice results in elevated oxidative stress in brain tissue as well as sensorimotor deficits [23]. Neurological disorders, such as ischemic stroke, Alzheimer's disease, Parkinson's disease, and Huntington's disease usually involve oxidative stress. Vitamin C as an important antioxidant in brain has thus been implicated in the protection against these diseases in animal models [24]. Indeed, an early study showed that when monkeys were given 1 g/day of vitamin C parenterally for six days before middle cerebral artery occlusion, brain infarct size was decreased by ~50% in the vitamin C-treated group compared with the control group [25]. Administration of vitamin C also results in amelioration of experimental parkinsonism [26], β-amyloid peptide-induced oxidative damage in rat brain [27], as well as Huntington's behavioral phenotype in mice [28].

The neuroprotection by vitamin C is believed to result mainly from its antioxidant function. In addition, vitamin C is proposed to act as a neuromodulator of glutamatergic, dopaminergic, cholinergic, and GABAergic transmission and related behaviors. Such neurotransmission modulating activities may also contribute to the beneficial effects of vitamin C in neurological disorders [24].

3.1.5. Role in Pulmonary Diseases

Pulmonary protection is also a well-recognized biological function of vitamin C in experimental animals. Early work demonstrated that cigarette smoke depletes vitamin C levels in lung tissue, and either oral or intravenous administration of vitamin C prevents cigarette smoke-induced lung inflammation and oxidative stress [29]. A recent study shows that high-dose vitamin supplementation attenuates airway inflammation by modulating Th1/Th2 balance in a murine model of asthma [30]. Vitamin C may also play a role in pulmonary immune response to infections. In this regard, vitamin C deficiency in GULO knockout mice is found to be associated with an increased susceptibility to influenza virus-induced lung inflammation and tissue injury [31].

3.1.6. Role in Hepatic and Gastrointestinal Diseases

Administration of vitamin C or its derivatives to experimental animals ameliorates hepatic ischemia-reperfusion injury and improves survival of liver grafts following transplantation

[32-34]. Vitamin C supplementation also protects experimental animals from hepatotoxicity and oxidative stress induced by acetaminophen, ethanol, or carbon tetrachloride. Recently, vitamin C is shown to attenuate liver injury due to metabolic syndrome in a murine model [35]. Although the majority of studies suggest a protective function for vitamin C in various liver diseases, conflicting results have also been reported in the literature. For example, a recent study demonstrates that vitamin C deficiency attenuates liver fibrosis via upregulating peroxisome proliferator-activated receptor-γ expression in senescence marker protein 30 knockout mice [36]. However, the relevance of this finding to the human health remains to be further investigated. In addition to liver diseases, there is evidence showing that vitamin C treatment also protects against gastrointestinal disorders, including intestinal ischemia-reperfusion injury and aspirin-induced gastric mucosal damage [37].

3.1.7. Role in Renal Diseases

A number of studies independently demonstrate a beneficial role for vitamin C in renal ischemia-reperfusion injury and kidney transplantation [38, 39]. Such protective effects of vitamin C are associated with decreased oxidative stress and inflammation in renal tissue. Vitamin C alone or in combination with vitamin E also attenuates nephrotoxicity induced by cisplatin, cyclosporine A, or radiocontrast agents. Moreover, vitamin C administration in combination with vitamin E or desferrioxamine ameliorates diabetic nephropathy in animal models [40, 41]. Desferrioxamine is an iron-chelating agent.

3.1.8. Role in Skin Diseases

Ultraviolet (UV) irradiation is found to decrease vitamin C levels in skin tissue, suggesting a potential involvement of this antioxidant vitamin in suppressing UV light-mediated skin damage. Indeed, topical application of vitamin C protects against UV light-induced skin inflammation and oxidative DNA damage. Vitamin C is also effective in protecting against acne vulgaris as well as minocycline pigment formation [42, 43]. Notably, co-application of vitamin C and other antioxidant compounds, such as vitamin E and fullerene (fullerene is a nanomaterial, which is discussed in Chapter 27) is highly effective in ameliorating oxidative and inflammatory skin injury induced by UV irradiation in animal models [44]. This suggests that vitamin C may synergize with other antioxidants in counteracting UV irradiation-induced oxidative stress.

3.1.9. Role in Sepsis

Oxidative stress and dysregulated inflammation are key events leading to multiple organ damage in sepsis. A number of studies in animal models demonstrate that administration of vitamin C protects against both lipopolysaccharide and bacteria-induced sepsis and the subsequent multiple organ damage [45]. Several mechanisms have been proposed to account for the beneficial effects of vitamin C in sepsis. These are listed below.

- Direct scavenging of ROS/RNS formed during sepsis
- Inhibition of activation of inducible nitric oxide synthase (iNOS) and the subsequent formation of nitric oxide during sepsis [46]
- Inhibition of activation of NAD(P)H oxidase and the subsequent formation of ROS as well as attenuation of activation of phosphatase type 2A during sepsis [47]

- Modulation of the functionality of inflammatory cells, resulting in decreased inflammatory responses [48, 49]

3.1.10. Role in Cancer

Early studies by E. Cameron and L. Pauling (a two-time Nobel Laureate) reported that high pharmacological doses of vitamin C could prolong survival of patients with terminal cancer [50, 51] (also see Section 3.2.3). Pharmacological millimolar concentrations of vitamin C are found to selectively kill cancer cells via an ROS-dependent mechanism [10, 52]. High doses of vitamin C given via intravenous injection also exhibit antitumor activities in many animal models of human cancer [10, 13, 53], supporting the early findings by E. Cameron and L. Pauling. The anticancer activities of pharmacological doses of vitamin C in animal models are also in line with the results from mechanistic studies, which are outlined below.

- Vitamin C-mediated inhibition of HIF-1α, a transcription factor involved in tumor cell growth under hypoxic conditions [11]
- Vitamin C-mediated inhibition of angiogenesis [12, 13]
- Vitamin C-mediated stabilization of p53 tumor suppressor [54]
- Vitamin C-mediated apoptosis [55, 56]
- Vitamin C-mediated inhibition of NF-κB, a transcription factor involved in inflammation and oncogenesis [57]

It should be noted that the above molecular and cellular mechanisms underlying vitamin C-mediated antitumor activities are not mutually exclusive. In fact, many of them are closely interrelated. For example, inhibition of HIF-1α may also lead to suppression of angiogenesis. Similarly, stabilization of p53 may promote this tumor suppressor-dependent cell cycle arrest and apoptosis.

3.2. Human Studies and Clinical Perspectives

3.2.1. Study Approaches

The role of vitamin C in human disease conditions has been investigated by applying various study approaches, including observational epidemiological studies, randomized controlled clinical trials, and case reports. Although treatment of scurvy has been so far the only fully established therapeutic use of vitamin C with a proven mechanism of action in humans, there are increasing numbers of studies suggesting a potential beneficial effect for vitamin C supplementation in the intervention of many common human diseases, such as cardiovascular disorders and cancer.

3.2.2. Role in Human Cardiovascular Diseases

It has long been recognized that high dietary intake of vegetables and fruits is associated with decreased risk of developing cardiovascular diseases. This association is particularly attributable to antioxidant compounds, such as vitamins C and E, present in these foods. Many observational epidemiological studies and randomized clinical trials have examined the relationship between antioxidant vitamin supplementation and incidence of cardiovascular

diseases [58]. However, the results and conclusions of these studies are not consistent. While observational epidemiological studies suggest an association between high vitamin C intake and decreased incidence of cardiovascular diseases, large-scale randomized, placebo-controlled trials in non-selected populations largely fail to conclude a beneficial effect of vitamin C supplementation in cardiovascular diseases [58]. The findings from these studies have been systematically reviewed and analyzed, and the potential causes of their discrepancy discussed [16, 59]. Among the potential causes are doses, duration of intervention, and confounding effects. In addition, these studies often did not consider the specific physiological conditions (e.g., vitamin C status) of the subjects. Because vitamin C may have opposing effects (antioxidant versus pro-oxidant activities) under different physiological conditions, cancellation of positive and negative outcomes within a pooled large sample population may result in lack of treatment effects. In this context, multiple relatively small-scale clinical trials in selected subpopulations of patients with significant oxidative stress have yielded promising results, supporting a potential therapeutic role for vitamin C alone or in combination with vitamin E in certain forms of cardiovascular diseases, including hypertension and ischemic heart disease [60-62].

3.2.3. Role in Human Cancer

Similar to the findings related to cardiovascular diseases, observational epidemiological studies have also suggested that consumption of diets rich in vitamin C is associated with decreased risk of developing cancer. However, large-scale randomized, placebo-controlled interventional trials have mostly concluded that dietary antioxidant vitamin supplementation does not reduce the incidence of cancer or cancer-related mortality [63]. This section focuses on discussion of the findings related to the utilization of high intravenous doses of vitamin C in therapeutic intervention of human malignancies.

3.2.3.1. A Historical Overview on High-Dose Vitamin C Therapy

As noted earlier in Section 3.1.10, studies conducted by E. Cameron and L. Pauling in the 1970s suggested that large doses (10 g/day via intravenous infusion for about 10 days and orally thereafter) of vitamin C were effective in increasing survival and improving quality of life of terminal cancer patients [50, 51]. Similar findings were also reported by clinical trials in Japan [64]. However, subsequent studies on large doses of vitamin C given orally (10 g/day) at the Mayo Clinic did not reach the same conclusion [65, 66], and the routes of administration (intravenous infusion versus oral intake) may explain the discrepant results. Indeed, as discussed above in Section 1.2, oral vitamin C intake produces plasma concentrations that are tightly controlled, and the maximal plasma concentration attainable by oral intake of vitamin C has been estimated to be approximately 200 μM. In contrast, intravenous administration of pharmacological doses of vitamin C results in millimolar plasma concentrations, and such millimolar concentrations of vitamin C have been shown to selectively kill cancer cells in vitro [52].

3.2.3.2. Recent Clinical Studies on High-Dose Vitamin C Therapy

Recently, S.J. Padayatty and coworkers reported three well-documented cases of advanced malignancies in which patients had unexpectedly long survival times after receiving high doses of vitamin C via intravenous administration [67]. The high intravenous dosing

regimens for the 3 patients were (1) 65 g twice per week for 10 months, (2) 30 g twice per week for 3 months, followed by 30 g once every 1-2 months for 4 years, interspersed with periods of 1-2 months during which the patient had more frequent infusion, and (3) 15 g twice per week for about 7 months followed by 15 g once every 2-3 months for about one year. Although the findings from this study supported a potential therapeutic role for high intravenous doses of vitamin C in cancer, other possibilities could not be excluded to account for the unexpectedly prolonged survival of the patients. In this context, a recent phase 1 clinical trial reported that high-dose intravenous vitamin C (0.4-1.5 g/kg body weight, three times weekly) was well-tolerated but failed to demonstrate anticancer activity when administered to patients with previously treated advanced malignancies [68]. Thus, large-scale randomized, placebo-controlled trials are needed to establish a possible therapeutic value for high intravenous doses of vitamin C in human cancer.

3.2.4. Role in Other Human Diseases

Multiple clinical trials have also suggested potential benefits of vitamin C supplementation in the intervention of some other human diseases, including sepsis, gut disorders, and metabolic syndrome.

3.2.4.1. Sepsis

Parenteral administration of vitamin C may decrease morbidity and mortality in critically ill patients who are septic or at risk of becoming septic. In a randomized double-blind, placebo-controlled trial with 216 critically ill patients, intravenous infusion of vitamin C and vitamin E was associated with a decreased mortality [69]. Another randomized trial with 595 critically ill surgical patients also found that a combination of vitamin C and vitamin E administration begun within 24 hours of traumatic injury or major surgery decreased relative risk of pulmonary edema and multiple organ failure [70]. These two clinical trials were not designed to distinguish between the effects of vitamin C and vitamin E. A third randomized trial on vitamin C alone reported decreased morbidity (as evidenced by significant reductions in edema formation, fluid resuscitation volume, and respiratory dysfunction) for severely burned patient who received high-dose vitamin C therapy intravenously [71].

3.2.4.2. Gut Disorders

Dietary vitamin C supplementation may exert beneficial effects in certain gastrointestinal disorders. A randomized controlled trial with 244 Japanese subjects having atrophic gastritis reported that taking vitamin C supplement for 5 years was associated with decreased oxidative stress markers in serum [72]. Multiple clinical trials demonstrate that addition of vitamin C to the standard antibiotic treatment regimens for *Helicobacter pylori* infections may increase *H. pylori* eradication rate [73] or reduce the dosage of the antibiotic clarithromycin without compromising the high eradication efficacy for clarithromycin-susceptible *H. pylori* infection [74].

3.2.4.3. Metabolic Syndrome, Anemia, Asthma, and Skin Disorders

There have also been clinical trials examining the potential therapeutic value of vitamin C supplementation alone or in combination with other antioxidants in metabolic syndrome, anemia, asthma, and skin disorders. A small-scale randomized, placebo-controlled trial reported that vitamin C infusion was associated with attenuated oxidative stress-mediated ar-

terial dysfunction in patients with metabolic syndrome [75]. Meta-analysis of clinical studies suggests a beneficial role of vitamin C for anemia management in hemodialysis patients [76]. Systematic review and meta-analysis reveal that relatively low dietary intake of vitamin C and vitamin A is associated with significantly increased odds of asthma and wheeze [77]. The therapeutic activities of topical application of vitamin C in skin disorders, such as acne vulgaris and UV light-induced dermatitis have also been demonstrated by randomized controlled clinical trials [42, 78].

4. Conclusion and Future Directions

The antioxidant effects of vitamin C have been well-documented in diverse experimental systems. This antioxidant vitamin exerts beneficial effects in various oxidative stress-associated disease processes in animal models. While multiple large-scale randomized clinical trials in non-selected populations fail to show a benefit of vitamin C supplementation in the intervention of cardiovascular diseases and cancer, many small-scale clinical trials in selected subpopulations of patients with overt oxidative stress have yielded promising results, suggesting a potential value for using vitamin C in disease intervention. Future studies should put more emphasis on developing reliable biomarkers to assess the status of antioxidants and oxidative stress in selected patient subpopulations, and test the efficacy of vitamin C supplementation in patient groups with marked oxidative stress and/or antioxidant deficiency in well-designed clinical trials. There is also a need to further characterize the pharmacokinetics and pharmacodynamics of vitamin C not only in normal individuals, but also in selected patient groups under oxidative stress and/or with antioxidant deficiency. Such studies will provide important insights into the pharmacological basis of vitamin C-based modalities in both preventive and therapeutic intervention of human diseases.

5. References

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Chapter 21

Vitamin E

Abstract

Vitamin E is a nutritional term, and natural dietary vitamin E comprises eight different forms: the α-, β-, γ-, and δ-tocopherols and the α-, β-, γ-, and δ-tocotrienols. Among the eight forms of vitamin E, α-tocopherol has received the most extensive investigations, accounting for over 90% of the total literature on vitamin E research. Recently, the non-α-tocopherol forms of vitamin E have also received attention due to their significant biological activities. The antioxidative and anti-inflammatory effects of vitamin E have been demonstrated in various in vitro and in vivo experimental models. In addition, both tocopherols and tocotrienols also possess other novel biological functions, including modulation of cell signaling, antiproliferation, induction of apoptosis, and antiangiogenesis. These diverse biological activities obviously contribute to the well-established beneficial effects of vitamin E in a variety of disease conditions in animal models. However, large-scale randomized controlled clinical trials on vitamin E (predominantly α-tocopherol) in non-selected populations have mostly failed to show a benefit in the intervention of human diseases, including cardiovascular disorders and cancer. In contrast, small-scale clinical trials in selected patient populations provide evidence for a possible beneficial role of vitamin E supplementation in disease intervention.

1. Overview

1.1. Definition and Dietary Sources

Vitamin E was discovered by H.M. Evans and K.S. Bishop in 1922 as an essential dietary factor for reproduction in rats [1]. Vitamin E is a nutritional term, and the major dietary sources of vitamin E include vegetable oils, seeds, and nuts. Natural dietary vitamin E comprises of eight different forms: α-, β-, γ-, and δ-tocopherols and α-, β-, γ-, and δ-tocotrienols. Tocotrienols have unsaturated side chain, whereas tocopherols contain a phytyl tail with three chiral centers that naturally occur in the *RRR* configuration. Among the eight forms of dietary vitamin E, α-tocopherol is the predominant form in human tissues due to efficient retention by liver and distribution to peripheral tissues (see Section 1.4 below). α-Tocopherol is also the most studied vitamin E isoform in animals and humans, presently accounting for over

90% of the total literature on vitamin E research. Other forms of vitamin E, such as γ-tocopherol and tocotrienols have also recently received attention due to their significant biological activities in mammalian systems [2].

1.2. Blood Levels and Tissue Distribution

The normal average plasma concentration of α-tocopherol ranges from 22 to 28 μM, which is about 10 and 100 times higher than that of γ-tocopherol (2.5 μM) and δ-tocopherol (0.3 μM), respectively. In tissues, the highest concentrations of α-tocopherol are found in adipose tissue and the adrenal glands. In contrast to α-tocopherol, the uptake and distribution of tocotrienols in the body are not very efficient with the plasma concentration usually below 1 μM [3].

1.3. Additional Nomenclatures

The natural form of α-tocopherol is designated as *RRR*-α-tocopherol (formerly called d-α-tocopherol; structure shown in Figure 21-1). As mentioned above, each tocopherol contains a phytyl tail with three chiral centers, thus giving eight stereoisomers. In contrast to the natural *RRR*-α-tocopherol, the synthetic α-tocopherol, designated as *all-rac*-α-tocopherol (formerly called dl-α-tocopherol) contains ~12.5% of the *RRR* form together with seven other stereoisomers that are less biologically active. As such, the natural form of α-tocopherol is believed to be more biologically active than the synthetic form. Recently, a novel form of α-tocopherol, namely, α-tocopheryl phosphate has been reported to be present in tissues and possess biological activities, including modulation of cell signaling [4, 5].

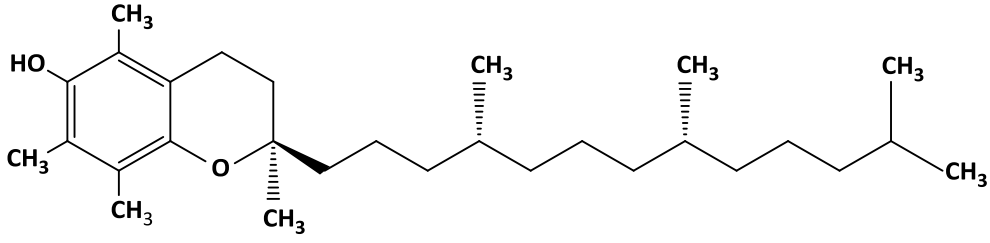

Figure 21-1. Structure of *RRR*-α-tocopherol. See text (Sections 1.1. and 1.3) for description of α-tocopherol and other forms of vitamin E.

1.4. Deficiency

As noted above, α-tocopherol is efficiently retained by liver and distributed to peripheral tissues via circulation. This is due to the presence of a liver protein that selectively recognizes α-tocopherol absorbed from the diet and is involved in the secretion of α-tocopherol into plasma for distribution to peripheral tissues. This protein is named α-tocopherol transfer pro-

tein (α-TTP) [6]. A defect in the gene for α-TTP in humans causes a disease known as ataxia with vitamin E deficiency [7] (see Section 3.2.2 below). In addition to the above genetic defect, insufficient dietary intake may also lead to vitamin E deficiency.

2. Biochemical Properties and Functions

The biological activities of α-tocopherol as well as other forms of vitamin E can be summarized into three general categories, as listed below.

- Antioxidant and pro-oxidant activities
- Anti-inflammatory activities
- Other novel effects

2.1. Antioxidant and Pro-oxidant Effects

Due to high lipophilicity, α-tocopherol and other forms of vitamin E are believed to be the major antioxidants for protecting against lipid peroxidation [8]. In various in vitro systems, both tocopherols and tocotrienols are able to scavenge peroxyl radicals. Indeed, the reaction rate between α-tocopherol and peroxyl radicals is much greater than that between peroxyl radicals and polyunsaturated fatty acid side chains of the membrane lipids. α-Tocopherol also reacts with others reactive oxygen and nitrogen species (ROS/RNS), such as singlet oxygen and peroxynitrite in certain in vitro systems. In addition, α-tocopherol has been shown to decrease ROS formation from NAD(P)H oxidase [9].

Pro-oxidant activities of α-tocopherol have been reported in vitro. α-Tocopherol is able to reduce redox-active metal ions, such as copper and iron. The α-tocopherol radical formed during reaction of α-tocopherol with ROS/RNS is also reactive and can cause hydrogen abstraction from polyunsaturated fatty acid side chains, leading to lipid peroxidation.

2.2. Anti-inflammatory Effects

Various forms of vitamin E have been demonstrated to suppress inflammatory responses in both in vitro and in vivo models. The anti-inflammatory effects of vitamin E may result from the inhibition of NF-κB, a transcription factor critically involved in the expression of proinflammatory cytokines [10]. The long-chain carboxychromanol metabolites of various forms of vitamin E also potently inhibit cyclooxygenases, important enzymes in the process of inflammation [11].

2.3. Other Novel Effects

α-Tocopherol and other forms of vitamin E possess many novel activities irrelevant to the antioxidant effects. These include modulation of enzyme activities (e.g., inhibition of pro-

tein kinase C), cell signal transduction, and gene expression. These non-antioxidant effects are believed to be the molecular basis of certain vitamin E-mediated biological actions, including antiproliferation, induction of apoptosis, and antiangiogenesis [2, 3]. In addition, tocotrienols also decrease cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase, the key enzyme in cholesterol biosynthesis [2]. Moreover, vitamin E isoforms are able to induce the gene expression of enzymes involved in xenobiotic biotransformation [12]. These non-antioxidant functions along with the aforementioned antioxidative and anti-inflammatory properties may explain the diverse roles of vitamin E in health and disease.

3. Role in Health and Disease

3.1. Animal Studies

3.1.1. Experimental Approaches

The biological activities of vitamin E have been extensively investigated in experimental animals via administration of natural vitamin E isoforms, including tocopherols and tocotrienols. Since natural vitamin isoforms are relatively unstable and not soluble in water, several vitamin E derivatives with increased stability and water solubility have been synthesized and studied in biological systems. These include α-tocopherol acetate, α-tocopherol succinate, and α-tocopheryl phosphate [13]. As noted earlier in Section 1.3, α-tocopheryl phosphate may also be formed endogenously. These relatively stable vitamin E derivatives are used in food supplements and cosmetics. In addition, α-tocopherol has been modified by coupling it to a lipophilic triphenylphosphonium cation through an alkyl linker to increase its selective accumulation in mitochondria. This mitochondria-targeted vitamin E derivative (MitoVitE) has been used to protect against pathophysiological conditions involving oxidative mitochondrial damage [13].

As mentioned above (Section 1.4), a defect in the gene for α-tocopherol transfer protein (α-TTP) in humans causes a disease known as ataxia with vitamin E deficiency. Targeted disruption of α-TTP gene in mice has been reported, and the knockout mice develop α-tocopherol deficiency and delayed-onset ataxia, which can be prevented by α-tocopherol supplementation [14]. This α-TTP knockout mouse model has been used to investigate the biological functions of α-tocopherol in disease conditions, including cardiovascular disorders.

3.1.2. Role in Cardiovascular Diseases

As a major form of vitamin E in the body, α-tocopherol has been extensively studied with respect to its benefit in cardiovascular diseases in animal models. There is substantial evidence showing an important role for α-tocopherol in protecting against cardiovascular pathophysiology, including atherosclerosis, hypertension, and myocardial ischemia-reperfusion injury [15-17]. Recently, other forms of vitamin E, such as α-tocopheryl phosphate and tocotrienols are also found to provide significant protection against cardiovascular diseases in animal models [18, 19]. The cardioprotective functions of vitamin E in experimental animals have been related to the following major mechanisms.

- ROS/RNS scavenging activity
- Inhibition of ROS formation from NAD(P)H oxidase
- Anti-inflammatory activity
- Augmentation of endothelial nitric oxide bioavailability
- Inhibition of cholesterol synthesis
- Modulation of cell signaling

3.1.3. Role in Diabetes and Metabolic Syndrome

Oxidative stress and inflammation are critical events underlying diabetes and metabolic syndrome. A large body of evidence suggests a role for natural vitamin E and derivatives in suppressing the development of diabetes in animal models [20, 21]. Various vitamin E isoforms are shown to also attenuate oxidative stress and inflammation in diabetic complications [22]. Supplementation of vitamin E isoforms ameliorates oxidative stress and inflammatory response as well as insulin resistance in animal models of metabolic syndrome [23]. A recent study further demonstrates that tocotrienols improve insulin sensitivity through activating peroxisome proliferator-activated receptors in experimental animals [24].

3.1.4. Role in Neurological Diseases

Depletion of brain α-tocopherol due to targeted disruption of α-TTP gene in mice causes lipid peroxidation of brain tissue along with neurodegeneration and ataxia [14]. These α-tocopherol-deficient mice also exhibit augmented β-amyloid accumulation and aggregation likely due to increased lipid peroxidation in brain tissue [25]. These findings indicate that α-tocopherol acts as a neuroprotective molecule. In addition to α-tocopherol, other forms of vitamin E (e.g., γ-tocopherol, tocotrienols) have also been shown to exert potent neuroprotective effects in animal models of neurological disorders, including stroke, Parkinson's disease, and diabetic neuropathy [2, 26, 27].

3.1.5. Role in Pulmonary Diseases

A beneficial role for vitamin E in animal models of pulmonary diseases has been demonstrated by multiple studies. Supplementation of both vitamin C and α-tocopherol attenuates acute lung inflammatory response elicited by cigarette smoke in mice [28]. Administration of α-tocopherol alone also protects experimental animals from lung oxidative stress and inflammation elicited by pulmonary toxicants and allergens [29-31]. The role of α-tocopherol in lung diseases has also been examined in α-TTP knockout mice. Severe deficiency of α-tocopherol in α-TTP knockout mice leads to exacerbation of acute hyperoxic lung injury [32]. In contrast, as compared with wild-type mice the α-TTP knockout mice with severe α-tocopherol deficiency are shown to have blunted airway allergic inflammatory response in an ovalbumin-induced asthma model [33]. The mechanisms by which severe α-tocopherol deficiency differentially modulates airway inflammation in different animal models warrant further investigations.

Recently, the role of γ-tocopherol in pulmonary protection has received more attention. This vitamin E isoform is shown to reduce ozone-induced exacerbation of allergic rhinosinusitis in rats [34]. Administration of γ-tocopherol also prevents airway inflammation in ovalbumin-elicited rhinitis and asthma [35]. Similarly, ozone-mediated enhancement of lower

airway allergic inflammation can be prevented by γ-tocopherol treatment in experimental animals [36]. These observations indicate that supplementation with γ-tocopherol may be a novel complementary therapy for allergic airway diseases.

3.1.6. Role in Hepatic and Gastrointestinal Diseases

α-Tocopherol protects against certain liver disease conditions in experimental animals. These include hepatic ischemia-reperfusion injury, liver transplantation, nonalcoholic fatty liver disease, and toxicant-induced hepatotoxicity [37-40]. The antioxidative and anti-inflammatory activities of α-tocopherol apparently contribute to the above protective effects on liver pathophysiology.

Administration of α-tocopherol or its derivatives has been demonstrated to ameliorate experimental colitis and intestinal oxidative stress in murine models [41]. Dietary supplementation of α-tocopherol also suppresses gastric mucosal damage elicited by *Helicobacter pylori* infection through, at least partially, inhibition of inflammatory cell infiltration [42]. As significant amounts of vitamin E isoforms, including tocotrienols occur in the gut following dietary intake, vitamin E may serve as an important defense against gut inflammation and oxidative stress.

3.1.7. Role in Renal Diseases

Multiple studies demonstrate that administration of α-tocopherol or tocotrienols results in amelioration of the pathophysiological processes underlying various renal diseases, including ischemia-reperfusion injury [43], chronic renal allograft nephropathy [44], diabetic nephropathy [45], and drug/xenobiotic-induced nephrotoxicity [46, 47]. The protective effects are associated with attenuation of oxidative stress and inflammation in renal tissue under these pathophysiological conditions.

3.1.8. Role in Skin Diseases

Similar to vitamin C (Chapter 20), α-tocopherol is present in mammalian skin and may serve as an important line of dermal antioxidant defenses. Topical application of vitamin E isoforms, including tocopherols and tocotrienols exerts dermal protection against certain skin disease conditions, such as ultraviolet (UV) irradiation-induced skin injury, contact dermatitis, and chemically-induced oxidative stress in animal models [48-50]. Notably, both vitamin C and vitamin E have been used in cosmetics [51]. Multiple mechanisms have been proposed to account for the dermal protective effects of vitamin E, which are outlined below.

- Direct ROS/RNS-scavenging effects
- Suppression of cytokine production and inflammatory cell infiltration
- Stabilization of both keratinocytes and mast cells [49, 52]

In addition, administration of an α-tocopherol-derived analog alone or in combination with celecoxib (a selective cyclooxygenas-2 inhibitor) has been shown to reduce the multiplicity of UV irradiation-induced skin cancer in mice [53].

3.1.9. Role in Cancer

Both natural vitamin E and vitamin E derivatives have been extensively investigated with

respect to their anticancer activities. A large body of evidence suggests a critical role for vitamin E in cancer protection in animal models [54, 55]. Early studies have focused on the anticancer activities of α-tocopherol and the potential clinical applications. Recent studies also demonstrate the importance of γ-tocopherol as well as tocotrienols in cancer protection. In fact, under certain conditions these non-α-tocopherol forms of vitamin E have more pronounced anticancer activities than α-tocopherol [2]. The anticancer effects of vitamin E in animal models most likely result from mechanisms involving multiple targets [54-56], as listed below.

- Inhibition of cell cycle
- Induction of apoptosis
- Suppression of angiogenesis
- Regulation of immunity and augmentation of tumor immunosurveillance

3.1.10. Role in Other Diseases and Conditions

Studies in experimental models also suggest a protective role for vitamin E supplementation in some other disease conditions. These include endotoxemia [57], arthritis [58], erectile dysfunction [59], cataract [60], and HIV infection [61]. Notably, a recent study shows that severe deficiency of α-tocopherol in α-TTP knockout mice confers resistance to malarial infection. This increased resistance may result from augmented oxidative damage to the parasites due to diminished antioxidant action of α-tocopherol [62].

3.2. Human Studies and Clinical Perspectives

3.2.1. Study Approaches

The potential beneficial effects of vitamin E in human disease conditions have been investigated by three major approaches: (1) observational epidemiological studies to assess the association between dietary vitamin E intake and risk of disease development, (2) randomized controlled clinical trials to determine the benefits of vitamin E supplementation in preventive or therapeutic intervention of diseases, and (3) clinical case reports on genetic variations or disorders that may potentially affect the metabolism and biological activities of vitamin E. In this context, as described below, a genetic disorder known as ataxia with vitamin E deficiency has been identified in humans. Studies on this genetic disorder and the underlying pathophysiology have provided valuable insights into the biological significance of vitamin E in human health and disease.

3.2.2. Ataxia with Vitamin E Deficiency

Ataxia with vitamin E deficiency (AVED), an autosomal recessive neurodegenerative disease, is caused by a defect in the gene for α-TTP, a protein critically involved in selectively transporting α-tocopherol from liver to plasma and its subsequent distribution to peripheral tissues. The neurological symptoms of AVED include ataxia, dysarthria, hyporeflexia, and decreased vibration sense, sometimes associated with cardiomyopathy and retinitis pigmentosa. Vitamin E supplementation to patients with this disease improves symptoms and prevents disease progression [7].

Pathophysiologically, patients with AVED develop neuropathy characterized as a dying back of the large caliber axons in the sensory nerves. The dying back of the sensory neurons is likely caused by nerve deficiency of α-tocopherol and the subsequent apoptosis [8].

3.2.3. Role in Human Cardiovascular Diseases

Observational epidemiological studies suggest an inverse relationship between consumption of vitamin E and risk of developing cardiovascular diseases. However, randomized controlled interventional trials have been controversial, with some positive findings, many null findings, and some suggestion of harm in certain populations [63-65]. Possible reasons for the controversies include the doses and forms (natural versus synthetic) of the vitamin E used, the time-window of intervention, lack of reliable biomarkers to assess both the oxidative stress and antioxidant status in the human subjects, as well as the general populations included in the trials. It has been suggested that vitamin E may be beneficial to those individuals who are antioxidant-deficient or exposed to increased levels of oxidative stress, such as smokers, diabetics, and elderly patients, emphasizing the importance of subpopulation targeting. In this regard, recent smaller interventional studies with carefully chosen subpopulations, such as those under high levels of oxidative stress, have yielded largely positive results [63]. For example, a randomized controlled clinical trial reported that vitamin E supplementation (400 IU daily) significantly reduced cardiovascular events in a subgroup of middle-aged individuals with both type 2 diabetes and the haptoglobin 2-2 genotype [66]. Nevertheless, additional large-scale randomized controlled trials in selected populations are needed to better understand the effectiveness of vitamin E supplementation in the intervention of human cardiovascular diseases.

It should be noted that almost all of the major clinical trials on vitamin E have only involved the use of α-tocopherol. As discussed above, other isoforms of vitamin E also possess important biological activities, and their beneficial effects have been demonstrated in various animal models of human diseases, including cardiovascular diseases, cancer, and neurological disorders [2]. Thus, future clinical trials should also include the non-α-tocopherol forms of vitamin E for disease intervention.

3.2.4. Role in Human Cancer

Similar to what was found with cardiovascular diseases, observational epidemiological studies also show an inverse association between consumption of vitamin E and risk of cancer development. However, most large-scale randomized controlled trials have failed to show a beneficial role for α-tocopherol in cancer intervention [67-69]. The same reasons as described for the failure of the clinical trials on vitamin E and cardiovascular diseases (Section 3.2.3) could also account for the failure of clinical trials on α-tocopherol and cancer. Of note is the possibility that α-tocopherol may not be the most effective form of vitamin E with respect to cancer intervention. Indeed, as described above, the non-α-tocopherol forms of vitamin E exhibit marked anticancer activities in a variety of animal models of human cancer (Section 3.1.9). Thus, future studies should also focus on the non-α-tocopherol isoforms of vitamin E in human cancer intervention.

Although the majority of the large-scale randomized controlled trials have failed to show a benefit for α-tocopherol in cancer intervention, results from multiple small-scale studies have provided supporting evidence for a possible role of both α-tocopherol and γ-tocopherol

in primary prevention of certain specific types of cancer, including gastric noncardia adenocarcinoma, pancreatic cancer, ovarian cancer, and breast cancer [5]. The efficacy of vitamin E supplementation in human cancer intervention awaits further investigations in large-scale randomized controlled trials with better study design, including (1) choosing of the right vitamin E isoforms and the appropriate dosage and duration of intervention, (2) selection of the appropriate patient populations with marked antioxidant deficiency or oxidative stress, and (3) development of reliable biomarkers for assessing both the antioxidant and oxidative stress status in the human subjects.

3.2.5. Role in Other Human Diseases

3.2.5.1. Nonalcoholic Steatohepatitis

A recent randomized controlled trial in a total of 247 adults with nonalcoholic steatohepatitis reported that as compared with placebo, intake of vitamin E (*RRR*-α-tocopherol) at a dose of 800 IU daily for 96 weeks was associated with a significantly higher rate of improvement in disease condition as assessed by histological features of nonalcoholic steatohepatitis, inflammation, and serum enzyme levels [70]. The trial concluded that vitamin E is superior to placebo for the treatment of nonalcoholic steatohepatitis in adults without diabetes. This study also examined the effect of pioglitazone, an antidiabetic drug, at a dose of 30 mg daily for 96 weeks, and found no benefit of pioglitazone over placebo for the primary outcome (i.e., the histological features of nonalcoholic steatohepatitis). However, significant benefits of pioglitazone were observed for some of the secondary outcomes (e.g., improvement in insulin resistance) [70].

The above randomized controlled trial did not examine the effectiveness of combined therapy of vitamin E and pioglitazone. In this context, a previous pilot study reported that a combination therapy of vitamin E (400 IU/day) and pioglitazone (30 mg/day) produced marked improvement in nonalcoholic steatohepatitis histology although vitamin E (400 IU/day) alone had no significant effects [71]. The lack of effectiveness of vitamin E therapy in this pilot study could be due to the lower dose of vitamin E (400 IU/day in the pilot study versus 800 IU/day in the randomized controlled trial), shorter duration of treatment, as well as limitations in sample size and study design. The efficacy of vitamin E therapy in nonalcoholic steatohepatitis observed in the randomized controlled trial described above is also in line with the finding in experimental animals that administration of α-tocopherol protects against nonalcoholic fatty liver disease (Section 3.1.6).

3.2.5.2. Additional Diseases and Conditions

In addition to nonalcoholic steatohepatitis, small-scale clinical trials also suggest a possible beneficial role for vitamin E (α-tocopherol or γ-tocopherol) in several other pathophysiological conditions. These include severe sepsis [72], active ulcerative colitis [73], oxidative stress in normal and asthmatic subjects [74], digital ulcers in systemic sclerosis [75], UV irradiation-induced skin injury [76], and HIV infection [77]. Due to the relatively small numbers of patients involved as well as other design limitations associated with these small-scale clinical trials or pilot studies, the potential beneficial effects of vitamin E therapy in the above disease processes need to be further evaluated in well-designed, large-scale randomized controlled trials.

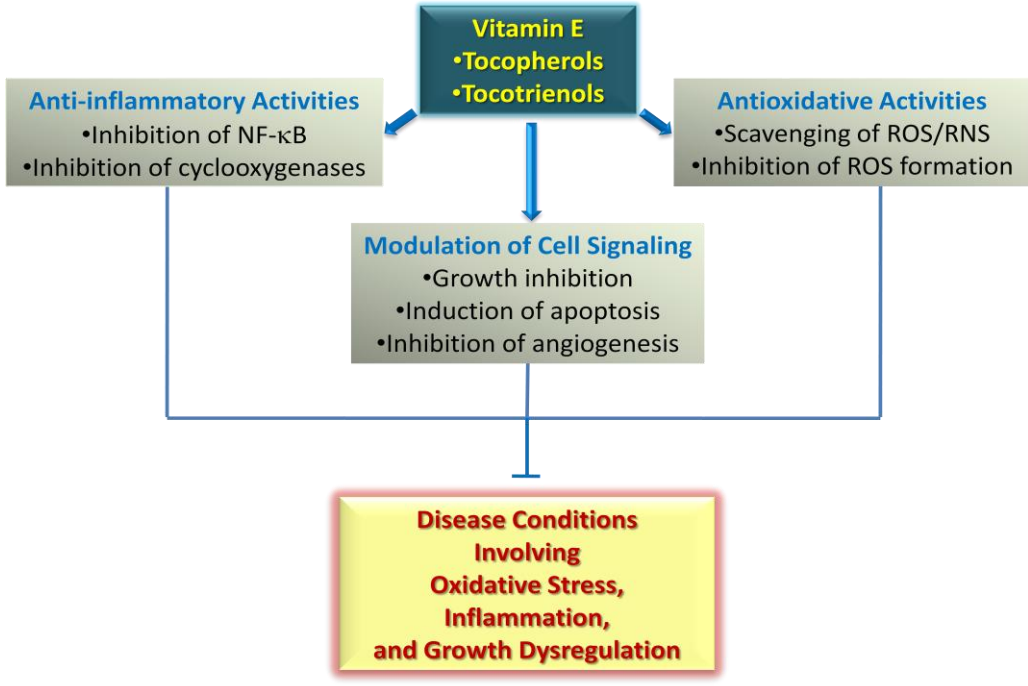

Figure 21-2. Vitamin E isoforms as multitasking molecules. As illustrated, vitamin E isoforms have antioxidative and anti-inflammatory activities. They also participate in modulating cell signal transduction, leading to growth inhibition, induction of apoptosis, and inhibition of angiogenesis. These mechanisms contribute to the beneficial effects of vitamin E in disease conditions involving oxidative stress, inflammation, and growth dysregulation.

4. Conclusion and Future Directions

Vitamin E isoforms are multitasking molecules that possess antioxidative and anti-inflammatory activities, and participate in regulating cell signaling, cell proliferation, and cell death (Figure 21-2). The disease-protective effects of vitamin E, especially α-tocopherol have been well-documented by extensive studies in animal models of human diseases involving oxidative stress and inflammation. The benefits of vitamin E (primarily α-tocopherol) have also been suggested by many observational epidemiological studies and small-scale interventional trials in selected patient groups. However large-scale interventional trials in non-selected populations have largely concluded the ineffectiveness of vitamin E (primarily α-tocopherol) supplementation in disease intervention. Possible reasons for the controversies include the doses and forms of the vitamin E used, the time-window of intervention, the lack of reliable biomarkers to assess both the oxidative stress and antioxidant status in the human subjects, as well as the general populations included in the trials. Indeed, nearly all major clinical trials on vitamin E have only investigated α-tocopherol. As discussed earlier, other forms of vitamin E, such as γ- and δ-tocopherol as well as tocotrienols also possess significant disease-protective activities. Hence, more research efforts should be devoted to the investigations of these vitamin E isoforms with regard to their biological activities, pharmacokinetics, and efficacy in disease intervention.

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Chapter 22

Carotenoids

Abstract

Carotenoids represent a large family of compounds of over six hundred fat-soluble plant pigments that provide much of the color that we see in nature. The major carotenoids in normal human diets include β-carotene, α-carotene, lycopene, lutein, zeaxanthin, β-cryptoxanthin, and astaxanthin. Some carotenoids, such as β-carotene have pro-vitamin A activity. Carotenoids are effective scavengers of singlet oxygen and peroxyl radicals. They also possess anti-inflammatory activities and other novel functions in mammalian systems. The health benefits of carotenoids are evidenced by their protective effects in a wide variety of disease processes that involve oxidative stress and inflammation in animal models. The beneficial role of carotenoids in various human disease conditions, including cardiovascular disorders and cancer has been suggested by extensive observational epidemiological studies and many small-scale interventional trials. However, large-scale randomized controlled trials on β-carotene supplementation have failed to show a beneficial effect in cardiovascular diseases and cancer. The exact value of carotenoid supplementation in human disease intervention awaits further investigations in well-designed clinical trials.

1. Overview

1.1. Definition and Dietary Sources

Carotenoids are a family of lipophilic pigmented compounds that are synthesized by plants. In plants, carotenoids contribute to the photosynthetic machinery and protect plant tissues from photodamage [1]. Carotenoids are responsible for the yellow, orange, and red colors of fruits and vegetables, which constitute the major sources of human dietary carotenoids. Over six hundred carotenoids have so far been identified in nature. The major ones in human diet include β-carotene, α-carotene, lycopene, lutein, zeaxanthin, β-cryptoxanthin, and astaxanthin. As shown in Figure 22-1, notable major dietary sources for the above carotenoids include carrot (β-carotene and α-carotene), tomato and watermelon (lycopene), kale and spinach (lutein and zeaxanthin), papaya and peach (β-cryptoxanthin), and salmon (astaxanthin).

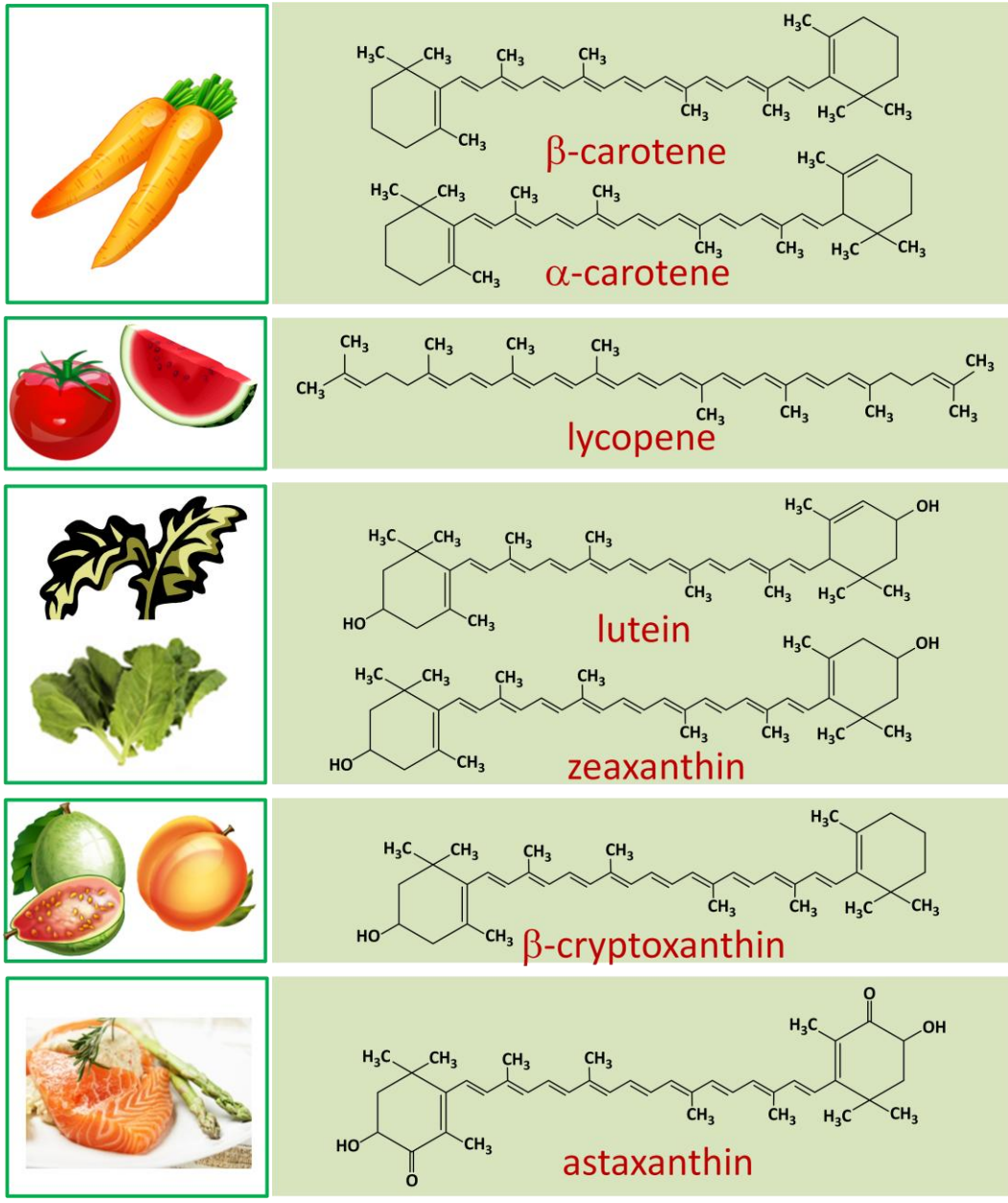

Figure 22-1. Major carotenoids in human diet and their dietary sources. See text (Sections 1.1 and 1.2) for description of carotenoids. (courtesy of Jason Z. Li)

1.2. Classification

Based on their chemical composition, carotenoids are classified into two groups: (1) carotenes which are composed of only carbon and hydrogen atoms and (2) xanthophylls which

also carry at least one oxygen atom. β-Carotene, α-carotene, and lycopene are predominant members of the carotene group, whereas lutein, zeaxanthin, β-cryptoxanthin, and astaxanthin belong to the xanthophylls group.

1.3. Blood Levels and Tissue Distribution

The tissue and blood levels of carotenoids in humans vary with diet. Carotenoids in human plasma are estimated to be at low micromolar or submicromolar concentrations. The plasma concentrations of carotenoids are considered useful biomarkers of dietary intake of fruits and vegetables. Lutein and zeaxanthin are also present in the retina.

Among the carotenoids, β-carotene, lycopene, lutein, zeaxanthin, and astaxanthin have been extensively studied with respect to their biological activities in both experimental animals and human subjects.

2. Biochemical Properties and Functions

The biochemical properties and functions of carotenoids can be summarized into four general categories, as listed below.

- As a precursor of vitamin A
- Antioxidant activities
- Anti-inflammatory activities
- Other novel activities, such as modulation of cell signaling

2.1. As a Precursor of Vitamin A

β-Carotene is best known as a precursor of vitamin A. Carotenoids that can be converted to vitamin A also include α-carotene and β-cryptoxanthin [2]. These carotenoids may thus prevent vitamin A deficiency. Vitamin A is essential for growth and development, as well as visual function. In contrast, lycopene, lutein, zeaxanthin, and astaxanthin lack pro-vitamin A activity.

2.2. Antioxidant Activities

Carotenoids are potent quenchers of singlet oxygen [2]. They also scavenge other reactive species, such as peroxyl radicals, nitrogen oxides, and peroxynitrite. In addition, carotenoids, especially lycopene are found to induce cellular antioxidant enzymes via Nrf2-dependent signaling mechanism [3]. Due to the extremely low levels of carotenoids in tissues and blood, the in vivo antioxidant effects of these compounds under physiological conditions remain to be established. Carotenoids at physiologically or pharmacologically relevant concentrations have been shown to exert non-antioxidant activities, as described below.

2.3. Anti-inflammatory Activities

Carotenoids suppress inflammatory responses in both in vitro and in vivo systems. The anti-inflammatory activities may result from multiple mechanisms, including detoxification of reactive oxygen and nitrogen species (ROS/RNS) and suppression of proinflammatory cytokine formation. In this regard, carotenoids, such as lycopene and astaxanthin are shown to potently inhibit NF-κB signaling [4, 5]. NF-κB is a critical transcription factor involved in the regulation of proinflammatory cytokine gene expression. Carotenoids may also inhibit other pathways involved in inflammation, such as cyclooxygenases [6].

2.4. Other Novel Activities

Besides the antioxidant and anti-inflammatory activities, carotenoids have been shown to possess other biological functions, such as inhibition of growth factor signaling and tumor cell growth, modulation of androgen metabolism, induction of apoptosis, immunomodulation, and antiangiogenesis [1, 2, 7, 8]. These novel molecular events also contribute to the beneficial activities of carotenoids in health and disease.

3. Role in Health and Disease

3.1. Animal Studies

The beneficial effects of carotenoids in health and disease have been extensively investigated in experimental animals. The following sections summarize recent findings on the role of carotenoids in protecting against diverse disease processes in animal models.

3.1.1. Role in Cardiovascular Diseases

Substantial evidence from animal studies supports a beneficial role of dietary supplementation of carotenoids in cardiovascular pathophysiology involving oxidative stress and inflammation. These include atherosclerosis [9, 10], hypertension [11], myocardial ischemia-reperfusion injury [12], cardiac hypertrophy and heart failure [13], as well as drug-induced cardiotoxicity [14]. The protective carotenoids include β-carotene, lycopene, lutein, zeaxanthin, astaxanthin, and crocetin. It is important to note that different carotenoids may exhibit different cardiovascular protective effects. In this context, astaxanthin has recently received great attention because of its marked cardiovascular protection in animal models [15, 16]. Several mechanisms have been suggested to account for the cardiovascular protective activities of carotenoids, which are outlined below.

- Detoxification of ROS/RNS
- Decreased ROS formation from cellular sources, such as NAD(P)H oxidase
- Increased vascular nitric oxide bioavailability
- Anti-inflammation
- Inhibition of low-density lipoprotein (LDL) oxidation

3.1.2. Role in Diabetes and Metabolic Syndrome

A number of studies have reported a beneficial role for various carotenoids, including lycopene, lutein, zeaxanthin, and astaxanthin in amelioration of experimental diabetes and diabetic complications. Treatment with carotenoids is associated with decreased oxidative stress and inflammation in the pancreatic islets, which apparently contribute to the improvement of diabetic conditions, including insulin resistance, hyperglycemia, as well as development and progression of diabetic complications. Notably, the retina-associated carotenoids lutein and zeaxanthin exhibit a marked protection on diabetes-associated eye disorders, such as cataract and retinopathy [17, 18].

Carotenoids, especially the recently identified seaweed carotenoid fucoxanthin have been shown to protect against obesity and other metabolic abnormalities in animal models of metabolic syndrome [19]. The antiobesity effect of fucoxanthin is mediated, at least partially, by its altering lipid-regulating enzymes and mitochondrial uncoupling proteins of visceral adipose tissue in mice [20]. A beneficial effect of astaxanthin in controlling obesity, hypertension, insulin resistance, and blood glucose levels in animal models has also been reported in the literature [21, 22].

3.1.3. Role in Neurological Diseases

Oxidative stress and inflammation are critical mechanisms underlying various neurological disorders, including cerebral ischemia-reperfusion injury, Parkinson's disease, and Alzheimer's disease. The neuroprotective effects of retinoic acid in the above disorders have been well-recognized in animal models. There is also evidence suggesting a neuroprotective role for carotenoids in experimental animals. For instance, administration of lycopene or dietary intake of tomato powder rich in lycopene is shown to protect experimental animals from cerebral ischemia-reperfusion injury as well as toxin-induced parkinsonism [23-25]. Other carotenoids, including astaxanthin and crocetin have also been reported to exhibit neuroprotection in cerebral ischemia-reperfusion injury and toxin-induced parkinsonism in animal models [26-28].

3.1.4. Role in Pulmonary Diseases

Multiple studies have demonstrated a beneficial role for lycopene in airway inflammatory disorders. Administration of lycopene ameliorates ovalbumin-induced airway inflammation in a murine model of asthma [29]. It is further shown that lycopene supplementation suppresses Th2 responses and lung eosinophil infiltration [30]. In cultured airway epithelial cells, lycopene decreases proinflammatory responses induced by rhinovirus infection and lipopolysaccharide [31]. Oral intake of lycopene or tomato extracts rich in lycopene also prevents cigarette smoke-induced emphysema and alleviates bleomycin-elicited lung fibrosis and inflammation [32, 33]. A recent study in β-carotene 15,15'-monooxygenase-1 knockout mice demonstrates that genetically altered β-carotene metabolism leads to lung inflammation, which can be suppressed by dietary β-carotene supplementation, suggesting a critical involvement of β-carotene in controlling lung inflammation [34].

3.1.5. Role in Hepatic and Gastrointestinal Diseases

The hepatoprotective effects of carotenoids have been demonstrated by numerous studies in animal models of human liver disorders, including ischemia-reperfusion injury [35, 36],

nonalcoholic fatty liver disease [37, 38], viral hepatitis [39], and drug/xenobiotic-induced hepatotoxicity [40]. Carotenoids also exhibit a beneficial role in gastrointestinal disorders, such as drug-induced peptic ulcer [41], *Helicobacter pylori* infection [42], inflammatory bowel disease [43], and intestinal ischemia-reperfusion injury [44]. The benefits of carotenoids in liver and gastrointestinal diseases most likely result from their antioxidative and anti-inflammatory activities. In addition, dietary administration of carotenoids, such as lycopene and astaxanthin also results in marked induction of multiple antioxidant enzymes in target tissues, which may further contribute to the protective effects of carotenoids.

It should be mentioned that high doses of carotenoids (e.g., lycopene) are able to induce phase 1 biotransformation enzymes, such as P4502E1, which may lead to bioactivation of alcohol, causing exacerbation of alcoholic liver injury [45]. In addition, the apoptosis-promoting activity of lycopene is found to worsen dextran sulfate sodium-induced acute colitis, an experimental model of human ulcerative colitis [46].

3.1.6. Role in Renal Diseases

Administration of β-carotene is shown to suppress renal ischemia-reperfusion injury in experimental animals, as evidenced by amelioration of renal dysfunction and oxidative stress [47, 48]. In db/db mice, a rodent model of type 2 diabetes, treatment with astaxanthin prevents the progression of diabetic nephropathy [49]. In vitro, astaxanthin is found to protect against oxidative stress, inflammation, and apoptosis in high glucose-exposed proximal tubular epithelial cells [50]. It also protects renal mesangial cells from high glucose-induced oxidative stress and proinflammatory cytokine expression [51]. Notably, astaxanthin accumulates in the mitochondria of mesangial cells and suppresses high glucose-induced mitochondrial ROS formation [51]. These biochemical and cellular mechanisms may contribute to the beneficial effects of astaxanthin in ameliorating diabetic nephropathy observed in experimental animals.

The protective effects of dietary carotenoids, such as lycopene and astaxanthin on drug/xenobiotic-induced nephrotoxicity and oxidative stress have been documented by many studies. The drugs/xenobiotics whose nephrotoxicity can be ameliorated by carotenoids include cisplatin [52], cyclosporine A [53], gentamicin [54], and mercury chloride [55].

3.1.7. Role in Eye Diseases

The macular region of the retina is yellow in color due to the presence of two diet-derived carotenoids lutein and zeaxanthin, which are thus also called macular pigments [56]. These two carotenoids absorb blue light and possess potent antioxidative and anti-inflammatory activities. Lutein and zeaxanthin have been demonstrated to protect against age-related macular degeneration in animal models [56]. They also exert beneficial effects in other eye disorders, including diabetic retinopathy [17], cataract [18], and retinal ischemia-reperfusion injury [57]. In addition to the macular pigments, other carotenoids, such as astaxanthin and lycopene also protect against eye disorders, including age-related macular degeneration and cataract in experimental animals [58, 59]. Notably, the protective effects of carotenoids in the above eye disorders are strongly associated with their ability to suppress inflammation and oxidative stress, as well as modulation of cell signaling. Consistent with the above in vivo findings, carotenoids are shown to protect retinal or lens cells from oxidative and inflammatory stress in vitro [58-61].

3.1.8. Role in Skin Diseases

Carotenoids as light-harvesting pigments play an important part in protecting plants from excessive light and photo-oxidative stress. Carotenoids are also present in mammalian skin tissue. As noted earlier in Section 2.2, these pigments are effective scavengers of singlet oxygen, peroxyl radicals, and other reactive species that participate in photodamage. The role of carotenoids in skin photoprotection has been investigated in animal models and human subjects [2, 62]. There is ample evidence supporting that carotenoids when given via dietary intake can increase their levels in skin tissue and afford protection against ultraviolet (UV) irradiation-induced skin damage, as evidenced by amelioration of erythema, inflammation, and oxidative stress. Carotenoids are shown to also protect skin from environmental pollutant-induced oxidative damage and inflammation [63].

Carotenoids may have a role in skin carcinogenesis. Dietary intake of lutein and zeaxanthin protects against UV irradiation-induced epidermal hyperproliferation and skin carcinogenesis [64, 65]. Dietary administration of β-carotene also inhibits chemically-induced skin carcinogenesis [66]. In contrast, β-carotene is shown to enhance photocarcinogenesis in animal models under certain conditions [67]. Hence, β-carotene may possess both anti- and procarcinogenic activities depending on the experimental conditions.

3.1.9. Role in Cancer

Carotenoids suppress spontaneous development of cancer in various animal models, and protect against multistage carcinogenesis induced by chemical carcinogens [1, 68-70]. The anticancer activities of carotenoids have been observed with almost all of the major types of human cancer in animal models, including lung, liver, breast, prostate, and brain cancer. Carotenoids not only decrease cancer growth, but also suppress cancer invasion and metastasis. Several potential mechanisms have been proposed to account for the anticancer activities of carotenoids in experimental animals [1, 7, 68, 69, 71]. These are listed below.

- Antiproliferation
- Induction of apoptosis
- Antiangiogenesis
- Induction of phase 2 enzymes involved in the detoxification of carcinogens
- Antioxidative stress
- Anti-inflammation
- Induction of gap junctional communication

Antiproliferation, induction of apoptosis, and antiangiogenesis are obviously important mechanisms involved in the anticancer activities of carotenoids. Induction of phase 2 enzymes results in enhanced detoxification of electrophilic carcinogens and diminished formation of carcinogen-DNA adducts, thus suppressing the initiation step of multistage carcinogenesis. As oxidative stress and inflammation are important events in cancer development, suppression of these events by carotenoids may also contribute to inhibition of carcinogenesis. Gap junctional communication allows small molecular signaling between cells through channels that are formed by gap junction proteins, such as connexin 43. Virtually all human tumors are deficient in gap junctional communication, and restoration of gap junctional communication by forced expression of connexins suppresses the phenotypes of neoplasia.

Carotenoids by inducing connexin 43 have been found to augment gap junctional communication, which may play an important role in their in vivo anticancer activities [71].

3.2. Human Studies and Clinical Perspectives

3.2.1. Role in Human Cardiovascular Diseases

There have been a number of observational epidemiological studies showing an inverse relationship between dietary intakes or blood levels of carotenoids and risk of developing cardiovascular diseases, including atherosclerosis, hypertension, and coronary heart disease [1, 72, 73]. These observations are in line with the experimental findings that dietary carotenoids afford significant cardiovascular protection in animal models of human cardiovascular diseases (Section 3.1.1). The results from observational epidemiological studies are also consistent with the well-recognized ability of carotenoids to suppress oxidative stress and inflammation in experimental systems. In this context, oxidative stress and dysregulated inflammation have been increasingly recognized as important mechanisms underlying human cardiovascular diseases.

Although the potential cardiovascular benefits of carotenoids have been suggested by extensive observational epidemiological studies, large-scale randomized controlled trials have mostly failed to show an efficacy of β-carotene supplementation in the intervention of human cardiovascular diseases [1, 72, 73]. The lack of benefits in these trials could be due to the relatively short duration of intervention, use of non-optimal dosages of β-carotene, and the general populations involved in the studies. Furthermore, β-carotene may not be the most effective carotenoid in cardiovascular protection. In this context, other carotenoids, such as lycopene and astaxanthin have been shown to exhibit marked cardiovascular protective effects in animal models (Section 3.1.1).

Notably, recent randomized controlled trials in selected subgroup populations suggest a possible beneficial effect of carotenoids in hyperlipidemia, hypertension, and vascular inflammation [74-77]. Large-scale randomized controlled trials in selected patient groups are needed to further investigate the potential role of carotenoid supplementation in the intervention of cardiovascular diseases.

3.2.2. Role in Human Cancer

As described above, animal studies provide substantial evidence supporting that carotenoids are protective against various types of cancer (Section 3.1.9). Results from these animal studies are in line with the findings from observational epidemiological studies demonstrating that increased consumption of diet rich in carotenoids is associated with decreased risk of many types of cancer [1, 2, 78]. In addition, blood levels of β-carotene have been inversely associated with risk of developing lung cancer. However, most large-scale randomized controlled trials have failed to show any beneficial effects of β-carotene supplementation in cancer intervention. Two interventional trials even reported an increased risk for lung cancer when high doses of β-carotene were supplemented to a population who were smokers or exposed to asbestos [2]. Several possible reasons for the unexpected results have been proposed, among which is that β-carotene at high doses may act as a pro-oxidant, thereby promoting carcinogenesis [2].

The failure of β-carotene supplementation for cancer intervention has prompted investigations of other dietary carotenoids, especially lycopene as potential protective agents against human cancer. Multiple observational epidemiological studies have found an inverse relationship between dietary intakes or circulation levels of lycopene and risk of prostate cancer [79, 80]. Several relatively small-scale randomized controlled trials also suggest a beneficial effect of lycopene supplementation in slowing the progression of prostate cancer. However, large-scale well-designed interventional trials are warranted to ascertain the efficacy of lycopene in the intervention of prostate cancer as well as other types of human cancer [79, 80]. In addition, well-designed randomized controlled trials are also needed to assess the potential value of other dietary carotenoids in human cancer intervention, including both primary and secondary prevention.

3.2.3. Role in Other Human Diseases

Cardiovascular diseases and cancer have been the major focus of studies related to the health benefits of carotenoids. Carotenoids have also been studied with regard to their potential effects in many other human diseases. The sections below provide a brief summary of the recent findings from both observational epidemiological studies and interventional trials on the benefits of carotenoids in these diseases.

3.2.3.1. Eye Diseases

The macula lutea, also known as yellow spot is part of the retina and the area of maximal visual acuity. The carotenoids lutein and zeaxanthin are the pigments responsible for the color of this tissue. As aforementioned, these two macular pigments are protective against age-related macular degeneration and cataract in animal models (Section 3.1.7). Consistently, both observational epidemiological studies and small interventional trials have suggested a beneficial role for lutein and zeaxanthin in patients with age-associated macular degeneration or cataract [81-83]. Supplementation of a mix of β-carotene, vitamins C and E, and zinc was also found to attenuate the development of advanced age-related macular degeneration in a randomized controlled trial [84].

3.2.3.2. Skin Diseases

Carotenoids accumulate in skin tissue following dietary intake. Multiple interventional trials have shown a protective effect of dietary supplementation of carotenoids, such as β-carotene, lycopene, lutein, and zeaxanthin in UV irradiation-induced skin injury [62, 85]. This is consistent with the findings in animal studies that carotenoids are protective against skin photodamage by UV light (Section 3.1.8). It has been suggested that dietary carotenoids may contribute to life-long protection against UV irradiation in humans. In addition to dietary intake, topical application of carotenoids has also been shown to afford protection against skin oxidative stress and aging in human subjects [62].

3.2.3.3. Additional Diseases and Conditions

Observational epidemiological studies demonstrate that plasma levels of lycopene are significantly lower in asthmatic patients than in healthy control subjects, suggesting a potential involvement of this carotenoid in asthma [86, 87]. An early randomized controlled interventional trial reported a beneficial effect of lycopene supplementation on exercise-induced

asthma and oxidative stress [88]. However, this finding was not replicated in a subsequent interventional trial [89].

As discussed above in Section 3.1, extensive animal studies have demonstrated astaxanthin as an effective protective agent in various disease conditions via its potent antioxidative and anti-inflammatory activities. In line with this, a recent randomized controlled trial reports that supplementation of astaxanthin decreases oxidative stress and inflammation in healthy human subjects [90]. Randomized controlled interventional trials also show a beneficial role for astaxanthin supplementation in improving the symptoms in patients with *Helicobacter pylori* infection [91], and in ameliorating oxidative stress, inflammation, and vascular dysfunction in renal transplant patients [92]. These observations indicate the feasibility of using astaxanthin in the intervention of human diseases involving oxidative stress and dysregulated inflammation.

There is substantial evidence supporting that inflammation and oxidative stress contribute to the development of human diabetes and metabolic syndrome. An inverse association between blood levels of carotenoids and risk of diabetes or metabolic syndrome has been demonstrated by observational epidemiological studies in various populations [93-97]. However, randomized controlled clinical trials on β-carotene supplementation so far have not produced positive results with regard to the intervention of diabetes and metabolic syndrome in human subjects [98-100].

4. Conclusion and Future Directions

Carotenoids are an important group of chemicals found in human diet that have antioxidative and anti-inflammatory activities. In addition, they also possess other novel biological functions, such as antiproliferation and modulation of cell signaling. Carotenoids are protective against various disease processes in animal models. Consistently, many observational epidemiological studies and multiple small-scale interventional trials in selected patient groups suggest a beneficial role for carotenoids in human diseases, including cardiovascular disorders and cancer. However, most large-scale randomized controlled trials predominantly on β-carotene supplementation in non-selected populations have failed to show a benefit in cardiovascular diseases and cancer. Large-scale randomized controlled trials with selected subgroup targeting are warranted to ascertain the value of carotenoid supplementation in the intervention of human diseases. Future efforts should also be focused on investigating the involvement of carotenoids other than β-carotene in human health and disease. It should be kept in mind that human diet contains many other bioactive compounds, such as polyphenols (Chapter 23), which may interact with carotenoids to influence the development of human diseases. In this context, future research including both basic and clinical research should be directed to study the combined effects of carotenoids with other diet-derived phytochemicals.

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Chapter 23

Phenolic Compounds

Abstract

Phenolic compounds are an important bioactive component of human diet rich in fruits and vegetables. Dietary phenolic compounds are usually classified into five groups, namely, flavonoids (e.g., genistein), stilbenes (e.g., resveratrol), phenolic acids (e.g., caffeic acid), lignans (e.g., secoisolariciresinol), and others (e.g., curcumin). Dietary phenolic compounds possess diverse important biochemical properties and functions, including antioxidative and anti-inflammatory activities as well as other novel effects on cellular processes. These important biochemical properties and functions are translated into their critical role in protecting against disease pathophysiology in many animal models of human diseases. Both observational epidemiological studies and interventional clinical trials also suggest potential benefits of dietary phenolic compounds in various human disease processes.

1. Overview

1.1. Definition and Dietary Sources

Phenolic compounds, also known as phenols or phenolics, are a class of chemicals consisting of one or more hydroxyl groups (-OH) bonded directly to one or more six-membered aromatic rings. The simplest of the class is phenol (C_6H_5OH), which is a monophenol. Monophenols contain one such aromatic hydroxyl group, whereas compounds containing two such hydroxyl groups are called biphenols. Polyphenols are those that contain more than two such hydroxyl groups. However, the polyphenol literature often includes discussion of biphenols and monophenols because they often possess similar biological activities as do polyphenols. Notably, biphenols are also frequently considered as polyphenols in the literature. But, strictly this is not correct.

Phenolic compounds, particularly polyphenols are widely distributed in plants. Fruits and vegetables as well as wine and tea constitute the major dietary sources of phenolic compounds, with an average consumption of approximately one gram per day for a normal human adult [1, 2].

1.2. Classification

Dietary phenolic compounds are generally classified into five major groups, which are listed below.

- Flavonoids (e.g., genistein)
- Stilbenes (e.g., resveratrol)
- Phenolic acids (e.g., caffeic acid)
- Lignans (e.g., secoisolariciresinol)
- Others (e.g., curcumin)

Flavonoids, the most commonly studied dietary phenols, share a common structure consisting of two aromatic rings that are bound together by three carbon atoms that usually form an oxygenated heterocycle (Figure 23-1). Flavonoids are further classified into the following six subclasses.

- Flavonols (e.g., quercetin)
- Flavones (e.g., luteolin)
- Isoflavones (e.g., genistein)
- Flavanones (e.g., taxifolin)
- Anthocyanidins (e.g., delphinidin)
- Flavanols (e.g., epigallocatechin-3-gallate)

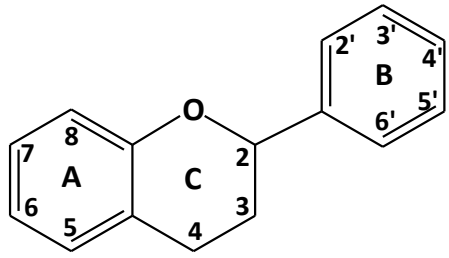

Figure 23-1. The basic chemical structure of flavonoids. This shared structure of flavonoids consists of two aromatic rings A and B that are bound together by the third ring C. See text (Section 1.2) for description of flavonoids.

The structures of some commonly studied phenolic compounds in biology and medicine and their major dietary sources are shown in Figure 23-2.

1.3. Pharmacokinetic Properties

Following dietary intake, the levels of phenolic compounds in tissues and blood are usually in the submicromolar range. Administration of pharmacological doses of pure phenol-

ic compounds may produce low micromolar plasma concentrations in animals and human subjects. Due to the hydroxyl groups, phenolic compounds undergo rapid conjugation reactions catalyzed by phase 2 biotransformation enzymes in vivo. It has been shown that gastrointestinal tissues may be exposed to high levels of phenolic compounds following their oral supplementation or dietary intake [3].

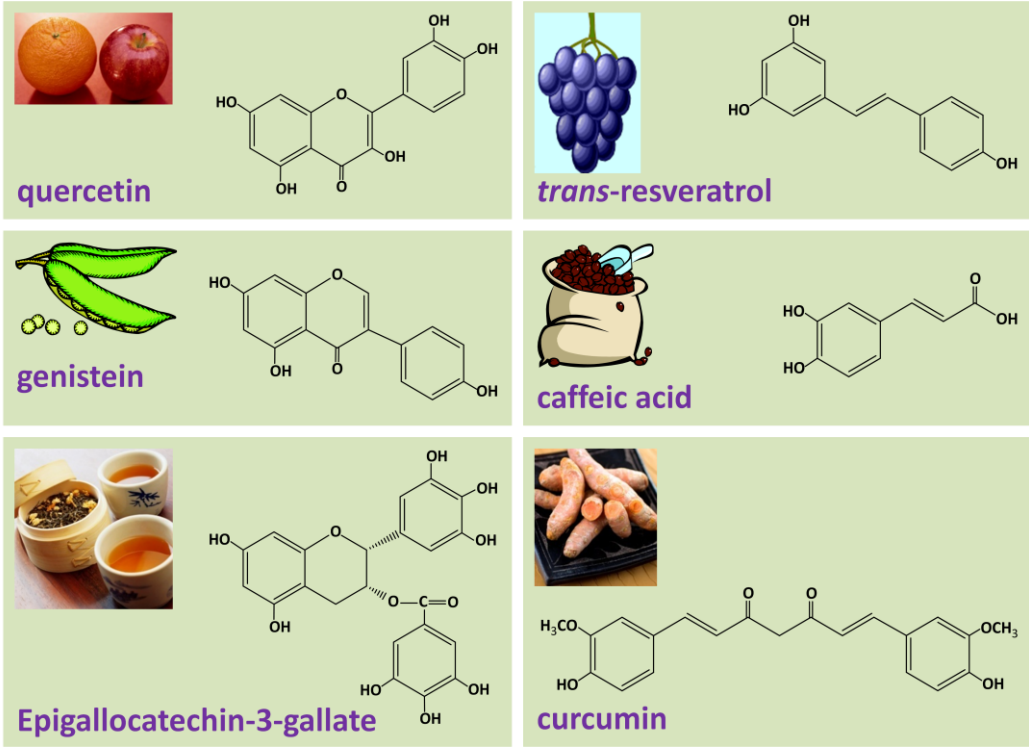

Figure 23-2. Structures of some commonly studied phenolic compounds in biology and medicine and their major dietary sources. Quercetin is mainly found in citrus fruits and apples. Soy beans and tea are major sources of genistein and epigallocatechin-3-gallate, respectively. Resveratrol is present in grape seeds and skin. Caffeic acid is a major phenolic compound in coffee. Curcumin is the principal curcuminoid of the popular Indian spice turmeric.

2. Biochemical Properties and Functions

Dietary phenolic compounds have been shown to possess various biochemical properties and functions. These are summarized into four general categories, as listed below.

- Antioxidant and pro-oxidant activities
- Anti-inflammatory activities
- Effects on nitric oxide bioavailability and endothelial function
- Other novel functions

2.1. Antioxidant and Pro-oxidant Activities

The antioxidant activities of phenolic compounds may result from the following mechanisms: (1) scavenging of reactive oxygen and nitrogen species (ROS/RNS) and other reactive species, (2) inhibition of ROS formation from cellular sources, and (3) induction of endogenous cellular antioxidant enzymes. Notably, these mechanisms may operate coordinately to account for the efficient antioxidative activities observed with certain phenolic compounds. In addition to the antioxidant functions, phenolic compounds may also exert pro-oxidant properties under certain conditions.

2.1.1. Scavenging of ROS/RNS and Other Reactive Species

In many in vitro systems, plant phenolic compounds have been shown to possess antioxidant activities. At high micromolar concentrations, they are able to scavenge various ROS/RNS as well as other reactive species, such as reactive carbonyls [4]. In addition, many phenolic compounds chelate redox active metal ions, thus preventing these metal ions from mediating the formation of more reactive forms of ROS, such as hydroxyl radical.

Due to the extreme low concentrations reached in blood from regular dietary intake, phenolic compounds are generally considered to exert insignificant ROS/RNS-scavenging effects in vivo. However, supplementation of pharmacological doses of phenolic compounds may result in markedly elevated levels of these compounds in vivo that are high enough to directly scavenge ROS/RNS and other reactive species.

2.1.2. Inhibition of ROS Formation

In cultured cells, dietary phenolic compounds at micromolar concentrations have been shown to decrease superoxide formation from NAD(P)H oxidase, an important cellular source of ROS involved in disease pathophysiology. The decreased ROS formation may result from their suppression of NAD(P)H oxidase expression, disruption of signaling pathways leading to NAD(P)H oxidase activation, or direct inhibition of the enzymatic activity of the oxidase. Both parent phenolic compounds and their metabolites may be directly involved in the inhibition of ROS formation from NAD(P)H oxidase. Indeed, the metabolites of flavonoid compounds have recently been found to potently inhibit NAD(P)H oxidase in cells, leading to decreased formation of superoxide [5]. In addition to NAD(P)H oxidase, other ROS-producing enzymes, such as xanthine oxidoreductase may also be inhibited by dietary phenolic compounds.

2.1.3. Induction of Endogenous Antioxidant Enzymes

At micromolar concentrations, many phenolic compounds have been found to upregulate endogenous cellular antioxidant enzymes in cell cultures. Administration of pharmacological doses of certain phenolic compounds has also been reported to induce tissue antioxidant enzymes in experimental animals. The transcription factor Nrf2 appears to be the key regulator involved in the upregulation of antioxidant enzymes by phenolic compounds both in vitro and in vivo [6, 7].

2.1.4. Potential Pro-oxidant Activities

Under certain in vitro conditions, many phenolic compounds at high concentrations exhi-

bit pro-oxidant activities, particularly in the presence of redox-active metals, including copper and iron ions. Due to the presence of redox metal ions in culture media, phenolic compounds undergo auto-oxidation to form ROS in cell cultures [8]. There is also evidence that certain phenolic compounds, such as epigallocatechin-3-gallate at pharmacological doses may increase ROS levels in vivo [9]. Such pro-oxidant effects have been proposed as an underlying mechanism of phenolic compound-mediated anticancer activities in animal models (see Section 3.1.8 below).

2.2. Anti-inflammatory Activities

Phenolic compounds are effective inhibitors of inflammatory responses in both in vitro and in vivo systems [10, 11]. They may affect various cascades of inflammation, including (1) inhibition of NF-κB, a critical transcription factor regulating expression of proinflammatory cytokines, (2) inhibition of cyclooxygenases, lipoxygenases, and phospholipase A2, enzymes involved in the formation of inflammatory mediators, such as prostaglandins and leukotrienes, and (3) inhibition of expression of adhesion molecules and other proinflammatory factors. In addition, the antioxidant activities of phenolic compounds may contribute to their anti-inflammatory functions as oxidative stress and inflammation are two intimately related processes.

2.3. Effects on Nitric Oxide Bioavailability and Endothelial Function

As decreased nitric oxide bioavailability and compromised endothelial function are crucial events underlying cardiovascular diseases, the beneficial role of phenolic compounds in these events has recently received attention [12, 13]. Phenolic compounds, such as flavanols and resveratrol at low micromolar concentrations increase nitric oxide levels in cultured endothelial cells. These compounds and/or their metabolites inhibit NAD(P)H oxidase, thus reducing superoxide formation from this enzyme, a major source of vascular superoxide. As discussed in Section 2.3.1 of Chapter 1, superoxide reacts with nitric oxide at a near diffusion-limited rate to form peroxynitrite. Phenolic compounds also increase the expression of endothelial nitric oxide synthase (eNOS), leading to increased formation of nitric oxide. Through decreasing superoxide formation and induction of eNOS, phenolic compounds may significantly enhance endothelial nitric oxide bioavailability.

2.4. Other Novel Functions

At micromolar concentrations or pharmacological doses, phenolic compounds exhibit a variety of other biological activities. These effects include: (1) modulation of many cellular signaling molecules, leading to decreased cell proliferation, migration, and angiogenesis, (2) induction of apoptosis in tumor cells, (3) modulation of estrogen or androgen receptors, and (4) activation of sirtuins.

Activation of sirtuins by phenolic compounds, especially resveratrol has recently received great attention. Sirtuins are a highly conserved family of NAD^+-dependent enzymes

that regulate lifespan in lower organisms. To date, seven mammalian homologues (Sirt1-7) have been identified, and they are linked to many activities, including cellular stress resistance, genomic stability, tumorigenesis, and energy metabolism [14]. Activation of Sirt1 by pharmacological doses of resveratrol leads to protection against metabolic disease as well as improvement of survival in mice on a high-calorie diet [15] (see Section 3.1.2 below for more discussion). A number of other plant phenolic compounds, including butein, quercetin, fisetin, isoliquiritigenin and piceatannol have been shown to activate Sirt1 [16, 17].

In summary, as illustrated in Figure 23-3, phenolic compounds affect numerous cellular processes and possess diverse biochemical properties and functions. Increasing evidence suggests that these diverse biochemical properties and functions contribute to the beneficial role of dietary phenolic compounds in health and disease.

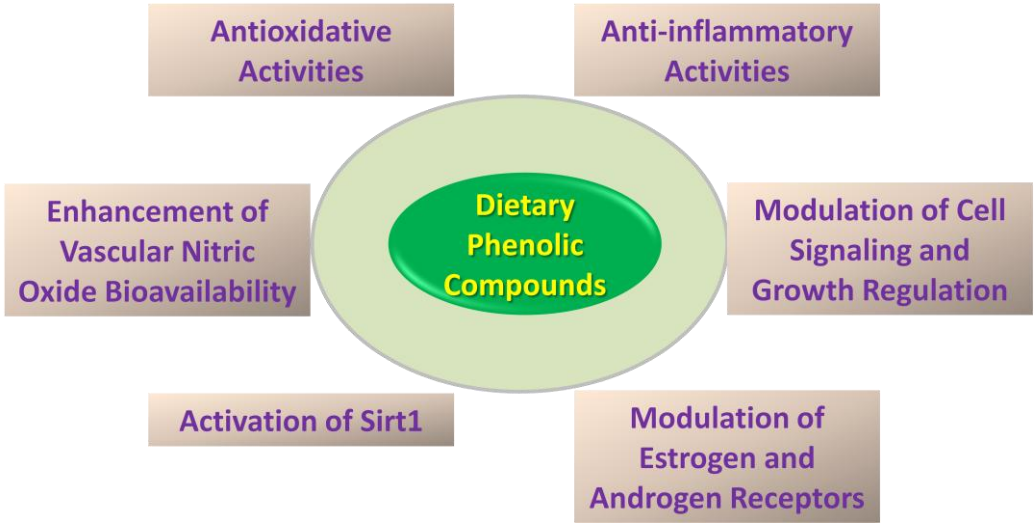

Figure 23-3. Dietary phenolic compounds as multitasking molecules**.** As illustrated, dietary phenolic compounds possess antioxidative and anti-inflammatory activities. In addition, they also participate in modulating cell signal transduction and growth regulation. Moreover, phenolic compounds are able to cause activation of Sirt1 as well as modulate the receptors of sex hormones. These diverse mechanisms contribute to the disease-protective effects of dietary phenolic compounds.

3. Role in Health and Disease

3.1. Animal Studies

The beneficial effects of phenolic compounds in disease pathophysiology have been extensively investigated in a wide variety of animal models over the last two decades. A large body of experimental evidence supports an important protective role for numerous phenolic compounds in animal models of human diseases involving nearly all organs and systems. It is impossible to include this vast amount of information in a single chapter. Hence, the sections below are intended to summarize the key findings from these studies.

3.1.1. Role in Cardiovascular Diseases

Cardiovascular diseases are among the best studied disorders with regard to the protection by dietary phenolic compounds. The most notable polyphenol with established cardiovascular benefits in animal models is probably resveratrol [18]. The cardiovascular protective effects of numerous other phenolic compounds have also been demonstrated in animal models [13, 19-21]. In addition to the individual pure phenolic compounds, fruit and plant extracts rich in polyphenols have been reported to be also cardiovascular protective. The disease entities in which dietary phenolic compounds exhibit a beneficial role include hypertension, atherosclerosis, myocardial ischemia-reperfusion injury, cardiac hypertrophy and heart failure, cardiac arrhythmia, and drug/xenobiotic-induced cardiotoxicity.

The cardiovascular protective effects of phenolic compounds are usually manifested as prevention of disease development and/or retardation of disease progression. Certain phenolic compounds, such as curcumin have been demonstrated to even reverse cardiac hypertrophy in a murine model induced by aortic banding and phenylephrine infusion. Although overwhelming evidence supports beneficial activities of phenolic compounds in cardiovascular pathophysiology, there are also studies suggesting deleterious effects of certain phenolic compounds under specific experimental conditions. For example, it was recently reported that late chronic catechin treatment could worsen endothelial dysfunction in low-density lipoprotein receptor-deficient mice with established atherosclerosis although the same catechin treatment reversed the age-related vascular dysfunctions in wild-type mice [22]. The significance of this observation to human health remains unclear.

The biochemical properties and functions of phenolic compounds described in Section 2 are apparently the molecular and cellular basis of cardiovascular protection by these compounds. Specifically, the following major mechanisms may particularly account for the beneficial effects of phenolic compounds in cardiovascular pathophysiology.

- Inhibition of oxidative stress and inflammation in cardiovascular tissues
- Augmentation of nitric oxide bioavailability in vasculature
- Antiplatelet activities
- Stimulation of Sirt1 signaling to augment cardiovascular function
- Stimulation of high-density lipoprotein-mediated cholesterol efflux from macrophages, preventing foam cell formation

Obviously, the antioxidative and anti-inflammatory activities are important in phenolic compound-mediated cardiovascular protection as oxidative stress and inflammation are key events of cardiovascular pathophysiology. Augmentation of nitric oxide bioavailability will improve vasodilation and endothelial function. In addition, vascular nitric oxide is able to suppress inflammation as well as thrombogenesis. Phenolic compounds are shown to also have direct antiplatelet activities via interference with signaling involved in platelet activation and aggregation. The antiplatelet activities may contribute to the in vivo inhibition of thrombogenesis by phenolic compounds [20]. As Sirt1 plays an important role in energy metabolism and cardiovascular function, stimulation of this signaling pathway by phenolic compounds, especially resveratrol has been shown to improve cardiac function of experimental animals with cardiomyopathy [23]. Recently, phenolic compounds from coffee are demonstrated to stimulate high-density lipoprotein-mediated cholesterol efflux from macrophages in

vitro and in vivo, providing a novel mechanism for the antiatherosclerotic effects of dietary phenolic compounds [24, 25].

In addition to the above listed mechanisms, phenolic compounds may target other cellular pathways or enzymes to exert cardiovascular protection. For example, inhibition of matrix metalloproteinases by phenolic compounds may attenuate cardiovascular remodeling, a critical event involved in hypertension, atherosclerosis, cardiac hypertrophy, and heart failure. Recently, the dietary polyphenol curcumin is found to protect against cardiac hypertrophy and heart failure via its inhibition of p300 histone acetyltransferase activity in cardiac tissue [26, 27]. Histone acetylation plays a critical role in the pathophysiology of cardiac hypertrophy and heart failure.

3.1.2. Role in Diabetes and Metabolic Syndrome

3.1.2.1. DIABETES

The antidiabetic effects of phenolic compounds are well-recognized in animal models of both type 1 and type 2 diabetes. Phenolic compounds have been found to prevent the development of diabetes as well as retard the progression of diabetic complications. Multiple mechanisms have been proposed to explain the protective effects of phenolic compounds in diabetes and diabetic complications in addition to their antioxidative and anti-inflammatory activities. These additional mechanisms, as listed below, involve multiple targeting sites, including gut, pancreas, liver, and peripheral tissues (e.g., muscle, adipose) [28, 29].

- Inhibition of carbohydrate digestion and glucose absorption
- Protection against pancreatic β-cell dysfunction, and stimulation of insulin secretion from pancreatic β-cells
- Suppression of glucose release from liver storage
- Improvement of glucose uptake in the insulin-sensitive tissues, such as muscle and adipose

A variety of phenolic compounds are able to inhibit the activity of α-amylase and α-glucosidase, enzymes involved in the digestion of dietary carbohydrates to glucose in the gut. Intestinal absorption of glucose is mediated by active transport via the sodium-dependent glucose transporter SGLT1 and by facilitated sodium-independent transport via the glucose transporter GLUT2. Numerous phenolic compounds have been demonstrated to inhibit these glucose transporting systems, thereby diminishing the absorption of glucose in the gut. Both inhibition of dietary carbohydrate digestion and suppression of glucose absorption in the gut may greatly contribute to the amelioration of postprandial hyperglycemia in diabetes.

Pancreatic β-cells are an important targeting site for the antidiabetic activities of many phenolic compounds, especially the soy-derived isoflavones. Phenolic compounds have been found to protect pancreatic β-cells from glucotoxicity as well as oxidative and inflammatory stress. They also stimulate insulin release from pancreatic β-cells possibly via modulation of Ca^{2+} signaling and AMP-activated protein kinase pathway.

Liver plays a major role in the regulation of blood glucose levels in tight cooperation with peripheral tissues. A large number of dietary phenolic compounds, including tea catechins and soy isoflavones have been demonstrated to reduce hepatic glucose output by sup-

pressing gluconeogenetic enzyme expression and increasing the activity of glucokinase to improve glycogenesis and glucose utilization. Activation of AMP-activated protein kinase pathway has been implicated in the above effects of phenolic compounds on hepatic glucose regulation [30].

There is also evidence suggesting that the antidiabetic activities of phenolic compounds may result from their stimulation of glucose uptake in insulin-sensitive peripheral tissues, such as muscle and adipose tissue. Phenolic compounds have been found to both increase the sensitivity of insulin receptor and augment the activity of glucose transporters in cultured skeletal cells and adipocytes.

3.1.2.2. Metabolic Syndrome

Substantial evidence supports a beneficial role for dietary phenolic compounds in metabolic syndrome in animal models. In particular, various phenolic compounds, including tea catechins, soy isoflavones, and resveratrol have been demonstrated to exhibit antiobesity effects in animal models of high fat diet-induced obesity [31-33]. The benefits of phenolic compounds in obesity and other manifestations of metabolic syndrome may involve multiple mechanisms related to lipid metabolism as well as the direct effects on adipocytes. In addition, the effects of phenolic compounds on carbohydrate metabolism as described above (Section 3.1.2.1) may also contribute to their antiobesity activity. Obviously, the antioxidative and anti-inflammatory effects of phenolic compounds are beneficial in metabolic syndrome as oxidative stress and inflammation are key events involved in obesity, insulin resistance, and other abnormalities associated with metabolic syndrome.

Several signaling pathways have been suggested to be the molecular targets of dietary phenolic compounds in metabolic syndrome. These include AMP-activated protein kinase and Sirt1 signaling. Activation of AMP-activated protein kinase has been demonstrated as an important protective mechanism in diabetes and metabolic syndrome [30]. Recently, activation of Sirt1 signaling by phenolic compounds, especially resveratrol has been found to improve the efficiency of energy metabolism and insulin resistance, as well as suppress obesity and prevent early mortality associated with obesity in animal models [34]. There is also evidence suggesting a close interaction between AMP-activated protein kinase and Sirt1signaling in ameliorating obesity and other metabolic abnormalities caused by high-fat diet in experimental animals [35].

3.1.3. Role in Neurological Diseases

Extensive studies in animal models have demonstrated a protective role for a variety of different dietary phenolic compounds in many neurological disorders, including stroke, Parkinson's disease, and Alzheimer's disease. [36-40]. The neuroprotection has been observed with both pure phenolic compounds and fruit or vegetable extracts rich in polyphenols. Furthermore, dietary supplementation of phenolic compounds attenuates aging-related motor and cognitive deficits in experimental animals.

The potential mechanisms underlying the neuroprotective effects of phenolic compounds may include their antioxidative and anti-inflammatory activities. An important aspect related to the neuroprotection of phenolic compounds is their ability to penetrate the blood-brain barrier and reach the brain tissue. In addition, certain phenolic compounds have been found to induce antioxidant enzymes in brain tissue [41]. As redox-active metal ions (e.g., iron, copper) are particularly involved in neurodegenerative disorders, such as Parkinson's disease,

chelation of these redox-active metal ions by phenolic compounds may also contribute to their neuroprotection [42].

Sirt1 signaling has been demonstrated to promote survival and stress tolerance in neurons of the central nervous system. Recently, a role for the activation of Sirt1 by dietary phenolic compounds in neuroprotection has been suggested in the literature [43]. For example, resveratrol pretreatment is found to increase the activity of Sirt1 in rat brain tissue, and protect against cerebral ischemic damage. The protective effect of resveratrol on cerebral ischemic injury is abolished by the Sirt1-specific inhibitor, sirtinol, suggesting that the neuroprotection occurs via activation of Sirt1 signaling pathway. Moreover, activation of Sirt1 by resveratrol leads to inhibition of mitochondrial uncoupling protein 2, which may also contribute to resveratrol-mediated neuroprotection [44]. Inhibition of uncoupling protein 2 improves mitochondrial ATP production.

3.1.4. Role in Pulmonary Diseases

A number of dietary phenolic compounds, including resveratrol, curcumin, and tea catechins have been shown to be protective against various pulmonary diseases in animal models, such as asthma, chronic obstructive pulmonary disease (COPD), and drug-induced pulmonary fibrosis.

Ovalbumin-induced murine allergic airway inflammation is a commonly used experimental model of asthma. In this murine model, a variety of phenolic compounds are reported to attenuate airway inflammation and oxidative stress. There is also evidence that phenolic compounds, such as quercetin protect experimental animals from ovalbumin-induced airway inflammation via regulating Th1/Th2 balance [45].

Treatment with phenolic compounds ameliorates cigarette smoke-induced COPD-like airway inflammation, oxidative stress, and lung tissue injury in experimental animals. Cigarette smoke is a major cause of COPD in humans. As asthma and COPD are characterized by inflammation and oxidative stress, the protective effects of phenolic compounds in these pulmonary diseases are thus most likely attributed to their antioxidative and anti-inflammatory functions. Phenolic compounds, such as resveratrol, curcumin, and tea polyphenols induce antioxidant enzymes in lung tissue, which may also contribute to the pulmonary protection [46, 47]. More recently, Sirt1 signaling pathway has been implicated as a protective mechanism in cigarette smoke-induced lung injury [48]. Thus, activation of Sirt1 by phenolic compounds, such as resveratrol may represent a novel mechanism for protection against the pathogenesis of COPD.

In addition to asthma and COPD, viral infection-elicited lung inflammation is attenuated by dietary administration of curcumin in mice. Curcumin, resveratrol, and epigallocatechin-3-gallate also protect experimental animals from bleomycin-induced pulmonary fibrosis, inflammation, and oxidative stress. It is further found that the beneficial effects of epigallocatechin-3-gallate in bleomycin-induced pulmonary injury are mediated, at least partially, by augmentation of antioxidant enzymes via Nrf2 signaling [47].

3.1.5. Role in Hepatic and Gastrointestinal Diseases

3.1.5.1. Hepatic Diseases

Many phenolic compounds are protective in diverse hepatic diseases, including ischemia-reperfusion injury, nonalcoholic fatty liver disease, alcoholic fatty liver disease, endotoxin-

elicited liver injury, drug/xenobiotic-induced hepatotoxicity, and viral hepatitis. While phenolic compound-mediated hepatoprotective effects are well-documented, the exact underlying mechanisms are often difficult to be delineated. This is due to the fact that phenolic compounds possess multiple biological activities, and often time these various activities all contribute to hepatoprotection. Nevertheless, the hepatoprotective effects of phenolic compounds are usually associated with attenuation of inflammation and oxidative stress in hepatic tissue. This suggests that the antioxidative and anti-inflammatory activities of phenolic compounds may primarily contribute to hepatoprotection. In addition, other novel pathways may also be involved. For example, the protective effects of resveratrol on both alcoholic and nonalcoholic fatty liver diseases in mice are shown to be mediated partially by activation of Sirt1 and AMP-activated protein kinase signaling pathways [49, 50].

3.1.5.2. Gastrointestinal Diseases

Although blood or tissue levels of phenolic compounds are usually in the range of low or submicromolar concentrations following dietary supplementation, the concentrations of these compounds in the gastrointestinal tract have been found to be much higher [3]. Thus, phenolic compounds may exert significant beneficial effects in gastrointestinal disorders that involve inflammatory and oxidative stress [51, 52]. Indeed, dietary supplementation of pure phenolic compounds, including resveratrol, curcumin, tea catechins, and soy isoflavones has been found to protect against inflammatory bowel disease, *Helicobacter pylori* infection, and intestinal ischemia-reperfusion injury in experimental animals. Protection against intestinal inflammatory and oxidative disorders has also been observed with dietary supplementation of plant extracts rich in polyphenols.

3.1.6. Role in Renal Diseases

Oxidative stress and inflammation play an important role in various kidney diseases, such as ischemia-reperfusion injury, diabetic nephropathy, and drug/xenobiotic-induced nephrotoxicity. A variety of phenolic compounds, including tea polyphenols, soy isoflavones, resveratrol, and curcumin have been shown to exert beneficial effects on the above pathophysiological processes. Dietary supplementation of plant or fruit extracts rich in polyphenols also results in protection against renal diseases, including diabetic nephropathy and drug-induced nephrotoxicity. In addition to the antioxidative and anti-inflammatory activities, other novel actions of phenolic compounds may participate in the renal protection. For instance, resveratrol and flavonoids (e.g., quercetin, daidzein) are shown to promote mitochondrial biogenesis in renal tubular cells via Sirt1-dependent signaling [53]. The improved energy metabolism via activating Sirt1 may contribute to phenolic compound-mediated renal protection against ischemia-reperfusion injury. More recently, Sirt1 activation is found to protect mouse renal medulla from oxidative injury [54]. How exactly Sirt1 activation leads to protection against oxidative stress remains to be elucidated. Possible mechanisms include upregulation of cellular antioxidants and improvement of mitochondrial function [50]. Targeting Sirt1 signaling may thus represent a novel approach to protecting against renal diseases involving oxidative stress and dysregulated energy metabolism.

3.1.7. Role in Skin Diseases

A wealth of data from animal studies has revealed beneficial effects of various phenolic compounds (e.g., green tea polyphenols) in skin disorders, especially ultraviolet (UV) irradia-

tion-induced skin injury [55]. The photoprotective effects of dietary phenolic compounds can be achieved by either oral administration or topical application. Green tea polyphenols also provide protection against UV irradiation-induced oxidative DNA damage, immunosuppression, and skin carcinogenesis in animal models [56, 57]. Notably, green tea polyphenols promote rapid repair of UV-induced DNA damage likely through stimulation of a nucleotide excision repair mechanism [58]. In addition, chemically-induced skin inflammation is attenuated by oral administration of green tea polyphenols in mice [59]. Moreover, green tea polyphenols have been demonstrated to promote keratinocyte differentiation and skin wound healing [60], protect hair follicles from γ-irradiation-induced apoptosis in vivo [61], and reduce psoriasiform lesions in the flaky skin mouse model [62]. The flaky skin mouse is a commonly used animal model of human psoriasis.

3.1.8. Role in Cancer

In addition to cardiovascular diseases, cancer is another most extensively studied disease with regard to phenolic compound-mediated protection in experimental animals [63-65]. Almost all of the classes of phenols listed in Section 1.2 are demonstrated to exhibit anticancer activities. Notable examples include resveratrol, curcumin, tea catechins, and soy flavonoids. The anticancer activities of phenolic compounds have been shown in animal models of both spontaneous cancer development and chemical carcinogenesis. It is known that cancer development involves multiple etiological factors as well as the complex interactions between genetic abnormalities and dysregulated cell signaling. As such, protective effects of phenolic compounds in cancer are unlikely due to their action on any specific pathway of cancer development. As described earlier in Section 2, phenolic compounds possess multiple biological activities, which may act together to contribute to the protection against cancer development. Largely based on studies in animal models and cell cultures, several mechanisms have been proposed to explain the beneficial effects of dietary phenolic compounds in cancer. These are listed below.

- Antioxidative effects
- Anti-inflammatory activities
- Antiproliferation
- Induction of apoptosis
- Antiangiogenesis
- Modulation of xenobiotic biotransformation enzymes

Dysregulated inflammation and oxidative stress are critical events in cancer development. Thus, suppression of oxidative stress and inflammation by phenolic compounds may contribute to cancer chemoprotection. Phenolic compounds, such as resveratrol, curcumin, and tea polyphenols are able to block cell cycle and induce apoptosis in cancer cells via modulating cancer cell signaling pathways. The pro-oxidant activities of phenolic compounds, such as epigallocatechin-3-gallate may also be involved in induction of apoptosis in cancer cells [9]. These effects along with antiangiogenesis may be primarily responsible for the suppression of cancer growth and metastasis. In this context, phenolic compounds, such as curcumin and tea polyphenols have been shown to potently block cancer invasion and metastasis in animal models [66, 67].

Phenolic compounds have long been known for their ability to protect against chemical carcinogenesis. Among the various mechanisms involved, induction of phase 2 enzymes has been demonstrated to play an important part in the detoxification of electrophilic carcinogens, thereby inhibiting the formation of carcinogen-DNA adducts, a critical event leading to initiation of the multistage carcinogenesis. In addition, some phenolic compounds, such as resveratrol can also inhibit P450 enzymes, resulting in decreased bioactivation of carcinogens to form DNA-damaging species [68]. Hence, modulation of carcinogen-metabolizing enzymes by phenolic compounds may be a particularly relevant mechanism for protection against chemical carcinogenesis.

3.1.9. Role in Other Diseases and Conditions

Substantial evidence suggests that dietary polyphenolic compounds also exert beneficial effects in various other pathophysiological conditions involving oxidative stress and inflammation in animal models. These include sepsis [69], arthritis [70, 71], HIV infection [72], and aging [73].

3.2. Human Studies and Clinical Perspectives

It has long been recognized that diet plays an important part in the maintenance of optimal health in humans. In this context, consumption of diet rich in fruits and vegetables has been associated with decreased risk of developing prevalent diseases, including cardiovascular disorders and cancer. As discussed in previous chapters, various bioactive components from diet rich in fruits and vegetables, including vitamin C, vitamin E, and carotenoids have been suggested to play a beneficial role in human diseases. Similarly, the health benefits of dietary phenolic compounds have also been extensively assessed over the last two decades in human subjects through both observational epidemiological studies and interventional clinical trials. Although cardiovascular diseases and cancer have been the focus of human studies, the potential beneficial effects of dietary phenolic compounds in other human diseases have also recently received attention. The sections below summarize the major findings from these human studies.

3.2.1. Role in Human Cardiovascular Diseases

A potential beneficial effect of dietary phenolic compounds in cardiovascular diseases was first suggested by an observational epidemiological study, the Zutphen Elderly Study, showing that flavonoid intake in regularly consumed foods was inversely associated with mortality from coronary heart disease in elderly men [74]. Since the Zutphen Elderly Study, there have been numerous observational epidemiological studies assessing the relationship between dietary intake of various classes of phenolic compounds and risk of developing cardiovascular diseases. Despite of null and inconsistent findings from some studies, the large majority of observational studies overwhelmingly suggest an inverse association between dietary intake of phenolic compounds and risk of developing different forms of cardiovascular diseases. In line with these findings, a number of small-scale interventional trials also suggest a potential benefit of supplementation of dietary phenolic compounds, such as cocoa polyphenols in the intervention of cardiovascular pathophysiology. The beneficial actions of polyphenols include: (1) improvement of hyperlipidemia and hypertension [75-77], (2) aug-

mentation of vascular nitric oxide formation and amelioration of endothelial dysfunction [21, 78], and (3) inhibition of platelet activation and aggregation [79]. These observations in human studies are also consistent with the findings from animal experiments, and point to a potential value of using dietary phenolic compounds in the intervention of human cardiovascular diseases.

3.2.2. Role in Human Cancer

The discovery that the wine polyphenol resveratrol protected against carcinogenesis in experimental animals in 1997 [80] sparked the recent surge of interest in the potential benefits of phenolic compounds in human cancer. The well-documented cancer chemoprotective effects of a variety of dietary phenolic compounds in animal models of human cancer have also prompted the studies on these compounds in protecting against cancer in human subjects. An increasing number of observational epidemiological studies have demonstrated an inverse association between dietary consumption of phenolic compounds, such as green tea polyphenols and soy flavonoids, and risk of cancer development.

In line with the findings in observational epidemiological studies, multiple small-scale interventional trials show a possible protective role for supplementation of dietary phenolic compounds in certain types of human cancer, such as prostate cancer. For example, in a recent randomized controlled trial, supplementation of tea catechins (600 mg per day) for one year was found to prevent the development of invasive prostate cancer in men with high-grade intra-epithelial neoplasia, suggesting that tea catechins may be effective for treating premalignant lesions of prostate [81]. A more recent interventional trial in prostate cancer patients reported that brief treatment with tea polyphenols (mainly epigallocatechin-3-gallate) resulted in a significant reduction of serum markers, including prostate-specific antigen, hepatocyte growth factor, and endothelial growth factor [82]. In addition to prostate cancer intervention, supplementation of green tea extract in a randomized controlled trial was found to improve clinical response rate in patients with high-risk oral premalignant lesions in a dose-dependent manner, indicating a potential value for using green tea polyphenols in oral cancer prevention [83]. In another pilot trial, supplementation of green tea extract in patients who had colorectal adenomas removed by polypectomy was shown to reduce the development of metachronous colorectal adenoma compared with a group of patients who did not take the green tea extract [84].

It should be noted that there are also many small-scale clinical trials that have failed to show an effectiveness of supplementation of phenolic compounds, including green tea polyphenols in human cancer intervention. Thus, at the present time the exact value of phenolic compounds in human cancer intervention remains inconclusive. Hence, well-designed large-scale interventional trials are needed to firmly establish the potential efficacy of various classes of dietary phenolic compounds in the intervention (both preventive and therapeutic) of common types of human cancer.

3.2.3. Role in Other Human Diseases and Conditions

Over the last two decades, the benefits of phenolic compounds in other human disorders have also been investigated through both observational epidemiological studies and small-scale interventional trials. Although many such studies have produced inconsistent results, some do point to a potential value of supplementation of dietary phenolic compounds in the intervention of certain disease conditions. These include metabolic syndrome [32, 33, 85, 86],

kidney transplantation [87, 88], bone loss in menopausal women [89-91], and UV irradiation-induced skin damage [92, 93].

4. Conclusion and Future Directions

Phenolic compounds represent an important component of human diet. In addition to having antioxidative and anti-inflammatory activities, these compounds also possess many other novel functions, such as modulation of cell signaling. The diseases-protective effects of dietary phenolic compounds have been well-recognized in various animal models of human diseases associated with oxidative stress and inflammation. Observational epidemiological studies and interventional clinical trials in selected patient groups also suggest potential health benefits of supplementation of dietary phenolic compounds in certain human diseases. However, the exact value of dietary phenolic compounds in human disease intervention remains to be established via large-scale well-designed interventional trials. Future studies should also emphasize the dose-response relationship of dietary phenolic compounds in disease intervention. As multiple classes of phenolic compounds as well as other antioxidant compounds coexist in regular human diet rich in fruits and vegetables, both basic and clinical studies should be directed to determine the interactions between the different groups of dietary antioxidant compounds with respect to their combined biological activities and role in disease intervention. Although dietary phenolic compounds have been shown to be well-tolerated in human subjects, potential adverse effects of these compounds, especially upon long-term supplementation at high doses should not be ignored. In this context, there are an increasing number of case reports of liver injury in human subjects associated with intake of green tea dietary supplements.

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SECTION IV
SYNTHETIC ANTIOXIDANTS

A number of synthetic antioxidant compounds have been developed over the last several decades. Many of them are mimetics of endogenous antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase. Some of these antioxidant enzyme mimetics possess certain specificities in terms of scavenging reactive oxygen and nitrogen species (ROS/RNS). The synthetic antioxidants also include glutathione precursors (e.g., *N*-acetylcysteine), spin traps (e.g., α-phenyl-*tert*-butylnitrone), and nanomaterials (e.g., cerium oxide nanoparticles). Due to the non-protein nature, synthetic antioxidant compounds are relatively stable and usually able to penetrate the cells, and as such some of them can be administered orally. Synthetic antioxidants are widely used in both cell cultures and experimental animals to study the protective effects on ROS/RNS-mediated damage or to help identify the ROS/RNS involved in particular pathophysiological processes. In addition, certain synthetic antioxidant compounds have been studied in clinical trials for the intervention of diseases involving an oxidative stress mechanism. The subsequent Chapters 24-27 describe the chemical and biochemical properties of the above four classes of synthetic antioxidants with an emphasis on their protective role in disease pathophysiology.

Chapter 24

Antioxidant Enzyme Mimetics

Abstract

Antioxidant enzyme mimetics are synthetic small molecular mass compounds that catalytically metabolize reactive oxygen and nitrogen species, including superoxide, hydrogen peroxide, and peroxynitrite. The commonly studied antioxidant enzyme mimetics in biology and medicine include mimetics of superoxide dismutase, catalase, and glutathione peroxidase, as well as the catalytic scavengers of peroxynitrite. The protective effects of the various classes of antioxidant enzyme mimetics in disease pathophysiology have been demonstrated in a variety of animal models of human diseases, including cardiovascular disease, diabetes and metabolic syndrome, neurological disorders, and cancer. In contrast to the extensive experimental animal studies, there have been very limited clinical investigations on the safety and efficacy of antioxidant enzyme mimetics in human subjects.

1. Overview

The recognition of the critical involvement of reactive oxygen and nitrogen species (ROS/RNS) in disease pathophysiology has sparked the surge of interest in the potential use of antioxidant enzymes for disease intervention. Due to the protein nature and the associated instability, limited bioavailability, and antigenicity, systematic administration of native antioxidant enzymes for disease intervention has been much restricted. To circumvent these limitations, a number of synthetic antioxidant compounds that mimic the catalytic activities of endogenous antioxidant enzymes have been developed over the last two decades. These include mimetics of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). Some of the mimetics possess certain specificities in terms of catalytically scavenging or decomposing ROS/RNS. Compared with the native enzymes, antioxidant enzyme mimetics are relatively stable, able to penetrate the cells, and can be administered orally. These compounds are widely used as protective agents against oxidative stress in vitro and in vivo. They are also commonly employed as tools to study the involvement of ROS/RNS in disease pathophysiology. This chapter focuses on discussion of the commonly used mimetics of SOD, catalase, and GPx. The peroxynitrite scavengers are also covered in this chapter.

2. Chemistry and Biochemistry

2.1. Superoxide Dismutase Mimetics

Superoxide is crucially involved in various disease processes, and SOD enzymes play an important role in protecting against superoxide-mediated cell and tissue injury (Chapter 3). However, as stated above, use of the native SODs for suppressing oxidative tissue injury has many drawbacks, including limited bioavailability, antigenicity, and high expense. As such, several low molecular mass SOD mimetics have been developed to overcome the above limitations. Commonly used SOD mimetics are compounds that contain manganese (Mn) as the redox-active center, and include the following [1, 2].

- Mn(III) metalloporphyrins
- Mn(III) salen complexes
- Mn(II) pentaazamacrocyclic ligand-based complexes

In addition to these Mn-containing mimetics, several other structurally distinct chemicals have also been found to possess SOD-like activity. These include the nitroxide compound 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (Tempol) and fullerene derivatives [3, 4]. Cerium oxide nanoparticles may also have SOD-like activity. Fullerenes and cerium oxide nanoparticles are covered in Chapter 27.

2.1.1. Mn(III) Metalloporphyrins

The Mn-based metalloporphyrin complexes scavenge superoxide, hydrogen peroxide, peroxynitrite, and lipid peroxyl radicals. Although being referred to as SOD mimetics in the literature, these compounds apparently do not selectively scavenge superoxide. The commonly investigated Mn(III) metalloporphyrins include Mn(III) tetrakis (4-benzoic acid) porphyrin (abbreviation: MnTBAP), Mn(III) tetrakis (1-methyl-4-pyridyl) porphyrin (abbreviation: MnTMPyP), and the AEOL series, such as AEOL10113. The chemical name for AEOL10113 is Mn(III) tetrakis (*N*-ethylpyridinium-2-yl) porphyrin (abbreviation: MnTE-2-PyP^{5+}). Due to its positive charge, MnTE-2-PyP^{5+} can accumulate in mitochondria [5]. The chemical structures for MnTMPyP, MnTBAP, and MnTE-2-PyP^{5+} are shown in Figure 24-1.

2.1.2. Mn(III) Salen Complexes

The Mn(III) salen complexes, also known as EUK series, scavenge superoxide and hydrogen peroxide, and thus have SOD and catalase activities. The members of this class include EUK-8, EUK-134, EUK-189, EUK-207, EUK-418, and EUK-423 [1].

2.1.3. Mn(II) Pentaazamacrocyclic Ligand-based Complexes

Mn(II) pentaazamacrocyclic ligand-based SOD mimetics, such as M40401 and M40403 are selective for superoxide, which is different from the other two classes of SOD mimetics described above. This selectivity for superoxide is believed to be due to the fact that the Mn atom at the center of the pentaazamacrocyclic ligand-based compounds is held by five coordination points and is only available for one-electron reduction [6]. In this regard, scavenging of hydrogen peroxide and peroxynitrite requires transfer of two electrons.

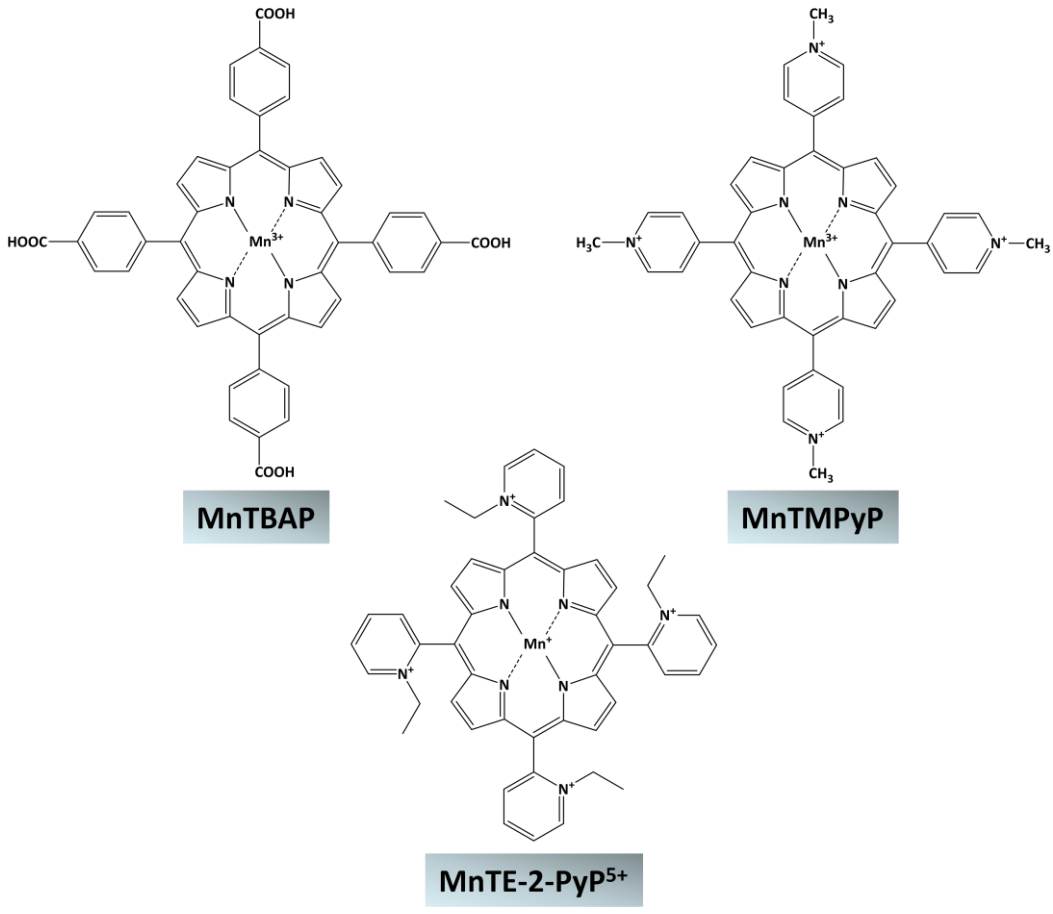

Figure 24-1. Structures of some commonly used superoxide dismutase (SOD) mimetics. See text (Section 2.1) for detailed description of various types of SOD mimetics. MnTBAP, Mn(III) tetrakis (4-benzoic acid) porphyrin; MnTMPyP, Mn(III) tetrakis (1-methyl-4-pyridyl) porphyrin; MnTE-2-PyP^{5+}, Mn(III) tetrakis (*N*-ethylpyridinium-2-yl) porphyrin. (courtesy of Jason Z. Li)

2.2. Catalase Mimetics

Catalase is a heme-containing protein that catalyzes the dismutation of hydrogen peroxide to form water and molecular oxygen. Catalase mimetics are compounds with redox-active metal centers that often contain either manganese (Mn) or iron (Fe). As discussed above, the SOD mimetics Mn(III) metalloporphyrins and Mn(III) salen complexes also have catalase activity [7]. As such, these compounds are frequently designated as SOD/catalase mimetics.

2.3. GPx Mimetics

Another class of endogenous catalytic hydrogen peroxide scavengers is the selenium-containing GPx (Chapter 6). One of the best studied GPx mimetics is the selenoorganic com-

pound 2-phenyl-1,2-benzisoselenazol-3(2H)-one, also known as ebselen (structure shown in Figure 24-2). Ebselen is one of the first selenium-based GPx mimetics developed. It catalytically scavenges hydrogen peroxide and other organic hydroperoxides in the presence of reducing cofactors, including glutathione, *N*-acetylcysteine, and dihydrolipoic acid. Ebselen also scavenges peroxynitrite and possibly other ROS/RNS. It is shown to be a substrate for thioredoxin reductase [8]. In addition to scavenging ROS/RNS, ebselen may also inhibit ROS-producing enzymes, including 5-lipoxygenase and NAD(P)H oxidase, and possess anti-inflammatory activity [7].

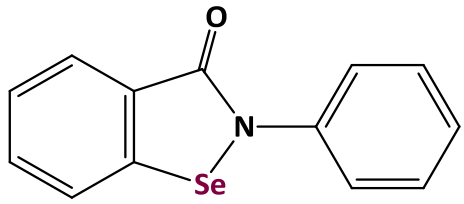

Figure 24-2. Structure of the glutathione peroxidase (GPx) mimetic ebselen. See text (Section 2.3) for detailed description of ebselen. The chemical name of ebselen is 2-phenyl-1,2-benzisoselenazol-3(2H)-one.

A number of diselenide and ditelluride containing compounds have been reported to catalytically scavenge peroxides with higher GPx-like activity than ebselen. For example, the diselenide compound 2,2'-diseleno-bis-beta-cyclodextrin (2-SeCD) can efficiently scavenge a variety of peroxides, including hydrogen peroxide using glutathione as a cofactor [7].

2.4. Peroxynitrite Scavengers

As noted above, peroxynitrite can be scavenged by manganese-based metalloporphyrin compounds with SOD and catalase activities as well as by GPx mimetics (e.g., ebselen). In contrast to manganese-based metalloporpyrins (e.g., MnTBAP, MnTMPyP), iron (Fe)-based metalloporphyrins display much higher rate of peroxynitrite decomposition and are relatively selective for peroxynitrite. Examples of commonly used iron-based peroxynitrite scavengers include 5,10,15,20-tetrakis (2,4,6-trimethyl-3,3-disulfonatophenyl) porphyrinato iron (III) (abbreviation: FeTMPS), 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrinato iron (III) (abbreviation: FeTPPS), and 5,10,15,20-tetrakis (*N*-methyl-4′-pyridyl) porphyrinato iron (III) (abbreviation: FeTMPyP) [9].

3. Role in Disease Intervention

3.1. Animal Studies

The critical involvement of superoxide and other ROS/RNS in disease pathophysiology has prompted the use of catalytic antioxidant enzyme mimetics for disease intervention in

experimental animals. As described above, many of the catalytic antioxidant enzyme mimetics lack selectivity for ROS/RNS. In addition, the catalytic scavenging ability of these compounds toward ROS/RNS as initially demonstrated in cell-free systems may not be the same in more complex biological systems. Moreover, these mimetics have been found to exert other biological activities, such as inhibition or induction of enzymes involved in ROS and xenobiotic metabolism [10, 11]. As such, interpretation of the protective effects of these antioxidant enzyme mimetics in disease pathophysiology should be done with caution. In this context, simultaneous assessment of the effects of the mimetics on ROS/RNS levels and oxidative stress markers in disease processes will help identify the mechanisms of action for these compounds in vivo.

Substantial evidence from studies in animal models shows beneficial effects of various antioxidant enzyme mimetics in disease conditions involving an oxidative stress mechanism. The sections below summarize the major experimental findings in this research area.

3.1.1. Role in Cardiovascular Diseases

A number of antioxidant enzyme mimetics are protective against hypertension, endothelial dysfunction, atherogenesis, myocardial ischemia-reperfusion injury, and heart failure. Notably, treatment with superoxide-selective Mn(II) pentaazamacrocyclic ligand-based SOD mimetics (e.g., M40401, M40403) is shown to improve endothelial function in atherosclerotic mice [12], ameliorate myocardial ischemia-reperfusion injury [13], and attenuate the development of heart failure [14]. Recently, the GPx mimetic ebselen is found to reduce atherosclerotic lesions in diabetic ApoE-deficient mice [15]. The above protective effects are correlated with decreased oxidative stress and inflammation in the target tissues, which support a critical role for superoxide as well as other ROS/RNS in the pathophysiology of cardiovascular diseases. These observations are also in line with the findings that genetic manipulations of endogenous antioxidant enzymes (e.g., SOD, catalase, GPx) significantly modulate the pathophysiology of the above cardiovascular diseases in animal models. Together, these studies support the notion that protection against cardiovascular diseases can be achieved by using catalytic antioxidant enzyme mimetics.

3.1.2. Role in Diabetes and Metabolic Syndrome

As discussed in previous chapters, genetic overexpression of endogenous antioxidant enzymes has been shown to protect against diabetes and diabetic complications in experimental animals. There is also substantial evidence for the beneficial effects of antioxidant mimetics, including Tempol, MnTMPyP, the superoxide-selective mimetics (e.g., M40401, M40403), and ebselen in diabetes and diabetic complications [16, 17]. Consistent with these in vivo findings, treatment with antioxidant mimetics protects the pancreatic β-cells from oxidative stress in vitro.

Oxidative stress and inflammation have been causally linked not only to diabetes, but also to obesity and obesity-associated complications. Treatment with antioxidant enzyme mimetics, such as Tempol reduces oxidative stress and improves insulin resistance in obese Zucker rats, indicating a potential value of these mimetics in the management of metabolic syndrome [18]. Obesity-associated vascular and renal dysfunction are also ameliorated by treatment with Tempol and other antioxidant enzyme mimetics [19]. Notably, supplementation of Tempol in the food of mice has been shown to cause significant weight loss and pre-

vent obesity without toxicity [20]. More recently, Tempol is demonstrated to inhibit adipogenesis in vitro, which may underlie, at least in part, some of its in vivo effects on body weight and obesity [21]. These observations are consistent with the notion that oxidative stress is an important pathophysiological component of adiposity.

3.1.3. Role in Neurological Diseases

The increasing recognition of the critical role of oxidative stress and inflammation in various neurological disorders has prompted studies on the neuroprotective effects of catalytic antioxidant enzyme mimetics in experimental animals. Administration of antioxidant enzyme mimetics, including mimetics of SOD, catalase, and GPx, as well as peroxynitrite scavengers and fullerene derivatives has been demonstrated to protect against many neurological disorders [22]. These include cerebral ischemic injury, Parkinson's diseases, Alzheimer's disease, and neuroexcitotoxicity. Notably, treatment with antioxidant enzyme mimetics has also been found to prevent or reverse aging-related learning deficits and brain oxidative stress, and extent the lifespan in experimental animals [23-25]. These observations from a different angle support the oxidative stress mechanism of neurodegeneration and aging, and point to the feasibility of using antioxidant enzyme mimetics in the intervention of neurological disorders that involve an oxidative stress component.

3.1.4. Role in Pulmonary Diseases

Lung is exposed to higher oxygen tension than most other organs under both physiological and pathophysiological conditions. Indeed, oxidative stress has long been recognized as a causative factor in various pulmonary diseases. The protective effects of antioxidant enzyme mimetics have been demonstrated in many animal models of human lung diseases, including hyperoxic lung injury, pulmonary ischemia-reperfusion injury, asthma, chronic obstructive lung disease, acute respiratory distress syndrome, as well as radiation- and drug-induced lung injury [6, 26]. A notable benefit of these mimetics in the above lung pathophysiological processes is marked attenuation of inflammatory responses and oxidative stress. In addition, there is also evidence supporting the ability of antioxidant enzyme mimetics to inhibit lung fibrosis in experimental animals [27].

3.1.5. Role in Hepatic and Gastrointestinal Diseases

Antioxidant enzyme mimetics are protective against many liver disorders in animal models. For example, treatment with metalloporphyrin SOD mimetics (e.g., MnTMPyP, MnTBAP) or the diselenide GPx mimetic 2-SeCD attenuates hepatic ischemia-reperfusion injury, as evidenced by decreased release of liver enzymes and reduced inflammation and oxidative stress in liver tissue [28, 29]. Administration of MnTBAP also prevents Fas-induced acute liver failure and oxidative stress in mice [30]. Both alcoholic and nonalcoholic fatty liver diseases are ameliorated by treatment with antioxidant enzyme mimetics, such as Tempol and ebselen [31, 32]. Some antioxidant enzyme mimetics, including MnTBAP, ebselen, EUK-8, and EUK-134 have been shown to also protect against endotoxin-elicited liver injury and drug/xenobiotic-induced hepatotoxicity [33-35]. In addition to liver diseases, treatment with Tempol or M40403 suppresses chemically-induced colitis in animal models [36, 37]. Tempol also exerts a beneficial effect on intestinal ischemia-reperfusion injury via inhibiting superoxide formation and inflammatory responses in the intestine [38]. Together,

these findings point to the potential value of catalytic antioxidant enzyme mimetics in the intervention of liver and gastrointestinal diseases associated with oxidative stress and dysregulated inflammation.

3.1.6. Role in Renal Diseases

The protective effects of antioxidant enzyme mimetics in kidney diseases have been investigated in experimental animals over the last decade. The renal disease conditions in which antioxidant enzyme mimetics have a protective role include ischemia-reperfusion injury [39], diabetic nephropathy [40], and drug/xenobiotic-induced nephrotoxicity [41, 42]. Treatment with antioxidant enzyme mimetics, such as Tempol also attenuates kidney inflammatory responses to acute sodium overload and protects against autoimmunity-induced glomerular injury [43, 44]. More recently, the SOD/catalase-mimetic MnTBAP has been shown to prevent the development of insulin resistance and glucose intolerance due to uremia in a mouse model of surgically induced chronic renal failure [45]. Treatment with MnTBAP also blocks the development of insulin resistance in normal mice after urea infusion [45]. These studies suggest broad applications of antioxidant enzyme mimetics in the management of diverse pathophysiological conditions involving kidney.

3.1.7. Role in Cancer

An early study by T.W. Kensler and coworkers in 1983 showed an inhibitory effect on tumor promotion by a copper-coordination complex with SOD mimetic activity [46]. This study provided the first evidence that a low molecular mass antioxidant enzyme mimetic may have an impact on multistage carcinogenesis in experimental animals. Subsequent studies have demonstrated that several other antioxidant enzyme mimetics, including MnTBAP, MnTE-2-PyP^{5+}, and ebselen are also able to suppress chemical carcinogenesis in animal models [47-49].

Antioxidant enzyme mimetics are also protective against carcinogenesis in animal models unrelated to chemical carcinogens. For example, treatment with antioxidant enzyme mimetics Tempol and EUK-189 prolongs the latency of thymic lymphoma and the animal survival, and reduces oxidative stress in Atm-deficient mice. [50-52]. The Atm-deficient mouse is a murine model of human ataxia telangiectasia, a hereditary disorder characterized by a high incidence of lymphoid malignancies, premature aging, and other abnormalities. Recently, treatment with MnTE-2-PyP^{5+} is shown to inhibit angiogenesis and tumor growth via suppressing oxidative stress in a mouse model of breast cancer [53]. These findings point to the possibility of utilizing antioxidant enzyme mimetics for the intervention of human cancer.

The potential effects of antioxidant enzyme mimetics on cancer therapy, including chemotherapy and radiotherapy have been investigated in experimental animals. In a mouse model of prostate cancer, co-treatment with MnTE-2-PyP^{5+} does not significantly affect radiation-induced inhibition of tumor growth, but protects against radiation-induced decreases in red blood cell counts, hemoglobin, and hematocrit [54]. This suggests a role for MnTE-2-PyP^{5+} in reducing adverse effects of radiation therapy. However, in a murine model of lymphosarcoma, pretreatment with Tempol significantly reduces cyclophosphamide-induced tumor-killing activity. It is further shown that pretreatment with Tempol upregulates aldehyde dehydrogenase in liver, an enzyme known to detoxify the active metabolites of cyclophosphamide [11]. Such active metabolites are believed to be responsible for the antitumor activity of cyclophosphamide. Hence, the concurrent use of antioxidant enzyme mimetics may

differentially affect the outcomes of cancer therapy depending on the types of the therapy as well as other experimental conditions.

3.1.8. Role in Other Diseases and Conditions

Catalytic antioxidant enzyme mimetics may also exert beneficial effects in other diseases and conditions in experimental animals. These include endotoxin-induced sepsis and multiple organ dysfunction [55], skin disorders (e.g., ultraviolet-induced injury) [56], arthritis [57], and total body irradiation-induced lethality [58].

3.2. Human Studies and Clinical Perspectives

In contrast to the substantial experimental evidence from animal studies, the clinical evidence for the effectiveness of antioxidant enzyme mimetics in disease intervention in human subjects is much limited. The GPx mimetic ebselen (Section 2.3 and Figure 24-2) is probably the only antioxidant enzyme mimetic whose potential therapeutic effects have been evaluated in multiple clinical studies. Several small-scale clinical trials in 1990s demonstrated a potential benefit of ebselen treatment in patients with acute ischemic stroke and patients with delayed neurological deficits after aneurysmal subarachnoid hemorrhage [59-61]. However, there are also trials reporting no beneficial effects of ebselen supplementation in patients with critical illness, including cerebrovascular events [62]. Thus, the neuroprotective effects of ebselen in humans remain to be established.

Topical application of antioxidant enzyme mimetics for disease intervention has been investigated in clinical trials. For example, a phase 1 trial reported that topical application of Tempol to the scalp before whole brain radiation was safe and might protect against radiation-induced alopecia in patients with metastatic cancer to the brain [63]. There is also evidence indicating that topical application of EUK-134 may suppress ultraviolet irradiation-induced oxidant formation on the skin surface in human subjects [64].

4. Conclusion and Future Directions

Antioxidant enzyme mimetics are able to catalytically metabolize ROS/RNS. While most of the available antioxidant enzyme mimetics lack selectivity for a particular ROS/RNS, some, such as Mn(II) pentaazamacrocyclic ligand-based SOD mimetics do show high specificity for superoxide. Antioxidant enzyme mimetics protect cells from oxidative stress. These compounds also exhibit beneficial effects in a variety of disease conditions involving oxidative stress and dysregulated inflammation in animal models. However, the potential usefulness of antioxidant enzyme mimetics in the intervention of human diseases currently remains largely unknown due to very limited clinical trials. Future studies should focus on the continued evaluation of both safety and efficacy of the existing antioxidant enzyme mimetics in preclinical models as well as developing more effective new compounds for preclinical assessment. These preclinical studies will lay a foundation for subsequent well-designed clinical trials to determine the potential effectiveness of antioxidant enzyme mimetics in human disease intervention, including both prevention and treatment.

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Chapter 25

Glutathione Precursors

Abstract

The reduced form of glutathione (GSH) is an important biomolecule in mammalian health and disease. Glutathione precursors include the compounds that directly give rise to GSH and those that provide substrates for de novo GSH biosynthesis. Thus, GSH precursors represent an important approach to increasing cellular and tissue GSH levels. GSH precursors may possess other novel biological activities in addition to augmenting cellular and tissue GSH levels. The protective effects of GSH precursors, especially *N*-acetylcysteine in disease pathophysiology have been well-recognized in a variety of animal models of human diseases. A number of clinical trials also suggest a potential beneficial role for *N*-acetylcysteine in the intervention of many human diseases, including cardiovascular disorders, diabetes, chronic obstructive pulmonary disease, and contrast medium-induced nephropathy.

1. Overview

The reduced form of glutathione (GSH) is a critical antioxidant in mammals, and increased GSH levels have been shown to provide protection against oxidative and electrophilic injury under various disease conditions (see Chapter 5 for discussion of GSH). However, direct administration of GSH to increase its tissue levels has proven inefficient due to the membrane impermeability and instability of GSH. As such, many membrane permeable and relatively stable compounds, known as GSH precursors or prodrugs have been developed, which can be administered to elevate GSH levels in vivo via various mechanisms. The term GSH precursors refers to synthetic compounds that can increase tissue GSH levels via either directly giving rise to GSH or providing substrates for de novo GSH biosynthesis. The GSH precursors acting via the former mechanism include GSH esters, whereas those acting through the latter mechanism consist of *N*-acetylcysteine and a number of other cysteine analogs. *N*-Acetylcysteine is an old drug approved by the United States Food and Drug Administration (FDA), which has been used as the specific antidote for acetaminophen overdose. It has also been extensively studied as a potential protective agent for the management of a variety of other pathophysiological conditions. This chapter discusses the basic chemical and biochemical properties of GSH precursors and their involvement in health and disease with an emphasis on *N*-acetylcysteine.

2. Chemistry and Biochemistry

2.1. Glutathione Esters

Since GSH is not significantly taken up by cells, an effective approach to increasing cellular GSH levels is to supply a membrane permeable GSH-containing compound that is well transported into cells and converted to GSH. In this regard, membrane permeable GSH esters have been developed to provide an effective way of delivering GSH into cells. There are two types of GSH esters: GSH monoethyl ester and GSH diethyl ester. When administered to experimental animals, GSH esters can be transported into many tissues and hydrolyzed by tissue esterases to release GSH, resulting in increased tissue levels of GSH [1].

2.2. *N*-Acetylcysteine

Another way of augmenting tissue GSH levels is to increase the availability of the rate-limiting substrate cysteine using the non-toxic precursor *N*-acetylcysteine (structure shown in Figure 25-1). Orally delivered *N*-acetylcysteine is readily absorbed and converted by esterases to cysteine in tissues (mainly liver), where it is used for synthesis of GSH. In addition to supplying cysteine for GSH synthesis, *N*-acetylcysteine also directly scavenges ROS/RNS and binds redox-active metal ions. *N*-Acetylcysteine has been shown to possess anti-inflammatory activities through mechanisms including inhibition of NF-κB activation and suppression of the expression of proinflammatory cytokines and adhesion molecules [2, 3]. Moreover, it has been reported that *N*-acetylcysteine inhibits platelet aggregation via increasing platelet nitric oxide [4]. These novel effects may also contribute to *N*-acetylcysteine-mediated protection against disease pathophysiology.

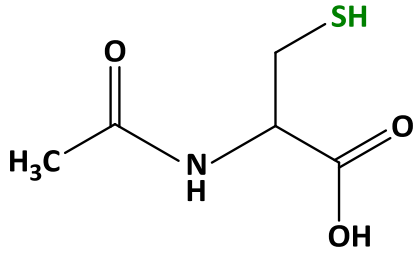

Figure 25-1. Structure of *N*-acetylcysteine. See text (Section 2.2) for description of *N*-acetylcysteine.

2.3. Other Glutathione Precursors

In addition to GSH esters and *N*-acetylcysteine, there are many other GSH precursors that can be utilized to increase tissue GSH levels. These include various analogs of cysteine, such as *S*-carboxymethylcysteine, *N*-isobutyrylcysteine, *N*-acystelyn, erdosteine, and fudosteine. In general, these cysteine-derived analogs have improved stability and bioavailability compared

with *N*-acetylcysteine. These compounds have also been investigated in animal studies and clinical trials for the intervention of certain diseases, such as chronic obstructive pulmonary disease [5].

3. Role in Disease Intervention

3.1. Animal Studies

3.1.1. Role in Cardiovascular Diseases

The protective effects of GSH precursors have been demonstrated in animal models of human cardiovascular diseases, including hypertension, atherosclerosis, myocardial ischemia-reperfusion injury, heart failure, and drug-induced cardiotoxicity. Notably, administration of either GSH monoethyl ester or *N*-acetylcysteine attenuates atherosclerotic plaque formation and reduces oxidative stress in vascular wall in experimental animals fed atherosclerotic diet [6, 7]. *N*-Acetylcysteine also prevents accelerated atherosclerosis, vascular inflammation, and oxidative stress in uremic ApoE-deficient mice [8]. These findings indicate GSH as an important protective molecule and point to the feasibility for using thiol compounds in the intervention of vascular degeneration.

3.1.2. Role in Diabetes and Metabolic Syndrome

GSH is a critical molecule in protecting the pancreatic β-cells from oxidative stress and inflammation, and in suppressing the development of diabetes in animal models (Chapter 5). There is substantial evidence supporting the beneficial effects of *N*-acetylcysteine and GSH esters in diabetic complications, including cardiomyopathy, neuropathy, vascular dysfunction, and cataract. It is of note that *N*-acetylcysteine protects against hyperglycemia-mediated myocardial hypertrophy likely through attenuation of PKC-β2 overexpression [9]. PKC-β2 overexpression is an important mechanism underlying diabetic cardiac hypertrophy. It has been reported that treatment with *N*-acetylcysteine restores myocardial manganese superoxide dismutase activity in diabetic animals, which may partially contribute to the improvement of diabetic cardiomyopathy [10].

N-Acetylcysteine is also beneficial in treating metabolic syndrome. For example, oral administration of *N*-acetylcysteine ameliorates arterial hypertension, insulin resistance, and oxidative stress in chronically glucose-fed rats [11]. Dietary supplementation of *N*-acetylcysteine also alleviates sucrose-induced oxidative stress and insulin resistance in rats. Notably, the beneficial effects of *N*-acetylcysteine on sucrose-induced oxidative stress and insulin resistance can be duplicated by consumption of a cysteine-rich diet [12]. More recently, *N*-acetylcysteine is found to prevent high-sucrose diet-induced obesity and metabolic disturbances in experimental animals [13].

3.1.3. Role in Neurological Diseases

The neuroprotective effects of GSH esters have been well-documented in various animal models of human neurological disorders. For example, infusion of GSH monoethyl ester into the third ventricle during middle cerebral artery occlusion and reperfusion is found to markedly reduce the infarct size in experimental animals [14]. Administration of GSH monoethyl

ester prevents mitochondrial GSH depletion during focal cerebral ischemia, which may be an important mechanism underlying its protection against stroke in animal models [15]. Intraperitoneal injection of GSH monoethyl ester improves functional recovery, enhances neuron survival, and stabilizes spinal cord blood flow after spinal cord injury in rats [16]. There is also evidence showing beneficial actions of both GSH esters and *N*-acetylcysteine in other neurodegenerative diseases, such as Parkinson's disease [17]. In this regard, GSH depletion in dopaminergic neurons is an early pathophysiological event of Parkinson's disease. Thus, repletion of GSH via GSH esters and *N*-acetylcysteine may represent an effective approach to the management of Parkinson's disease. In addition to Parkinson's disease, brain GSH deficiencies have also been observed in aged animals. Supplementation of GSH monoethyl ester is shown to restore the aging-related GSH deficiencies, oxidative stress, and neurochemical alterations [18-20].

3.1.4. Role in Pulmonary Diseases

The best studied pulmonary disease with respect to protection by GSH precursors is chronic obstructive pulmonary disease (COPD). There is substantial evidence showing a beneficial role for GSH precursors, including *N*-acetylcysteine and *S*-carboxymethylcysteine in COPD in experimental animals [5, 21, 22]. The protective effects of these GSH precursors in COPD have been associated with decreased pulmonary inflammation and oxidative stress. In line with this, GSH precursors, including *N*-acetylcysteine, *S*-carboxymethylcysteine, and GSH monoethyl ester have also been found to attenuate airway inflammation and oxidative stress in animal models of asthma [23, 24]. Administration of *N*-acetylcysteine or GSH esters is shown to alleviate hyperoxia-induced lung injury [25, 26]. Moreover, oral supplementation of *N*-acetylcysteine protects against bleomycin-elicited lung oxidative stress, inflammation, and fibrosis in experimental animals [27, 28]. In cultured bronchial epithelial cells, *N*-acetylcysteine treatment inhibits bleomycin-induced secretion of proinflammatory cytokines, which is consistent with the in vivo observations [29].

3.1.5. Role in Hepatic and Gastrointestinal Diseases

GSH precursors are protective in liver diseases, including ischemia-reperfusion injury, nonalcoholic fatty liver disease, and drug/xenobiotic-induced liver injury. Intravenous infusion of *N*-acetylcysteine or GSH monoethyl ester attenuates liver ischemia-reperfusion injury, as evidenced by decreased release of liver enzymes and reduced oxidative stress [30, 31]. In addition, GSH monoethyl ester treatment protects liver mitochondria from oxidative damage during ischemia-reperfusion. The beneficial effects of both *N*-acetylcysteine and GSH esters in nonalcoholic fatty liver disease have been well-documented in various animal models, including the nutritional models of either feeding a diet deficient in both methionine and choline or feeding a high-fat diet [32, 33].

N-Acetylcysteine is best known as the specific antidote for acetaminophen overdose. This is primarily due to the increased GSH levels in liver, leading to enhanced detoxification of the reactive metabolite of acetaminophen, *N*-acetyl-*p*-benzoquinoneimine (Figure 25-2). *N*-Acetylcysteine or GSH esters also ameliorate liver injury induced by other drugs/xenobiotics, such as cyclophosphamide, carmustine, ethanol, and carbon tetrachloride.

GSH precursors, such as *N*-acetylcysteine and GSH monoethyl ester exert beneficial effects in certain gastrointestinal disorders. These include *Helicobacter pylori* infection, inflammatory bowel disease, and drug-induced peptic ulcer disease. Notably, treatment with *N*-

acetylcysteine plus mesalamine, a first line drug for ulcerative colitis, caused a significantly greater reduction in colonic injury than either agent alone in a rodent model of colitis [34]. This suggests that *N*-acetylcysteine may have added benefits when combined with established therapy in the management of diseases involving oxidative stress and inflammation.

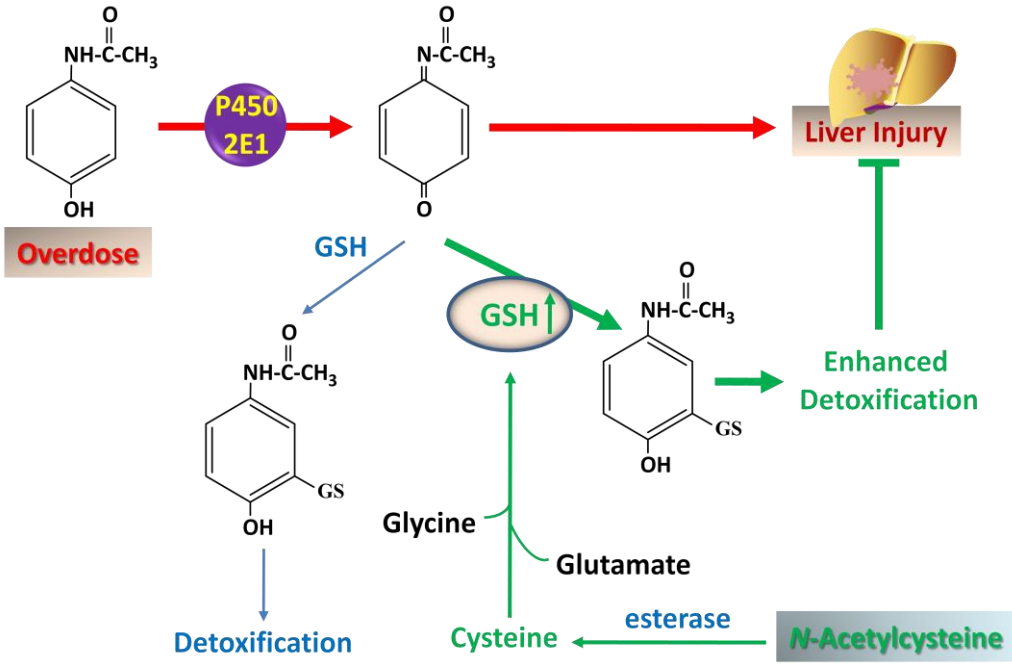

Figure 25-2. *N*-Acetylcysteine as the specific antidote for acetaminophen overdose. As illustrated, GSH-mediated conjugation reaction is a major detoxification mechanism for the toxic metabolite of acetaminophen, *N*-acetyl-*p*-benzoquinoneimine in liver cells. The cytochrome P450 2E1 enzyme is primarily responsible for the bioactivation of acetaminophen to produce the highly reactive *N*-acetyl-*p*-benzoquinoneimine. In acetaminophen overdose, a large amount of *N*-acetyl-*p*-benzoquinoneimine is produced, which overwhelms the normal GSH-dependent detoxification capacity of the liver cells, causing liver injury. By releasing cysteine, *N*-acetylcysteine increases liver GSH levels, leading to enhanced detoxification of *N*-acetyl-*p*-benzoquinoneimine.

3.1.6. Role in Renal Diseases

The most extensively studied renal disorder regarding *N*-acetylcysteine-mediated protection in experimental animals (as well as in human clinical trials) is probably contrast medium-induced nephropathy [35, 36]. While the exact mechanisms remain to be elucidated, the radical scavenging and anti-inflammatory activities of *N*-acetylcysteine seem to play an important part in the protection against contrast medium-induced nephropathy. Multiple studies also demonstrate a protective effect of *N*-acetylcysteine in renal ischemia-reperfusion injury [37, 38]. Treatment with *N*-acetylcysteine is found to attenuate oxidative stress and inflammation, and improve the graft function after kidney transplantation in experimental animals. *N*-acetylcysteine and GSH esters also ameliorate diabetic nephropathy and drug/xenobiotic-induced nephrotoxicity [39, 40].

3.1.7. Role in Cancer

Administration of *N*-acetylcysteine protects experimental animals from carcinogenesis induced by a variety of chemical carcinogens [41, 42]. Mechanistically, *N*-acetylcysteine treatment inhibits oxidative DNA damage as well as the formation of carcinogen-DNA adducts during chemical carcinogenesis. The anticancer activities of *N*-acetylcysteine have also been demonstrated in genetic models of spontaneous carcinogenesis. For example, long-term dietary supplementation of *N*-acetylcysteine is shown to reduce the incidence and multiplicity of lymphoma in Atm-deficient mice [43]. In the same animal model, *N*-acetylcysteine treatment inhibits oxidative DNA damage and reduces the frequency of DNA deletions, which may contribute to its cancer chemoprotection [44]. Supplementation of *N*-acetylcysteine also prevents chronic colitis-associated colorectal carcinoma in wild-type mice and hepatocarcinogenesis in transaldolase-deficient mice [45, 46]. Furthermore, it has been reported that *N*-acetylcysteine synergizes with the anticancer drug doxorubicin in the suppression of tumorigenesis and metastasis in murine models [47]. The major mechanisms that have been proposed to account for the anticancer activities of *N*-acetylcysteine in experimental animals are summarized below [48, 49].

- Direct reactions between *N*-acetylcysteine and carcinogens
- Increased levels of GSH, leading to enhanced detoxification of carcinogens
- Free radical-scavenging activities
- Anti-inflammatory effects
- Inhibition of angiogenesis

3.1.8. Role in Other Diseases and Conditions

N-Acetylcysteine, GSH esters, and other cysteine analogs have been shown to be protective in several other disease processes involving oxidative stress and inflammation in experimental animals. These include sepsis [50], ultraviolet irradiation-induced skin injury [51], wound healing [52], arthritis [53], viral (e.g., HIV, H1N1) infections [54-56], cataract [57, 58], and noise-induced hearing loss [59, 60].

3.2. Human Studies and Clinical Perspectives

3.2.1. N-Acetylcysteine as the Specific Antidote for Acetaminophen Overdose in Humans

Acetaminophen overdose is responsible for more than 40% of all cases of acute liver failure each year in the United States. *N*-Acetylcysteine has been used as the specific antidote for acetaminophen overdose [61]. In acetaminophen overdose, hepatic GSH is depleted by the toxic metabolite of acetaminophen, *N*-acetyl-*p*-benzoquinoneimine, and if left untreated, *N*-acetyl-*p*-benzoquinoneimine may accumulate, causing liver cell death (also see Figure 25-2). Timely administration of *N*-acetylcysteine prevents liver injury by replenishing hepatic GSH [62]. Notably, even if administered following the development of acetaminophen-induced fulminate hepatic failure, *N*-acetylcysteine still improves survival rates. Recently, it has been suggested that *N*-acetylcysteine be administered to all patients with acute liver failure regardless of its etiology [61].

3.2.2. Role in Human Cardiovascular Diseases and Diabetes

The potential beneficial effects *N*-acetylcysteine in human cardiovascular diseases and diabetes have been investigated in multiple small-scale clinical trials. In a randomized trial, *N*-acetylcysteine as an additive to blood cardioplegia in patients undergoing on-pump coronary artery bypass graft surgery was found to reduce coronary endothelial activation and myocardial oxidative stress [63]. A more recent randomized controlled trial demonstrated that high-dose intravenous *N*-acetylcysteine resulted in attenuation of oxidative stress, but did not provide an additional clinical benefit to placebo with respect to myocardial reperfusion injury in unselected patients with ST-segment elevation myocardial infarction undergoing primary percutaneous coronary intervention [64]. A meta-analysis reveals that *N*-acetylcysteine may reduce post-cardiothoracic surgery complications [65]. In a clinical trial with hypertensive patients having type 2 diabetes, long-term administration of *N*-acetylcysteine and L-arginine was found to reduce endothelial activation and systolic blood pressure [66]. However, this trial did not investigate the effects of *N*-acetylcysteine alone. A subsequent study by the same group demonstrated that oral supplementation of *N*-acetylcysteine alone was able to attenuate oxidative stress and endothelial activation after a high-glucose content meal in patients with type 2 diabetes [67]. While these findings suggest the potential protective effects of *N*-acetylcysteine in human cardiovascular pathophysiology and diabetes, large-scale randomized controlled trials are needed to further ascertain the clinical benefits of *N*-acetylcysteine-based intervention.

3.2.3. Role in Human Pulmonary Diseases

Clinical trials in patients with chronic obstructive pulmonary disease (COPD) collectively showed that orally administered *N*-acetylcysteine significantly improved responses to steroids, increased general wellbeing, and decreased exacerbation rates and emergency room visits [68]. In addition to *N*-acetylcysteine, *S*-carboxymethylcysteine has been recently recognized as a potentially effective and safe agent for COPD, as evidenced by its ability to reduce the incidence of exacerbation and improve quality of life. In a four-week, dose-escalating phase 1 trial, high-dose oral *N*-acetylcysteine was found to modulate lung inflammation in cystic fibrosis [69]. A subsequent phase 2 study reported that high-dose oral *N*-acetylcysteine didn't alter clinical or inflammatory parameters in patients with cystic fibrosis though it had a potential to increase extracellular GSH levels in the airways [70]. The therapeutic value of *N*-acetylcysteine and other GSH precursors in cystic fibrosis and COPD warrants further evaluation in large-scale well-designed clinical trials.

3.2.4. Role in Contrast Medium-induced Nephropathy

Approximately ten million procedures using radiological contrast agents are performed in the United States annually. The contrast medium-induced nephropathy remains a major clinical problem. The involvement of oxidative and inflammatory stress in contrast medium-induced nephropathy has prompted studies on using *N*-acetylcysteine as a potential protective agent in human subjects. In 2000, a randomized placebo-controlled trial showed that *N*-acetylcysteine was effective as prophylaxis against contrast medium-induced nephropathy [71]. Many subsequent clinical trials have attempted to replicate this report, but the results have been mixed and controversial. The inconsistent outcomes may be a result of the heterogeneity of the patient populations, different inclusion criteria, stage of kidney disease, and the

differences in doses and routes of administration of *N*-acetylcysteine [72, 73]. In this context, recent trials in selected patient populations reported beneficial effects of high-dose *N*-acetylcysteine alone or in combination with sodium bicarbonate in contrast medium-induced nephropathy [74-76]. Because of the dichotomous findings from clinical trials, prophylaxis of contrast medium-induced nephropathy with *N*-acetylcysteine is currently not considered standard care. Nevertheless, use of *N*-acetylcysteine has increased due to its demonstrated safety and potential effectiveness in the prevention of contrast medium-induced nephropathy in human subjects.

3.2.5. Role in Other Human Diseases

As an antioxidant and GSH precursor, *N*-acetylcysteine has potential uses in many other disease conditions involving oxidative stress and inflammation. Multiple pilot clinical trials suggest that *N*-acetylcysteine may be effective in the management of nonalcoholic steatohepatitis, kidney transplantation, ulcerative colitis, *Helicobacter pylori* infection, HIV infection, adenomatous colonic polyps, cigarette smoke-induced carcinogenesis, and addictive behaviors [68, 77-80]. Large-scale well-designed clinical trials are needed to further confirm the effectiveness of *N*-acetylcysteine in the intervention of these human diseases.

4. Conclusion and Future Directions

Glutathione precursors, including GSH esters, *N*-acetylcysteine, and other cysteine analogs can be administered to increase cellular and tissue levels of GSH. In addition to increasing GSH levels, GSH precursors, especially *N*-acetylcysteine also exert other novel biological effects, such as anti-inflammation, antiplatelet aggregation, and antiangiogenesis. The protective effects of GSH precursors, particularly *N*-acetylcysteine have been extensively demonstrated in animal models of human diseases, including cardiovascular diseases, diabetes and metabolic syndrome, neurological disorders, pulmonary diseases, liver and gastrointestinal disorders, kidney diseases, and cancer. In line with the findings in animal studies, multiple clinical trials have suggested beneficial effects *N*-acetylcysteine in various disease conditions. However, large-scale well-designed clinical trials are warranted to further establish the efficacy of *N*-acetylcysteine in human disease intervention. As *N*-acetylcysteine synergizes with other drugs in disease treatment, future clinical studies should put more emphasis on evaluating the added benefits of *N*-acetylcysteine in established treatment regimens for common human disease conditions. Future efforts should also focus on the development of novel GSH precursors as well as the preclinical assessment of their efficacy and safety in animal models of human diseases.

5. References

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Chapter 26

Spin Traps

Abstract

Spin traps are chemicals that react with unstable free radical species to form relatively stable free radical spin adducts that can be detected by electron paramagnetic resonance (EPR) spectroscopy. Nitrone compounds are the most commonly used spin traps with α-phenyl-*tert*-butylnitrone (PBN) as a notable example. Spin traps possess antioxidant activities due to their ability to scavenge free radicals. In addition, they also exert other novel biological activities, such as induction of cellular antioxidant enzymes, anti-inflammation, and antiangiogenesis. Animal studies have demonstrated a protective role for spin traps, especially PBN and its derivatives in the pathophysiology of a number of diseases. However, pooled analysis of clinical trials on the PBN derivative 2,4-disulfophenyl-*N*-*tert*-butylnitrone (also known as NXY-059) has concluded that this nitrone compound is ineffective for the treatment of acute ischemic stroke in human subjects though its efficacy in treating this neurological disorder is well-established in preclinical studies.

1. Overview

Spin traps are used to react with the short-lived free radicals to form relatively long-lived radical adducts that can be detected by electron paramagnetic resonance (EPR) techniques. EPR spin trapping has been considered the most specific way of detecting free radical species formed in biological systems. Some of the commonly used spin traps have been found to also exert antioxidant effects in both in vitro and in vivo systems. Notable examples include the nitrone compounds 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), α-phenyl-*tert*-butylnitrone (PBN), and PBN derivatives. These compounds have been demonstrated to protect against various pathophysiological conditions involving oxidative stress. As discussed below, in addition to directly scavenging free radicals, some spin traps are shown to upregulate cellular antioxidant enzymes and exert other novel biological effects. This chapter begins with a brief discussion of the chemical and biochemical properties of the antioxidant spin traps followed by a summary of the major findings with respect to the role of these compounds in disease intervention in both animal models and human clinical trials.

2. Chemistry and Biochemistry

2.1. Nitrone Spin Traps

Nitrones have been the most widely used spin traps for detecting free radicals, including oxygen radicals and carbon-centered radical species in biological systems. There are two classes of nitrone spin traps: the linear and the pyrroline-based cyclic nitrones. The linear nitrone spin traps include PBN (structure shown in Figure 26-1) and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN). The pyrroline-based cyclic nitrone spin traps consist of DMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), and 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (EMPO) [1]. These spin traps differ with respect to their affinity to free radicals and the stability of the radical adducts formed. In addition, the toxicity also varies with different spin traps. In this context, PBN and several PBN derivatives have been shown to be well-tolerated.

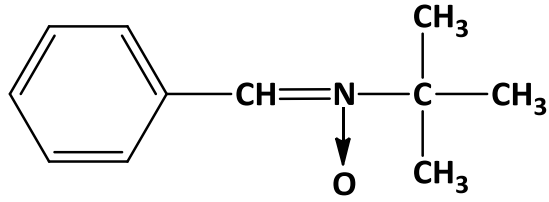

Figure 26-1. Structure of α-phenyl-*tert*-butylnitrone (PBN). See text (Section 2.1) for description of PBN.

The nitrone chemical structure in its simplest form can be written as X-CH=NO-Y. The nitrone trapping of a free radical intermediate is represented in the following simple reaction in which the free radical intermediate ($R^{\cdot}$) is trapped by the nitrone to form a nitroxyl free radical spin adduct (X-CHR-$NO^{\cdot}$-Y) (Reaction 1) [2].

$$X\text{-}CH{=}NO\text{-}Y + R^{\cdot} \longrightarrow X\text{-}CHR\text{-}NO^{\cdot}\text{-}Y \qquad (1)$$

The nitroxyl free radical spin adduct is usually much more stable than the original free radical intermediate ($R^{\cdot}$), therefore making it possible in principle to detect and characterize the original free radical intermediate using EPR spectroscopy. Likewise, the ability of nitrones to trap free radical intermediates to form relatively less reactive spin adducts makes it rational to use these compounds to protect against oxidative stress injury in biological systems. Indeed, the nitrone spin trap PBN has been well-recognized for its protective effects in oxidative stress-associated pathophysiology. Several PBN derivatives, including 2,4-disulfophenyl-*N*-*tert*-butylnitrone (also known as NXY-059; see Sections 3.1.3, 3.1.7, and 3.2 for more discussion of NXY-059) have been developed over the last two decades, which are shown to also possess potent antioxidant activities in biological systems. In addition to their well-recognized free radical-scavenging properties, nitrone spin traps have been demonstrated to possess other novel activities, such as induction of endogenous antioxidant enzymes, anti-

inflammation, and antiangiogenesis [3-5]. These novel effects may also contribute to nitrone spin trap-mediated disease protection.

2.2. Other Spin Traps

In addition to nitrones, nitroso compounds have also been used as spin traps, but the application of nitroso compounds as spin traps and potential antioxidant agents is limited by their instability and toxicity [1]. Iron-dithiocarbamate complexes are employed as spin traps for detecting and characterizing nitric oxide in biological systems [6]. The disease protective effects of the nitric oxide spin traps in animal models have recently been reported. Section 3 below summarizes the key findings regarding the beneficial effects of spin traps in disease pathophysiology with an emphasis on PBN and PBN derivatives.

3. Role in Disease Intervention

3.1. Animal Studies

3.1.1. Role in Cardiovascular Diseases

Spin traps, such as DMPO, PBN, and iron-dithiocarbamate complexes have been frequently utilized to study free radical formation in cardiovascular diseases. Consequently, these spin traps have been shown to protect against cardiovascular pathophysiology involving oxidative stress and inflammation. For example, DMPO, PBN, and PBN derivatives are able to mitigate myocardial ischemia-reperfusion injury in animal models [7-9]. It is further found that DMPO protects against myocardial ischemia-reperfusion injury through scavenging oxygen free radicals and preservation of mitochondrial electron transport chain [10]. PBN has been demonstrated to be beneficial in cardiac allograft transplantation via abrogation of inflammatory cytokine gene expression during alloimmune activation in rats [11]. Similarly, the nitric oxide spin trap iron-diethyldithiocarbamate complex is reported to attenuate inflammatory responses, prolong graft survival, and decrease histological rejection scores in a rat model of acute cardiac allograft rejection [12]. The protective effects of nitrone spin traps have also been observed in other cardiovascular disorders, including post-ischemic cardiac arrhythmias and hypertension in experimental animals [13, 14].

3.1.2. Role in Diabetes

There have been multiple studies on the potential effects of PBN in chemically-induced type 1 diabetes in animal models. An early study demonstrated that administration of PBN protects against streptozotocin-induced diabetes in mice, as evidenced by inhibition of hyperglycemia, hemoglobin glycation, nitric oxide formation, and pancreatic β-cell destruction [15]. These initial observations have been confirmed in a subsequent study, which further shows a protective role for PBN in alloxan-induced diabetes. PBN treatment attenuates both alloxan- and streptozotocin-induced free radical formation and activation of NF-κB in pancreatic tissue [16]. However, treatment with PBN fails to prevent spontaneous type 1 diabetes associated with autoimmunity in rats though lipid peroxidation in pancreatic tissue is inhi-

bited by the spin trap [17]. To date, the effects of nitrone spin traps on type 2 diabetes have not been reported in the literature.

3.1.3. Role in Neurological Diseases

Over the last two decades the neuroprotective effects of nitrone spin traps, especially PBN and its derivatives have been extensively investigated in various animal models of neurological disorders. The most studied neurological disease regarding the protection by nitrone spin traps in experimental animals is probably acute ischemic stroke [2, 18]. The beneficial effects of PBN and its derivative 2,4-disulfophenyl-*N*-*tert*-butylnitrone (NXY-059) in acute ischemic stroke have been demonstrated in both rodents and primates, which led to the initiation of clinical trials on NXY-059 for the treatment of acute ischemic stroke in humans [2, 18] (see Section 3.2 below). Although the exact mechanisms underlying nitrone spin trap-mediated stroke protection remain to be elucidated, attenuation of free radical damage and inflammation, and improvement of energy metabolism may play an important part. As oxidative stress and inflammation are common mechanisms of neurodegeneration, nitrone spin traps have also been studied for their beneficial effects in neurodegenerative disorders, including Parkinson's disease. Chemically-induced parkinsonism in mice is ameliorated by treatment with PBN [19]. PBN treatment also protects against toxin-induced seizure, excitotoxicity, and neuroinflammatory disorders in experimental animals [20-22].

3.1.4. Role in Pulmonary Diseases

The herbicide paraquat causes lung injury through an oxidative stress mechanism. Indeed, formation of oxygen free radicals has been identified by EPR spectroscopy with PBN-spin trapping in experimental animals following exposure to paraquat. Paraquat-induced acute broncoconstriction and edema in guinea pig lungs are markedly attenuated by PBN treatment [23]. Recently, it is reported that administration of PBN to neonatal rats protects these animals from hyperoxia-induced lung injury. It has been further observed that the hyperoxia-induced superoxide formation from NAD(P)H oxidase and gene expression of proinflammatory cytokines in lung tissue are inhibited by PBN treatment. This suggests that suppression of inflammation may play a part in PBN-mediated pulmonary protection against hyperoxic injury [24]. In line with this notion, PBN is shown to inhibit hyperoxia-induced formation of nitric oxide, an important event in hyperoxia-elicited inflammatory injury [25].

3.1.5. Role in Hepatic and Gastrointestinal Diseases

The protective effects of nitrone spin traps on chemically-induced liver disorders have been demonstrated in rat models, including carbon tetrachloride-induced hepatotoxicity and copper-induced fulminant hepatitis with jaundice [26, 27]. Recently, a new PBN derivative *N*-{[4-(lactobionamido)methyl]benzylidene}-1,1-dimethyl-2-(octylsulfanyl)ethylamine *N*-oxide is shown to suppress copper-induced fulminant hepatitis and oxidative stress at an effective dose 5-10 orders of magnitudes lower than that of PBN [28].

Treatment with PBN improves dextran sulfate sodium-induced colitis in mice, as evidenced by attenuated colonic inflammation, oxidative stress, and tissue necrosis. Notably, activation of NF-κB and NF-κB-regulated proinflammatory cytokine gene expression is diminished by PBN treatment in this murine model of colitis [29]. More recently, PBN treatment has been reported to reduce visceral pain associated with zymosan-induced colitis and

colonic oxidative stress in rats, further supporting a beneficial effect of PBN in experimental colitis [30]. In another murine model of colitis elicited by trinitrobenzene sulfonic acid, combination of PBN with mesalamine, a first line drug for treating ulcerative colitis, failed to improve the disease condition beyond that produced by mesalamine alone. However, the study did not report the effects of PBN alone on trinitrobenzene sulfonic acid-induced colitis [31].

3.1.6. Role in Renal Diseases

The nitrone spin trap PBN exerts beneficial effects in renal diseases, especially ischemia-reperfusion injury in animal models. Early studies reported that administration of PBN reduces ischemia-reperfusion-induced acute renal failure in rats, as revealed by improvement in creatinine and urea clearance [32]. Subsequently, studies have demonstrated that PBN treatment causes suprainduction of heme oxygenase-1 in renal tissue, which may mediate, at least partially, PBN's protection against ischemia-reperfusion injury [3]. In this context, transgenic overexpression of HO-1 is highly protective against renal ischemia-reperfusion injury (see Chapter 15 for discussion of heme oxygenase).

3.1.7. Role in Cancer

The anticancer activities of PBN were first observed by D. Nakae and coworkers in a rat model of hepatocarcinogenesis induced by a diet deficient in choline [33, 34]. In this model, chronic administration of PBN is found to inhibit the development of hepatocellular carcinoma. PBN treatment also inhibits the formation of oxidative DNA damage as well as the activity of cyclooxygenase-2 in the target tissue [33]. In addition, PBN is shown to selectively induce apoptosis in preneoplastic lesions during the early phase of hepatocarcinogenesis induced by the choline-deficient diet [35]. Notably, the metabolite of PBN, namely, 4-hydroxyl-PBN, also possesses anticancer activities [35].

Recently, PBN and its derivatives have been demonstrated to exert anticancer effects in other animal models of cancer. For example, administration of PBN leads to marked induction of tumor regression, inhibition of angiogenesis, and prolongation of survival in a C6 rat glioma model [5]. The PBN derivative 2,4-disulfophenyl-*N*-*tert*-butylnitrone (also known as OKN007 or NXY-059) also decreases tumor volumes and increases survival in the C6 rat glioma model [36]. More recently, PBN is shown to be protective in a murine model of colorectal carcinoma [37].

3.1.8. Role in Other Diseases and Conditions

There are a number of other diseases and conditions in which nitrone spin traps have been shown to exert beneficial effects in experimental animals. These include sepsis [38], ionizing radiation injury [39], light-induced retinal degeneration [40], acute acoustical trauma [41], and aging [42].

3.2. Human Studies and Clinical Perspectives

The enormous amount of data obtained from preclinical studies on nitrone spin trap-mediated disease protection has spurred the interest in utilizing these compounds for the intervention of human diseases. Indeed, as noted earlier in Section 3.1.3, the PBN derivative

NXY-059 was developed as a potential neuroprotectant for human stroke. Supported by the available preclinical data on its efficacy in treating stroke as well as the safety profiles in human subjects, a randomized double-blind, placebo-controlled trial (SAINT I) was initiated in 2003, and the SAINT I trial involving 1722 patients with acute ischemic stroke reported in 2006 that administration of NXY-059 within 6 hours after the onset of acute ischemic stroke significantly improved the primary outcome (reduced disability at 90 days) [43]. However, a subsequent larger trial (SAINT II) involving 3306 patients with acute ischemic stroke reported in 2007 that NXY-059 was ineffective for the treatment of acute ischemic stroke given within 6 hours after the onset of the symptoms [44]. Furthermore, a pooled analysis of the SAINT I and II trials concluded that NXY-059 lacks efficacy in the treatment of acute ischemic stroke [45]. As such, NXY-509 was not further developed as a drug for human ischemic stroke.

4. Conclusion and Future Directions

In addition to their free radical-scavenging activities, spin traps, especially the nitrone spin traps also possess other important biological properties, including upregulation of endogenous antioxidant enzymes, downregulation of inflammatory responses, and inhibition of angiogenesis (Figure 26-2).

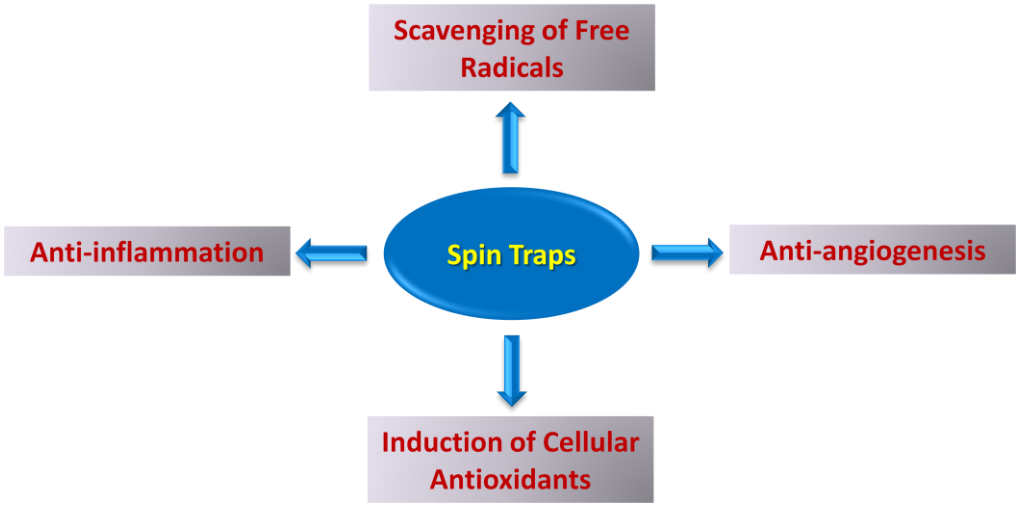

Figure 26-2. Biological activities of spin traps in disease intervention. As illustrated, in addition to their free radical-scavenging ability, spin traps also possess other novel biological activities, including induction of endogenous antioxidant enzymes, anti-inflammation, and antiangiogenesis. These diverse mechanistic events contribute to the biological role of spin traps in disease intervention.

The beneficial effects of spin traps, such as PBN and the PBN derivative NXY-059 have been demonstrated in many animal models of human diseases, including both rodent and primate models of acute ischemic stroke. However, pooled analysis of the SAINT trials con-

cluded that NXY-059 lacks effectiveness in treating human acute ischemic stroke. There have been many discussions and critiques of the SAINT trials as well as the preclinical studies on NXY-059, which provide suggestions for future studies on neuroprotective agents. These include (1) better characterization of drug candidate-target interaction and its relationship to pharmacodynamic treatment endpoints, (2) combination therapies with different compounds targeting different pathways of disease pathophysiology to achieve a better chance of success than single medications, and (3) testing candidate drugs in more carefully selected patient groups and better designed trials. In view of the demonstrated safety of NXY-059 in human subjects, this nitrone spin trap should be further investigated for its potential benefits in the intervention of other human diseases in well-designed clinical trials.

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Chapter 27

Nanomaterials

Abstract

Nanomaterials have received increasing attention in biology and medicine due to their unique interactions with biological systems and their potential applications in both diagnostics and therapeutics. Several kinds of nanomaterials, including cerium oxide nanoparticles, platinum nanoparticles, and fullerene derivatives have been shown to possess antioxidative and anti-inflammatory activities. Extensive studies have demonstrated a protective role for these antioxidant nanomaterials in a variety of animal models of human diseases, including cardiovascular diseases, neurological disorders, aging, and cancer. On the other hand, nanomaterials, like any existing drugs, also have the potential to cause adverse effects in biological systems. Future studies are warranted to further determine the efficacy and safety of the antioxidant nanomaterials in animal models as well as in clinical trials.

1. Overview

Nanomedicine, a combinatorial approach using nanotechnology and medicine, has become an increasingly important field of research in both diagnostics and therapeutics [1]. The field of nanomedicine involves the design and development of novel nanomaterials, such as metal nanoparticles, liposomal nanoparticles, fullerenes, carbon nanotubes, dendrimers, and nanoshells. Once engineered, these novel nanomaterials are investigated for their potential value in the diagnosis and treatment of diseases. Drug delivery, diagnostic imaging, and antioxidant activities are among the major areas of focus in nanomedicine.

Regarding the antioxidant properties of nanomaterials in biological systems, two scenarios may exit: (1) nanomaterials directly scavenge reactive oxygen and nitrogen species (ROS/RNS) via the unique redox chemistry of the surface and (2) nanomaterials serve as carriers for known antioxidant compounds or enzymes. Many antioxidants (either non-protein antioxidant compounds or antioxidant enzymes) exhibit low bioavailability due to limited solubility, instability, and first-pass effect. These limitations can be potentially circumvented by encapsulating the antioxidants to form antioxidant-loaded polymeric materials (nanocapsules or nanospheres). Certain antioxidant-loaded nanomaterials have been reported to protect

against oxidative damage in cultured cells and animal models [2-4]. Hence, the second scenario represents a form of drug delivery. This chapter focuses on the antioxidant activities related to direct scavenging of ROS/RNS by the redox-active nanomaterials, which mainly include cerium oxide nanoparticles, platinum nanoparticles, and fullerenes.

2. Chemistry and Biochemistry

2.1. Cerium Oxide Nanoparticles

Cerium is a rare earth element of the lanthanide series. The oxide form (CeO_2) is used as a support material in automotive catalysis. Cerium oxide nanoparticles, also known as nanoceria have been shown to scavenge superoxide possibly via the redox properties of Ce^{3+}/Ce^{4+} (Reactions 1 and 2) [5, 6].

$$O_2^{\cdot-} + Ce^{4+} \longrightarrow O_2 + Ce^{3+} \quad (1)$$

$$O_2^{\cdot-} + Ce^{3+} + 2H^+ \longrightarrow H_2O_2 + Ce^{4+} \quad (2)$$

Because of the above redox reactions, cerium oxide nanoparticles have been suggested to possess superoxide dismutase (SOD) mimetic activity, i.e., the autocatalytic dismutation of superoxide. It is further reported that the reaction rate constant for cerium oxide nanoparticle-catalyzed dismutation of superoxide may exceed that determined for the enzyme SOD [5]. More recently, cerium oxide nanoparticles are found to exhibit redox state-dependent catalase mimetic activity [7]. There is also evidence for the ability of cerium oxide to catalytically reduce nitrogen dioxide ($^{\cdot}NO_2$) to nitric oxide ($^{\cdot}NO$), and $^{\cdot}NO$ to N_2 [8, 9]. In contrast to the ROS/RNS-scavenging activities, formation of hydroxyl radicals from a Fenton-type reaction between cerium and hydrogen peroxide has been observed in vitro [10]. It remains to be further elucidated if the above redox reactions also occur in vivo. Figure 27-1 illustrates the redox chemistry of cerium oxide nanoparticles in relation to metabolism of ROS/RNS.

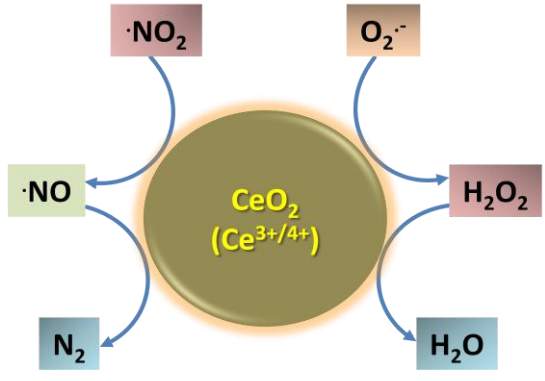

Figure 27-1. Metabolism of reactive oxygen and nitrogen species (ROS/RNS) catalyzed by cerium oxide nanoparticles**.** See text (Section 2.1) for description of the redox chemistry of cerium oxide nanoparticles in relation to catalytic metabolism of ROS/RNS.

2.2. Platinum Nanoparticles

Synthetic platinum nanoparticles have recently been shown to scavenge both superoxide and hydrogen peroxide in cell-free systems [11, 12]. The hydrogen peroxide-scavenging activity of platinum nanoparticles is much higher than that of EUK-8, a superoxide dismutase/catalase mimetic (Section 2.1.2 of Chapter 24). In addition, platinum nanoparticles scavenge peroxyl radicals and inhibit peroxyl radical-induced lipid peroxidation [13]. More recently, it is reported that platinum nanoparticles may have an activity similar to mitochondrial NADH:ubiquinone oxidoreductase [14]. This suggests a unique opportunity for using the nanoparticles to protect against disease conditions, such as Parkinson's disease, in which increased ROS formation and decreased mitochondrial complex I activity are critical pathophysiological events.

2.3. Fullerenes

The fullerene revolution began with the discovery by a team of scientists in 1985 of the buckyball (C_{60}) composed of 60 carbon atoms arranged in a hollow soccer ball shape [15] (structure shown in Figure 27-2). Because of this discovery, three of the team members were awarded the 1996 Nobel Prize in Chemistry. C_{60} is also known as buckminsterfullerene (or fullerene for short), named after Richard Buckminster Fuller, the architect who created the dome in 1967 with the same shape as that of the carbon cluster. Now, it is known that fullerenes represent a family of carbon allotrope molecules in the form of a hollow sphere, ellipsoid, tube, or plane with various numbers of carbon atoms [16, 17]. Fullerenes, especially C_{60} have received great attention for their potential usefulness in biomedicine. A number of derivatives of C_{60} fullerene, especially the polyhydroxylated C_{60} fullerenes (also known as fullerenols) have been developed to improve their biocompatibility (e.g., water solubility) and activities in biological systems. These functionalized C_{60} fullerenes have been shown to exert a number of biological activities, including enzyme inhibition, antimicrobial activity, and scavenging of free radicals [16, 17]. C_{60} fullerene derivatives may possess SOD activity, in that the fullerenes catalytically convert superoxide to hydrogen peroxide and molecular oxygen [18-20]. In addition, C_{60} fullerene derivatives are found to scavenge peroxyl radicals, singlet oxygen, and hydroxyl radicals [21, 22]. The free radical scavenging properties of fullerene derivatives may result from the capacity of the carbon-cluster to absorb electrons as well as the redox behaviors of the surface structures, including the hydroxyl groups introduced in the derivatives [18-20, 23].

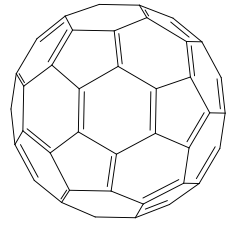

Figure 27-2. Structure of C_{60} fullerene. See text (Section 2.3) for description of fullerenes.

2.4. Other Nanomaterials

The free radical-scavenging properties of several other metal nanomaterials have also been reported in the literature. For example, both Nano-Se (also known as nano red element selenium) and nickel oxide nanoparticles are shown to scavenge carbon-centered free radicals in vitro [24, 25]. Nano-Se may also scavenge superoxide and singlet oxygen [25]. Gold-poly(amidoamine) dendrimer nanocomposites are found to potently scavenge hydroxyl radicals generated from the reaction between hydrogen peroxide and iron [26]. The redox chemistry underlying the potential free radical scavenging activities of these metal nanomaterials warrants investigations.

3. Role in Disease Intervention

3.1. Animal Studies

The increasing recognition of ROS-scavenging activities of cerium oxide nanoparticles, nickel oxide nanoparticles, and fullerenes in both cell-free and cell culture systems has prompted studies on using these nanomaterials for the intervention of diseases involving an oxidative stress mechanism. In addition, cerium oxide nanoparticles and fullerenes have been found to also possess anti-inflammatory activities, as evidenced by their ability to reduce the expression of inflammatory cytokines, adhesion molecules, and inducible nitric oxide synthase [27-29]. As oxidative stress and inflammation are intimately related, the ROS-scavenging ability of cerium oxide nanoparticles and fullerenes may primarily contribute to their anti-inflammatory activities. The sections below summarize the recent findings regarding the protective effects of nanomaterials on oxidative and inflammatory stress-mediated disease pathophysiology in various animal models of human diseases.

3.1.1. Role in Cardiovascular Diseases

Multiple in vitro experiments have demonstrated an important role for cerium oxide nanoparticles and C_{60} fullerene derivatives in suppressing oxidative stress injury and pro-inflammatory responses in cultured cardiovascular cells [30, 31]. The in vivo cardioprotection of cerium oxide nanoparticles was first studied by J. Niu and coworkers in a transgenic murine model of ischemic cardiomyopathy [32]. They demonstrated that treatment with cerium oxide nanoparticles attenuates the progressive cardiac dysfunction and remodeling as a result of inhibition of myocardial oxidative stress, endoplasmic reticulum stress, and inflammatory processes. The autoregenerative antioxidant properties of cerium oxide nanoparticles are suggested to account for the above beneficial effects. In addition to cerium oxide nanoparticles, fullerene derivatives may also have a protective effect on oxidative cardiomyopathy. As described in Chapter 3, mice with homozygous deletion of MnSOD die in utero or within a few days of birth due to catastrophic oxidative damage to mitochondria and subsequent development of dilated cardiomyopathy. Notably, treatment with a tris-malonic derivative of C_{60} fullerene dramatically increases the lifespan of the $MnSOD^{-/-}$ mice [18]. This observation coupled with the evidence that the C_{60} fullerene derivative is localized to mitochondria suggests that this fullerene derivative may function as MnSOD to protect against disease patho-

physiology associated with mitochondrial oxidative stress [18]. Recently, a polyhydroxylated fullerene derivative $C_{60}OH_{24}$ is shown to also protect against doxorubicin-induced acute and chronic cardiotoxicity as well as myocardial oxidative stress in rats [33, 34].

3.1.2. Role in Neurological Diseases

Neuroprotection is an effect that may result in salvage, recovery, or regeneration of the nervous system under disease conditions. A promising neuroprotective approach involves mitigating oxidative and inflammatory stress, which are believed to be key pathophysiological events underlying many types of neurological diseases, including ischemia, trauma, and degenerative disorders. Hence, recent efforts have been focused on determining the potential neuroprotective effects of nanomaterials that have antioxidant and anti-inflammatory properties. A number of in vitro studies demonstrate that cerium oxide nanoparticles and fullerene derivatives protect cultured neurons from oxidative stress injury elicited by exogenous ROS, excitotoxic compounds, and β-amyloid peptides [35-39]. In line with the in vitro findings, in vivo studies show that administration of a carboxyfullerene or a water-soluble hexasulfonated C_{60} fullerene derivative protects against cerebral ischemia-reperfusion injury and oxidative stress in experimental animals [40-42]. Treatment with carboxyfullerene derivatives also results in prolongation of survival and amelioration of oxidative functional degeneration in a murine model of familial amyotrophic lateral sclerosis [38]. In addition, carboxyfullerene derivatives suppress iron-induced oxidative stress in nigrostriatal dopaminergic system in vivo [43]. Concomitant local injection of a carboxyfullerene derivative also attenuates oxidative degeneration of substantia nigra induced by intranigral infusion of 1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of the parkinsonism-inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [44]. In contrast, concomitant systemic administration of the carboxyfullerene and MPTP leads to potentiation of MPTP-induced dopaminergic degeneration. An increase in the striatal MPP^+ level is observed in the carboxyfullerene and MPTP-cotreated mice, which may explain the above potentiation effect [44]. These findings implicate that fullerene-based nanomaterials may exert both beneficial and detrimental effects on disease pathophysiology depending on the experimental conditions.

3.1.3. Role in Pulmonary Diseases

Lung can be readily exposed to nanomaterials via inhalation, and as such the potential harmful effects of nanomaterials on respiratory system have been the focus of research in nanotoxicology [45]. On the other hand, the potent antioxidative and anti-inflammatory activities of certain nanomaterials may also have implications for protection against pulmonary diseases involving a mechanism of oxidative and inflammatory stress. Early studies demonstrated that administration of water-soluble fullerene derivatives leads to attenuation of lung ischemia-reperfusion injury and associated oxidative stress [46, 47]. More recently, fullerene derivatives are shown to inhibit IgE-dependent activation of human mast cells and peripheral basophils possibly as a result of the fullerene-mediated free radical scavenging [48]. This suggests that antioxidant fullerenes may be useful for the intervention of airway inflammatory disorders, such as asthma. In addition to fullerenes, the antioxidant cerium oxide and platinum nanoparticles also possess pulmonary protective activities. For example, intraperitoneal administration of cerium oxide nanoparticles to mice prevents the onset of radiation-induced pneumonia and prolongs the animal survival [49]. Intranasal administration of platinum na-

noparticles prior to exposure to cigarette smoke attenuates pulmonary inflammation, oxidative stress, and histological damage in mice [50]. Notably, nasal administration of platinum nanoparticles does not elicit pulmonary inflammation and toxicity. These observations suggest a potential value for using nanoparticles to prevent inflammatory pulmonary injury caused by environmental toxicants.

3.1.4. Role in Hepatic and Gastrointestinal Diseases

Pretreatment with C_{60} fullerene and its derivatives protects against carbon tetrachloride- or doxorubicin-induced hepatotoxicity and oxidative stress in experimental animals [51, 52]. Administration of a water-soluble fullerene derivative also inhibits intestinal ischemia-reperfusion injury via its free-radical scavenging activities [53]. Recently, it is shown that cerium oxide nanoparticles protect gastrointestinal epithelium from radiation-induced damage by reduction of ROS and upregulation of MnSOD in mice [54]. In cultured normal human colon cells, cerium oxide nanoparticles also inhibit radiation-induced ROS formation and cell death. Similar to the in vivo findings, treatment with cerium oxide nanoparticles increases the expression of MnSOD in cultured colon cells [54]. As MnSOD is a critical antioxidant defense, the above observations indicate a novel mechanism by which cerium oxide nanoparticles suppress oxidative stress. The molecular mechanism underlying cerium oxide nanoparticle-mediated induction of MnSOD awaits further studies.

3.1.5. Role in Eye Diseases

Retinal degeneration is a common cause of blindness. Photoreceptor cells of the retina are incessantly bombarded with photons of light, which, along with the cells' high rate of oxygen metabolism, continuously expose them to elevated levels of ROS. Indeed, accumulating evidence supports a critical involvement of oxidative stress in retinal degeneration. The potent autocatalytic properties of cerium oxide nanoparticles in detoxifying ROS have led to the use of the nanoparticles for treating oxidative retinal degeneration in experimental models [55-57]. Treatment of cultured retinal neurons with cerium oxide nanoparticles reduces intracellular accumulation of ROS. In vivo injection of cerium oxide nanoparticles also prevents loss of vision due to damaging light-induced degeneration of photoreceptor cells in mice. Notably, even when injected after light damage, the cerium oxide nanoparticles are still partially effective, suggesting that as long as the cells have not died, they may be prevented from entering or preceding down a death pathway [55]. These observations indicate that cerium oxide nanoparticles may be an effective modality for preventing and/or treating eye disorders associated with oxidative retinal degeneration.

3.1.6. Role in Aging

Exposure to cerium oxide nanoparticles prolongs the longevity of *Drosophila melanogaster*, suggesting a possible beneficial effect of nanomaterials in aging [58]. Chronic administration of a carboxyfullerene having SOD mimetic activity to mice not only reduces aging-associated oxidative damage and mitochondrial free radical production, but also significantly extends lifespan [59]. The fullerene-treated mice also exhibit improved performance on the Morris water maze learning and memory task, suggesting that the antioxidant fullerene derivative also rescues aging-related cognitive impairment [59]. Recently, the antioxidant platinum nanoparticles are found to possess significant antiaging activities in a nematode *Caenorhabdi-*

tis elegans model [60, 61]. Notably, treatment with platinum nanoparticles also recovers the shortened lifespan of the mev-1 (kn1) mutant. The shortened lifespan of the mutant is due to excessive oxidative stress. Collectively, these findings point to the feasibility of using antioxidant nanomaterials to combat aging.

3.1.7. Role in Cancer

Using nanomaterials as a novel strategy for anticancer drug delivery has recently received much attention [62]. This strategy significantly enhances the effectiveness of the well-established anticancer drugs in animal models. On the other hand, there is evidence suggesting that certain nanomaterials having antioxidative and anti-inflammatory properties may directly play a role in protecting against cancer development in experimental models. In vitro studies demonstrate that treatment with platinum nanoparticles suppresses phorbol-12-myristate-13-acetate (PMA)-induced ROS formation and promotion of cell transformation in a two-stage cell transformation model [63]. PMA is a classical tumor promoting agent capable of eliciting inflammation and oxidative stress. In cell cultures, platinum nanoparticles also exhibit selective inhibition of carcinoma cells over normal cells [64]. More recently, polyhydroxylated C_{82} fullerene derivatives are shown to potently inhibit angiogenesis and tumor growth in a mouse model of breast cancer [65]. In contrast to water soluble polyhydroxylated fullerenes, nanocrystalline C_{60} fullerene is reported to augment tumor growth in a mouse model of melanoma [66]. Hence, the exact role of fullerenes in tumorigenesis and the underlying mechanisms need to be further investigated.

3.1.8. Role in Other Diseases and Conditions

Antioxidant nanomaterials, especially the polyhydroxylated fullerene derivatives also have a protective role in various other disease processes in experimental animals. These include ultraviolet irradiation-induced oxidative skin injury [67], loss of hair [68], inflammatory arthritis [69], ionizing irradiation-induced immune dysfunction and multi-organ damage [70], and infections [17].

3.2. Human Studies and Clinical Perspectives

As discussed above, nanomaterials, including cerium oxide nanoparticles, platinum nanoparticles, and fullerenes possess antioxidative and anti-inflammatory activities, and their administration to experimental animals affords protection against a variety of disease conditions that involve oxidative stress and inflammation. However, clinical studies on using these nanomaterials for the intervention of human diseases have not yet been reported in the literature. One likely reason is that nanomedicine is still in its infancy, and much remains to be learned from studies using experimental models. Nanomaterials as potential drugs need to be further investigated in animal models with respect to not only their pharmacological effects, but also their toxicological profiles. In this regard, nanomaterials, including the antioxidant nanomaterials discussed in this Chapter have the potential to cause adverse effects [45]. In fact, nanotoxicology has been established recently to specifically address the detrimental effects of nanomaterials in biological systems. Although still in its early stage, nanotoxicology has already yielded enormous amounts of information regarding the adverse effects of various kinds of nanomaterials. Knowledge on both efficacy and safety of antioxidant nanomaterials

in experimental models will lay a basis for subsequent clinical studies to determine their value in the prevention and/or treatment of human diseases.

4. Conclusion and Future Directions

It has been increasingly recognized that nanomaterials, such as nanoceria, platinum nanoparticles, and fullerenes possess antioxidant activities via scavenging various ROS/RNS and other related reactive species. Moreover, nanomaterials, including cerium oxide nanoparticles and fullerene derivatives, exhibit SOD mimetic activity, suggesting a potential for these nanomaterials to catalytically detoxify superoxide in biological systems. Indeed, accumulating evidence points to the effectiveness of the above nanomaterials in protecting against disease pathophysiology involving an oxidative stress mechanism in animal models. It should be borne in mind that nanomaterials may possess other biological activities irrelevant to free radical scavenging, and these non-antioxidant activities may also play a role in disease intervention. Future studies should focus on continued characterization of both efficacy and safety of the already existing antioxidant nanomaterials in preclinical studies. Such studies will lay a foundation for subsequent clinical trials to evaluate the potential benefits of these nanomaterials in human disease intervention. Future efforts should also be devoted to the development of novel nanomaterials or derivatives of the existing nanomaterials with improved antioxidant properties and reduced adverse effects.

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