Chapter 1
Development of the Gastrointestinal Tract in Pigs

Kang Yao, Zhihong Sun, Zikui Liu, Zhefeng Li, and Yulong Yin

Contents

K. Yao • Y. Yin (✉)
Chinese Academy of Sciences, Institute of Subtropical Agriculture, Research Center for Healthy Breeding Livestock & Poultry, Hunan Engineering & Research Center of Animal & Poultry Science, Key Laboratory of Agro-ecological Processes in Subtropical Region, Scientific Observing and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Changsha, Hunan 410125, People's Republic of China
e-mail: yinyulong@isa.ac.cn

Z. Sun
College of Animal Science and Technology, Southwest University, Chongqing 400715, China

Z. Liu
Veterinary Faculty, Hunan Agricultural University, Changsha, Hunan 410128, China

Z. Li
Hangzhou King Techina Technology Co., Hangzhou, China

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_1,

1.1 Introduction

Feeding intolerance and necrotizing enterocolitis are all two commonly encountered problems in neonatal animal that result in significant mortality and morbidity and significant costs of care. The extent to which gastrointestinal tract (GIT) dysfunction occurs in neonatal animal is related to the increased incidence of clinical complications such as feeding intolerance, poor growth, necrotizing enterocolitis, malabsorption syndromes, and diarrhea.

The characteristics of GIT development in mammals (including pigs) during prenatal and postnatal periods, the effects of early nutrients on GIT development, and the negative impacts of early malnutrition on GIT development are discussed in this chapter. One aim of this chapter is to bring a clear understanding of the characteristics and mechanisms of development pertaining to gastrointestinal morphology, digestion, absorption, immune and microbial community at all stages of prenatal and postnatal period.

1.2 Characteristics of the Development of the Gastrointestinal Tract in Mammals

1.2.1 Stomach

The patterns exhibited by the different species resemble each other, although the chronology and sequence of appearance of the specialized glandular cells may vary. In this paragraph, the sequence of development of the human stomach is briefly described. At about 4 weeks following fertilization, the foregut, caudal to the primitive lung buds, expands. At 6–9 weeks after fertilization, depressions, characterized by epithelial cells oriented in a radial fashion, appeared in the epithelium along the lesser curvature; and the stratified (or pseudo-stratified) epithelium is then replaced by a layer of cuboidal cells. At the age of 11 weeks the first differentiated glandular epithelial cells appears, with clear representation of the structure of parietal cells. Finally, the chief cells (or a "zymogenic cell") which release a precursor enzyme in the stomach appeared at 12–13 weeks of age (Deren 1971).

In the human infant at birth, well-defined parietal cells are visible, and mucous neck cells are clearly discernible. The surface parietal cells contain periodic acid-schiff-positive material similar to that found in the adult. Although the chief cells are sparsely distributed compared to the adult stomach, they do appear to be capable of secreting pepsin. However, the total thickness of both the mucosa and the other layers of the stomach are much thinner than that observed in the adult (Deren 1971). The subsequent development of the stomach is related to thickening of the glandular portion of the stomach, with the appearance of more mature zymogen cells (Salenius 1962).

1.2.2 Intestine

The intestinal development in the pig, like in the human, is initiated early in fetal development (Kelly and Coutts 2000). At 40 days of gestation, the intestinal morphogenesis has progressed to the point where recognizable villi are present. At that time, mRNA for enzymes and cytoskeletal proteins are readily detectable (Perozzi et al. 1993). As the GIT develops, tremendous growth occurs, with a doubling of intestinal length in the last trimester of pregnancy. However, the mucosal surface area increase is even more dramatic, largely because of the villus and microvillus growth during this period of development (Neu 2007a). In the weeks before parturition, the pig intestine grows more rapidly than the body as a whole and its relative weight increases 70–80 % over the last 3 weeks of gestation (Sangild et al. 2000). The intestine undergoes tremendous growth during fetal life. It elongates 1,000 folds from 5 to 40 weeks. The length doubles in the last 15 weeks of gestation reaching a mean length at birth of 275 cm (Neu 2007b). In the small intestine, fingerlike projections, the villi are already formed at 16 weeks gestation (Neu 2007b).

After birth, the mammalian intestine undergoes two main phases of development during the neonatal period (Kelly and Coutts 2000). The first phase involves the preparation for extrauterine life when maternal colostrum and milk will provide the sole nutrient source. The second phase of intestinal development is associated with a shift in the digestive capability of the epithelium from one which digests and absorbs a milk diet to one which efficiently utilizes complex solid feeds. With these both phases, it is recognized that extensive changes occur in the gross architecture, ultrastructure, growth and differentiation of the intestine (Kelly and Coutts 2000). During the immediate postnatal period a dramatic increase in the diameter, total weight and surface area of the intestine of pig occurs (Zhang et al. 1997). Total intestinal and mucosal weight of pig increased by 58 % and 80 %, respectively during the first 6 h of suckling, and the mucosal DNA content is 4.6-fold higher at 24 h after birth (Zhang et al. 1997). This increase in growth is driven by accelerated cell proliferation in the crypt stem cell compartment of the intestine (Kelly and Coutts 2000). Microvilli begin to cover the apical surface of the small intestinal epithelium up to the adulthood, and the intestinal surface provides the largest interface between the outside environment and the internal milieu (approximately 2,000,000 cm^2, which is about the size of a tennis court). The increase in surface area during development has significant implications in terms of nutrient absorptive capacity (Neu 2007b).

1.3 The Characteristics of Functional Development of Gastrointestinal Tract

1.3.1 Digestion and Absorption

The function of the GIT including the esophagus is, to a large degree, mechanical. Contents received from the esophagus and stomach are propelled further down the

intestine and mixed with secreted fluids to digest and absorb the food constituents. The peristaltic transport of material in tract is a neuromuscular function affected by a number of factors (Arndorfer et al. 1997). Prenatally, GIT functional maturation of pig may be more dependent on internal signals (genetic, endocrine) than on luminal stimuli (enteral nutrients and bioactive factors) (Sangild et al. 2002). Postnatally, the digestive and absorptive capacity of neonatal pig is highly sensitive to change in the enteral dietary input (Jensen et al. 2001).

Absorption of nutrients, as measured by the uptake of monosaccharides and amino acids by the intestinal mucosa, is low during the first half of gestation of fetal pig but increases rapidly thereafter (Buddington and Malo 1996; Kong et al. 2012). During the final weeks of gestation of fetal pig, there is a particularly rapid increase in glucose uptake capacity (Sangild et al. 1993), while the ability of the mucosa to take up most amino acids, including lysine, remains unchanged (Sangild et al. 2000).

The primary factors in feeding premature infants are dependent on the development and maturation of digestion and absorption. The maturation of digestive and absorptive functions of carbohydrates, proteins, fats, minerals, and vitamins in neonate are determined in relation to availability of hydrolytic enzymes, such as lipases, proteases, amylases, glucosidases, and lactase (Lebenthal and Lebenthal 1999). Most digestive-absorptive processes are well developed in late preterm infants, and there should be very few limitations in terms of digestion and absorption of lactose, long-chain lipid, or protein (Neu 2006).

1.3.2 Protein

Protein digestion is initiated within the stomach, with the secretion of both acid and proteolytic enzymes (pepsin and papain). The protease zymogens are produced mainly by the chief cells of the fundic mucosa, and their activation and activity in the gastric lumen are stimulated by an acidic environment (pH 2.0–4.0) (Deren 1971). At the first 24–48 h after birth, the intragastric pH remains at about 5.5–7.0 (whereas the gastric pH in normal children and adults is 2.0–3.0) (Neu 2007b).

The enzymes in the small intestine (trypsin, chymotrypsin, and pancreatic proteases) perform the main function of digesting protein (Mayer 2003). The digestive cascade of pancreatic protease is catalyzed by food-stimulated secretion of enterokinase from the upper small intestinal epithelium. Enterokinase catalyzes the activation of trypsinogen to trypsin (Neu 2007b). The breakdown of large polypeptides into small dipeptides and tripeptides accomplishes two tasks: it allows for the process of digestion and absorption of nutrients to occur, and it renders potentially immunogenic proteins nonimmunogenic (peptides $<$8–10 amino acids in length are little immunogenic) (Mayer 2003). Active luminal enterokinase is detectable at 24 weeks gestation of human fetus but its activity level is relatively low and reaches only 25 % adult activity at term (Antonowicz and Lebenthal 1977). Similar to a lack of gastric acid, low enterokinase activities may limit protein

digestion and may be responsible for an increased capability of larger antigens and/or microorganisms to pass into the intestine without breakdown by luminal enzymes. After luminal digestion, small peptides and amino acids are absorbed and transported to the villus capillaries. The proteases are usually present and fully active before 24 weeks gestation of human fetus and should not be limiting (Lebenthal et al. 1983). This suggests that hydrolyzed protein would be better tolerated.

Absorption of amino acids mainly occurs in the proximal region of the small intestine of pigs (Buraczewska 1981). The intestinal mucosal cells absorb amino acids via active transport, simple diffusion, and facilitated diffusion. There are at least four sodium-dependent amino acid transporters in the luminal plasma membrane of the intestinal mucosal cells responsible for transporting amino acids into the cytoplasm. The capacity for macromolecular absorption is very important in newborn pigs, which relies on passive immunity from colostral immunoglobulins (Lin 2006). The fetal type of enterocytes responsible for macromolecular uptake is present at birth.

There is little knowledge about the rates of amino acid absorption across the apical membrane during development. The rates of absorption for five amino acids using intact tissues from the proximal, mid-, and distal small intestines of pigs are ranged in age from 90 % of gestation to 42 days after birth (12 days after weaning) (Buddington et al. 2001). The results suggest that the rates of absorption (sum of carrier-mediated and apparent diffusion) were highest at birth (except for proline) and declined by an average of 30 % during the first 24 h of suckling.

1.3.3 Lipid

Fats are essential components of the diet and have a critical role in the growth and development of the neonate. The digestion of lipid can be split into several phases (Hamosh 1996). The luminal phase of lipid digestion involves lipase-catalyzed de-esterification of triglycerides to 3-monoglyerides and free fatty acids, and bile acid-mediated micellar solubilization. Bile acids synthesized in the liver are critical to efficient fat digestion and absorption (Neu 2007b). In the newborn and particularly in premature infants, pancreatic secretion of lipase and bile salt secretion are relatively inadequate (Carey and Hernell 1992). Briefly, the next phases, lipid absorption and assimilation, include the permeation of fatty acids and 2-monoglycerides from the lumen into the cell, intracellular re-esterification, chylomicron formation, and transport of the chylomicrons from the cell into the circulation (Neu 2007b).

Developmental deficiencies in luminal lipid digestion by pancreatic lipases and solubilization by bile acid micelles that result in “physiological” fat malabsorption in the newborn, as well as compensatory mechanisms such as gastric lipase digestion, have been well studied and are reviewed elsewhere (Black 2007).

1.3.4 Saccharide

During the final weeks of gestation of fetal pig, there is a particularly rapid increase in glucose uptake capacity (Sangild et al. 1993). Active glucose transport systems are operating in the intestine of 17-week-old human fetuses (Eggermont 1991). Maturation of mechanisms for carbohydrate absorption occurs in a defined sequence during human fetal development (Kien 1996). Disaccharidases are present by 8 gestational weeks of human fetuses, their concentrations increase around 14 weeks of gestation and then progress steadily to term values. Glucoamylase, a specific intestinal hydrolytic enzyme, develops very well by the 13 gestational weeks (Eggermont 1969). Trehalase appears by the 13 gestational weeks of human fetuses (Schmitz 1991).

The intestinal epithelial absorptive enzymes, such as lactase, sucrase, maltase, isomaltase, and glucoamylase, are at mature levels in the term newborn human neonate (Antonowicz and Lebenthal 1977). However, small intestinal lactase activity of human neonate is low relative to the other disaccharidases (Antonowicz and Lebenthal 1977). Despite this limitation, these infants appear to adapt rapidly to lactose feedings and also possess intestinal microflora that have the capability to ferment lactose into two and three carbon fragments that are absorbed in the distal intestine and utilized for ATP production (lactose salvage pathway) (Kien 1996).

Postnatal adaptive responses to ingested carbohydrates lead to efficient carbohydrate absorption. Inadequately absorbed carbohydrates are salvaged by colonic flora through fermentation of carbohydrates to hydrogen gas and short-chain fatty acids. The latter are readily absorbed through the colon epithelium. In this setting, carbohydrate tends to be absent from the stool. Noninvasive reflection of the status of carbohydrate absorption may be obtained from breath hydrogen testing, a technique of particular value in young infants (Mobassaleh et al. 1985).

1.3.5 Gastrointestinal Motility

There is a critical point of development at around 24–25 weeks of gestation when peristalsis is observed in all human fetuses (Sase et al. 1999). The small bowel motility patterns of human fetuses are poorly developed before 28 weeks gestation. The disorganized small intestine motility patterns of human fetuses between 27 and 30 weeks gestation, progressed to a more mature pattern so that the migrating myoelectric complexes are present at 33–34 weeks of gestation (Berseth 1996). Gastro-anal transit ranges from 8 to 96 h in premature human infants as compared with 4–12 h in adults (Neu 2007b). The motility of the small intestine is considerably less organized in premature human infants than in term infants (Berseth 1996). This is caused by an intrinsic immaturity of the enteric nervous system that delays transit, causing subsequent bacterial overgrowth and distension from gases that are the byproducts of fermentation (Neu 2007a). In preterm human infants, the motilin receptor is not present until 32 weeks gestation, and the cyclic release of motilin is not present (Neu 2007b).

1.3.6 Microbial Community

The resident microbiota confers many benefits to the gastrointestinal physiology of the host and is, therefore, an example of a truly symbiotic relationship (Hooper and Gordon 2001; Tang et al. 2013). Further benefits become visible in the period after birth, when the complexity of the intestinal environment increases considerably while changing from an exclusively milk-containing diet to an adult diet after weaning (Rumbo and Schiffrin 2005). During this period, the intestinal microbiota plays a crucial inductive role in the intestinal development. After birth, the intestinal microbiota takes some time before developing a stable community (Gaskins 2001). During the first few weeks after birth, microbial succession in the GIT of humans (Favier et al. 2002) and pigs (Moughan et al. 1992) is remarkably similar. After birth, the germ-free GIT is rapidly colonized by anaerobic and facultative anaerobic bacteria. Profound changes occur in the intestinal ecosystem when young mammals are weaned from their mother's milk (Neu 2007b). After weaning, clostridium, ruminococcus, enterococcus, and enterobacter spp. appeared, and microbial DGGE profiles became more complex and also more stable with increasing age (Bauer et al. 2006).

A cross-talk exists between microbes and the intestine whereby stimulation of the secretion of peptides by Paneth cells promotes angiogenesis, growth, and also an environment that prevents the growth of potentially pathogenic microorganisms (Hooper and Gordon 2001; Rumbo and Schiffrin 2005; Gaskins 2001; Favier et al. 2002; Moughan et al. 1992; Bauer et al. 2006; Neish 2002). Most very premature infants in the neonatal intensive care unit (NICU) are started on broad-spectrum antibiotic therapy shortly after birth during a "rule-out sepsis" workup. This can alter the normal flora with which the neonate would become colonized (Kosloske 1994). Rather than lactobacillus, bifidobacter, or other symbiotic microorganisms, resistant species indigenous to the NICU may colonize the infant's intestines. The commensal microflora may represent a key regulatory checkpoint for the intestinal inflammatory response. The intestinal epithelium partially relies on toll-like receptors (TLRs) to act as an interface between the luminal microflora and cellular signal transduction pathways. TLRs are cell surface receptors that recognize specific microbial ligands, from both pathogens and commensals, which enables the innate immune system to recognize non-self and activates both innate and adaptive immune responses (Takeda et al. 2003). Recent studies suggest that the epithelium and resident immune cells do not simply tolerate commensal microorganisms but are dependent on them (Rakoff-Nahoum et al. 2004). This is important for not only disease entities that we see in the NICU such as NEC, which has recently been shown to decrease with the use of probiotics (Vinderola et al. 2005), but also diseases that affect the infant later in life, such as allergy and atopy (Kalliomaki et al. 2001).

1.3.7 Immunology Function

The GIT is the largest organ in the immune system of the body, and as such is the location for the majority of lymphocytes and other immune effector cells. Most apparent are the functions of the Peyer's patches and other aggregated lymphoid follicles, which contain germinal centers where B and T lymphocytes proliferate and become antigen-specific lymphocytes, IgA secreting plasma cells, and memory cells (Koutsos and Arias 2006).

The immune system also acts to ensure tolerance to "self," to food and other environmental components, and to commensal bacteria. The development of tolerance is the result of active immune mechanisms requiring antigen-contact and acting in a T cell dependent fashion (Faria and Weiner 2005; Taylor et al. 2006). Both development and maintenance of tolerance are lifelong processes which start very early in life, even prenatally. Profound immunologic changes occur during pregnancy, involving a polarization of T helper (Th) cells towards a dominance of Th2 and regulatory T cell effector responses in both mother and fetus (Reinhard et al. 1998; Raghupathy 2001). Mature CD^{4+} and CD^{8+} T cells can be detected in the fetus as early as around week 20 in human pregnancy (Michaelsson et al. 2006). Therefore, during the third trimester of human pregnancy, fetal T cells are able to mount antigen-specific responses to environmental and food-derived antigens and antigen-specific T cells are detectable in cord blood in virtually all newborns indicating in utero sensitization (Szepfalusi et al. 2000). Although the fetus at term may be sensitized to certain antigens, it does lack a fully functional immune system and has a sterile GIT. Changes occur at, and soon after, birth in order to allow the immune system of the neonate to become competent and functional; and also to allow the gut to become colonized with bacteria. Pregnancy, the suckling period, and the periods during which formula and solid foods are introduced offer windows during which nutrition might affect the immunologic development of the fetus and young infants (Philip et al. 2006).

The mucosal immune system fulfills the primary function of defense against potential pathogens that may enter across vulnerable surface epithelia. However, a secondary function of the intestinal immune system is to discriminate between pathogen-associated and "harmless" antigens, expressing active responses against the former and tolerance to the latter. There are considerable evidences to indicate that the major functions of the mucosal immune systems are defective at birth (Adkins et al. 2004). Passive transfer of maternally derived antibody clearly has a protective effect and is likely to provide "cover" during a period when the neonatal immune system is insufficiently developed to respond actively (Bailey et al. 2005). Two important periods of maximum exposure to novel antigens occur in the young animal, immediately after birth and at weaning (Bailey et al. 2005). In both cases, the antigenic composition of the intestinal contents can shift suddenly, as a result of a novel diet and of colonization by novel strains of bacteria. Thus, birth and weaning are likely to represent hazard and critical control points in the development of appropriate responses to pathogens and harmless dietary and commensal antigens.

In the newborn animal, factors in maternal milk may regulate responses (TGFb) as well as providing passive effector molecules (Ig). At weaning these factors are removed and there is evidence that the balance may be disturbed. The magnitude and severity of this response are likely to be dependent on the extent to which the effector and regulatory arms of the immune system have been expanded during the preweaning period.

The intestinal barrier is critical in terms of preventing bacterial translocation and initiating the inflammatory response, which might affect the well-being of not only the intestine but distal organs such as the lung and central nervous systems as well (Liu et al. 2005; Speer 2004). The intestinal barrier consists of epithelial, immunologic, luminal, and mucosal factors that control antigen entry and the generation of immunologic phenomena in the gut (Lu and Walker 2001; Isolauri 2001). Probably the most significant barrier to antigen entry into the mucosa-associated lymphoid tissue is the presence of enzymes starting in the mouth and extending down to the stomach, small bowel, and colon (Mayer 2003). The next layer of the barrier is the epithelial cell (Mayer 2003). The microvillus membrane from newborn rabbit is inherently more disordered than microvillus membrane from adult animals. This difference in membrane organization might in part account for the increased attachment and penetration of macromolecules noted during the perinatal period (Pang et al. 1983).

The intrinsic barrier consists of the physical structures of the intestine. In particular, the tight junction between the epitheliums is a formidable barrier to entry of foreign material (Sanderson 2003). In inflamed states, as well as in the perinatal period, the tight junctions are less "tight," allowing for the passage of macromolecules into the underlying lamina propria (Mayer 2003). The components of the intestinal mucosal barrier are underdeveloped at birth and respond inappropriately to luminal stimuli (Walker 2002). As a result of this immature, inappropriate response to microbial stimuli, certain infectious diseases occur in greater frequency during infancy.

1.4 The Effects of Nutrients on Gastrointestinal Tract Development

The neonatal GIT requires nutrients to support their proliferation and differentiation as well as the secretion of enzymes, proteins and other materials (Koutsos and Arias 2006). The neonate is immunologically naive and is very susceptible to infection and damage from harmful antigens. Early nutrition plays an important role in protecting the developing intestine from harmful agents and in modulating immune responses following antigenic challenge (Kelly and Coutts 2000). Feeding represents a major change in the infant's internal environment, with the flow of swallowed amniotic

fluid being replaced by colostrum, breast milk, or formula. Changes in dietary composition and intake undoubtedly have many highly significant effects on the growth and function of GIT.

Maternal colostrum and milk. Colostrum is characterized by a high concentration of IgG and relatively lower concentrations of IgA and IgM (Curtis and Bourne 1971; Klobasa and Butler 1987). The concentration of IgG in colostrum is several-fold higher than in sow plasma and declines rapidly during the first 24 h of secretion. Maternal colostrum and milk not only provide the newborn with nutrition but also confer passive immunity. For larger animals, such as horse, cow, pig, and sheep, colostrum is necessary for getting passive immunity. For animals such as rabbit and Guinea pig, transition of the antibodies are finished before birth. In addition, maternal milk might have the capacity to directly stimulate immune function (Kelly and Coutts 2000). Maternal milk provides a diverse range of substances that are developmentally delayed in the neonate and are thought to play a critical role in gastrointestinal defending. Maternal milk contained substances, including growth factors and cytokines, promotes the maturation of the intestine and the associated immune system (Kelly and Coutts 2000).

Breastfeeding is often considered as superior to infant formula feeding. When milk was infused directly into the rumen of calf, resulting in SCFA production, papillary growth was stimulated (Tamate et al. 1962). The most significant advantage of breast milk on the neonatal intestine might not be related to growth per se, but mucosal barrier and immune function (Burrin and Stoll 2002). This idea is based on the mounting evidence that breast milk is clearly superior to formula, with regard to, the incidence of infection and sepsis in neonatal infants (Iiboshi et al. 1994). Cytokines and lymphocytes present in breast milk can influence the development of the immune system. This suggests an immunoregulatory role for breast milk that is absent in infants consuming formula (Rees 2005). In addition, milk contains a large number of other components with antimicrobial activity including complex carbohydrates, glycoproteins, glycolipids, glycosaminoglycans, mucins, and oligosaccharides (Kelly and Coutts 2000).

1.4.1 Dietary Protein

Dietary protein is major macronutrient required for GIT growth of fetus and neonate. Dietary protein is also important for the maintenance of the immune system and influences the response to infection through several mechanisms (Millward 1999). One mechanism is the maintenance of gut barrier function, through the provision of threonine, cysteine, and other amino acids involved in the synthesis of mucus glycoproteins (Gil and Rueda 2002). Another is maintenance of general immunocompetence through the provision of specific amino acids for synthesis of cellular proteins of the immune system (Gil and Rueda 2002).

1.4.2 Individual Amino Acids

Individual amino acids have been proposed to act as signaling molecules that serve to regulate mRNA translation. Arginine is generally considered essential in neonates, because the metabolic pathways that synthesize this amino acid are not fully developed in neonates (Wu et al. 2004). Arginine is the most abound nitrogen carrier in tissue protein and may be responsible for maximal growth of young mammals (Flynn et al. 2002). Glutamine is a unique amino acid in that it functions as a key respiratory fuel for rapidly proliferating cells such as erythrocytes and lymphocytes and also as a precursor of nucleic acids, nucleotides, amino sugars, amino acids, and glutathione (Souba 1991). The amide nitrogen of glutamine is thought to be critical in the biosynthesis of nucleotides and hexosamines. Glutamine and nucleotides appear to act synergistically in the intestinal epithelial proliferation and differentiation (Huang et al. 2003). Dietary glutamine, through conversion to glutamate, probably contributes significantly to the intestinal glutathione synthesis (Duggan et al. 2002).

1.4.3 Short-Chain Fatty Acids

There is considerable evidence for the specific intestinal trophic effects of short-chain fatty acids (SCFA) on GIT development. Intraluminal infusions of SCFA have a stimulatory effect on colonic mucosal proliferation of neonatal rat. This effect is dose-dependent and vary among the SCFA's (butyrate > propionate > acetate) (Sakata 1987). For normal development of the ruminal epithelium to progress, a viable ruminal fermentation must be established, suggesting that there is a requirement for the presence of SCFA in the ruminal lumen to promote normal papillary development (Sander et al. 1959). Similarly, Lane and Jesse (1997) reported that infusing 50 % of the lamb's estimated net energy requirement in the form of SCFA at physiological concentrations resulted in increases in papillae length. Infusions of sodium propionate and sodium butyrate, but not sodium acetate, sodium chloride, or glucose, resulted in marked development of the ruminal papillae in calves (Tamate et al. 1962). A mixture of SCFA salts (propionate and butyrate included) fed as 10 % (wt/wt) of a concentrate starter ration resulted in increased incidence of ruminal parakeratosis, and, in all treated animals, a thickening of the stratum corneum was reported in calves (Gilliland et al. 1962) and lambs (Rickard and Ternouth 1965).

1.4.4 Long-Chain Fatty Acids

The study of the role of dietary long-chain fatty acids (LCFA) in pediatric nutrition is mainly focused on whether some of the n-3 long-chain, polyunsaturated fatty acids (LC-PUFA) improve the health and development of infants (Heird 2001; Li et al. 2011).

The n-3 LC-PUFA's or omega-3 fatty acids, particularly docosahexanoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (AA), have pleiotropic biological effects on immune function, inflammation, hemodynamics, and bone metabolism. A series of studies have demonstrated that n-3 LC-PUFAs enhance intestinal adaptation after small-bowel resection and their effects are greater than those of less saturated oils (Vanderhoof et al. 1994). The mechanisms for these differences between fatty acids remain unclear, although studies have observed increased circulating PYY concentrations with fish oil feeding. In contrast to the evidence that LCFA's are beneficial to intestinal growth and well-being, studies with neonatal piglets indicated that the LCFA oleic acid can cause significant mucosal injury and increased permeability. The latter effect is more severe in newborn than in 1-month-old piglets (Velasquez et al. 1994).

The best described mediator of the responses to fatty acids is the peptide cholecystokinin (CCK), probably through enteroendocrine cells (EECs). However, it still remains to be proven that the sensing apparatus is intrinsic to EECs, which may function as conduits for transepithelial signals (McLaughlin 2007).

Indeed, the current basic model for nutrient sensing implicates EEC cells as transepithelial signal transduction units, detecting intraluminal nutrient signals apically and secreting regulatory peptides or biogenic amines basolaterally to affect appropriate physiological responses. This can include classical endocrine effects and paracrine effects on adjacent cells, especially afferent nerve terminals

1.5 The Negative Impacts of Early Malnutrition on Gastrointestinal Tract Development

The early nutrition including fetal and early postnatal periods is particularly important, and the lack of any nutrient consumption would delay the growth of the intestine and of the other tissues. The fetal hypothalamic–pituitary–adrenal (HPA) axis has numerous key roles in the development of organisms. The previous studies showed that maternal undernutrition could act as a "programming" factor to produce a reduction in responsiveness of the fetal HPA axis (Hawkins et al. 1999). A severe state of protein-energy malnutrition significantly diminished intestine length of rats when compared with control animals (Plagemann et al. 2000). Intestinal weight and total DNA were similarly diminished in the malnourished rats (Hatch et al. 1979). Length and weight of small intestine of rats feeding low protein diet were lower than those of rats feeding normal protein (Deng and Qiu 2003).

1.6 Summary and Future Perspectives

This chapter has described areas relating to the development of GIT. The interactions among the components of intestinal ecological system (that are host intestinal anatomy, microbial populations, immune systems, and nutrition of the animal) are

indeed extremely complex. The immature intestine and immune system leave the neonate susceptible to viral and bacterial attack. Maternal milk appears to have a host of factors that can mature the function of neonatal GIT. The early nutrients also have important impacts on the morphological and functional development of neonate. We need a better understanding of the characteristics and mechanisms of development pertaining to gastrointestinal morphology, digestion, absorption, immune and microbial community at all stages of prenatal and postnatal period, in especial on cell proliferation and functional maturity in pigs. Furthermore, we need to divide the early stage of life into different time stage according to the characteristics and mechanisms of development of gastrointestinal morphology and function, and identify the pertinent regulatory factors for the development of gastrointestinal morphology and function.

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Chapter 2
Development of Digestive Glands in Pigs

Zhiru Tang, Zheng Ruan, and Yulong Yin

Contents

2.1 Introduction

The digestive glands include the pancreas, liver, and the gallbladder. The pancreas plays a central role in the digestive system by synthesizing and secreting specific digestive enzymes, and thus has an integrative role for the absorption and metabolism of nutrients in the organism. The liver performs numerous physiological and metabolic functions for the pig's whole body, but the most important one is maybe metabolism of

Z. Tang
College of Animal Science and Technology, Southwest University, Chongqing 400715, China

Z. Ruan
State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, China

Y. Yin (✉)
Chinese Academy of Sciences, Institute of Subtropical Agriculture, Research Center for Healthy Breeding Livestock & Poultry, Hunan Engineering & Research Center of Animal & Poultry Science, Key Laboratory of Agro-ecological Processes in Subtropical Region, Scientific Observing and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Changsha, Hunan 410125, People's Republic of China
e-mail: yinyulong@isa.ac.cn

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_2,

nutrients and toxins arising from digestion and/or absorption in the intestines. The pig's liver also produces bile, involved in lipid digestion. Bile is secreted into the intestinal system via the gallbladder. In pig, as in other mammals, the liver is involved in protein synthesis, toxin and hormone breakdown, vitamin storage as well as blood cell and blood component processing. The gallbladder is a small sac involved in bile storage. Bile is concentrated in the gallbladder. The gallbladder is located at the back of the liver. It is connected with the liver via the cystic duct and the hepatic bile duct.

2.2 Development of the Pancreas in Pigs

The pancreas is both an exocrine and an endocrine organ. Digestive enzymes are conducted via a duct system to the duodenum, and the endocrine products enter the blood directly. The bulk of the tissue in the pancreas is exocrine in nature.

The exocrine pancreas produces pancreatic juice, which contains enzymes, nonenzymatic proteins, and salts. These substances reach the duodenum via a single pancreatic duct in the pig (van den Borne et al. 2007). Enzymes able to digest proteins, carbohydrates, lipids, and nucleic acids, respectively, are produced in the acinar cells of the exocrine pancreas (Malagelada 1981; Pitchumoni et al. 1986). Electrolytes capable of neutralizing the acidic stomach contents and establishing the proper pH for enzymatic action are produced mainly in the intra-acinar and ductal cells (Schulz 1980). The islet cells of the endocrine pancreas produce hormones (e.g., insulin, glucagon) that regulate the utilization of the absorbed digestion products (Sitbon and Mialhe 1978; Christensen and Just 1988; Motyl et al. 1986; Malmlef et al. 1990). In the embryo, the pancreas develops as an outgrowth of the endoderm; the cell layer that delineates the primitive gut. Pancreatic progenitors derived from the endoderm form two buds (dorsal and ventral) which later fuse to form a single organ. Within these buds, the progenitor cells give rise, through a stepwise process, to endocrine, acinar, and duct cells.

The neonatal period is a very important developmental stage in the life of pig. The pancreas, an important digestive organ responsible for production of various hydrolytic enzymes, also shows a marked tissue mass gain (up to 80 %) during the first 2 postnatal days in newborn pigs (Widdowson and Crabb 1976; Tarvid et al. 1994).

In naturally suckled piglets, both body weight and pancreas weight increased with age. The pancreatic weight increased much faster than the body weight, particularly during the first postnatal day. The ratio of pancreas weight to body weight is significantly greater in 1-day and 3-day-old piglets than in newborn piglets. Mubira (1998) demonstrates that the pancreas growth is disproportionally faster than the body taken as a whole, the protein concentration in the pancreatic tissue is lower in 1- and 3-day-old than in the newborn piglets, The concentration of DNA in the pancreatic tissue remains unchanged with age and the total contents of protein and DNA increase progressively with age. Mubira (1998) also demonstrates that pancreatic lipase activity expressed either as per gram tissue weight, per milligram

protein or per milligram DNA is significantly lower in 1- and 3-day-old piglets when compared with newborn piglets, the enlargement of the acini is apparently due to an increase in the cell numbers although the difference among the three groups of animals in the number of cells per acinus did not reach a significant level and the size of the acinar cells did not change significantly with age.

However, the mechanisms responsible for this rapid pancreatic tissue growth are far from being completely understood, but are apparently related to colostrum ingestion since starved, water-fed, or formula-fed newborns show little changes (Tungthanathanich et al. 1992; Burrin et al. 1992). Further studies have shown that porcine colostrum contains a number of hormones and growth-promoting peptides, such as insulin, cortisol, epidermal growth factor (EGF), bombesin, and insulin-like growth factors-I and-II (IGF-I and -II) (Jaeger et al. 1990) and epithelial-like cells (Cera et al. 1987). The size of acinar cells decreased significantly in piglets fed 5 % lactose solution while it increased significantly in piglets fed porcine colostrum. The acinar cell mitotic index estimated from the incorporation rate of Brdu increased significantly in piglets fed either porcine colostrum or trypsinized porcine colostrums, the index being even greater in the trypsinized-colostrum group than in the colostrum group (Mubira 1998).

The rapid pancreatic tissue growth in naturally suckling piglets is apparently related to ingestion of colostrum. Widdowson and Crabb (1976) found that rapid pancreatic growth would not occur if piglets were prevented from sucking and fed water only. Mubira (1998) study showed that pancreatic weight increased significantly in piglets bottle-fed porcine colostrum both in absolute and relative terms, but no increment was observed in the piglets bottle-fed lactose solution. All these observations indicate that colostrum may contain some trophic factors capable of stimulating pancreatic growth in newborn animals.

A number of growth-promoting peptides and hormones, such as EGF, IGF-I, IGF-II, bombesin, and insulin, have been found in the mammary gland secretions in pigs and in several other species (Jaeger et al. 1987; Donovan et al. 1994; Simmen et al. 1988; Read et al. 1984; Westrom et al. 1987). The concentration of these peptides is generally high in colostrum and often several fold greater than that in the maternal circulation (Donovan et al. 1994; Simmen et al. 1988; Read et al. 1984). There is evidence showing that these colostrum growth-promoting peptides are stable in the gastrointestinal lumen in suckling pigs (Shen and Xu 1996) and in suckling rats (Philipps et al. 1997) as it can be absorbed intact into the blood circulation in newborn pigs (Xu and Wang 1996) and in suckling rats (Thornburg et al. 1984). It has also been found in laboratory rodents that pancreatic cells possess specific receptors for EGF, IGF-I, and IGF-II (Potau et al. 1984; Korc et al. 1983; Williams et al. 1984). It was observed in an earlier study that newborn piglets fed infant milk formula supplemented with IGF-I or IGF-II had heavier pancreas and greater pancreatic RNA and DNA contents than did the control animals (Xu et al. 1994). Subcutaneous administration of bombesin and EGF stimulated pancreatic growth in rats (Papp et al. 1987; Dembinski et al. 1982) and EGF and insulin stimulated mouse pancreatic acinar cell growth in vitro (Logsdon 1986).

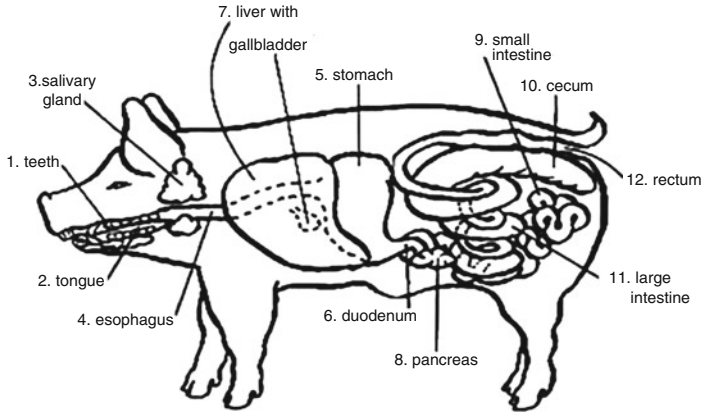

Fig. 2.1 The location of pig's liver and gallbladder

2.3 The Structure and Function of the Liver in Pigs

The liver is the largest internal organ, and the body' second largest, after the skin (Baruch 2000). The liver and its functions are referred to as the hepatic system, from the Greek word meaning liver. Schematically, liver metabolism can be partly view as the readjustment of molecules into a form usable by the pig's body tissues. In the case of toxins, liver metabolism converts poisonous molecules into less harmful molecules, finally eliminated into the urine.

2.3.1 The Structure of the Liver

As shown in Fig. 2.1, the liver in pigs is located behind the diaphragm in the abdominal cavity, connected to the digestive system through the bile ducts, gallbladder, and duodenum. Blood supply to the liver is approximately 75 % from the venous system, directly from the intestines. Pig livers have four main lobes for filtering toxins and nutrients from the bloodstream: the right, left, caudate and quadrate. Bile ducts connect the lobes to the gallbladder, where excess bile is stored until needed.

Four lobes are surrounded by a capsule of fibrous connective tissue called Glisson's capsule. The Glisson's capsule in turn is covered by the visceral peritoneum (tunica serosa), except where the liver adheres directly to the abdominal wall or other organs. The parenchyma of the liver is divided into lobules, which are incompletely partitioned by septa from Glisson's capsule (Ekataksin and Wake 1991). The parenchyma within the lobules is supported only by fine reticular fibers (which are discernible only with special preparations).

The blood vessels supplying the liver (portal vein and hepatic artery) enter at the hilum (or porta hepatis), from which the common bile duct (carrying bile secreted by the liver) and lymphatic vessels also leave (Fig. 2.2).

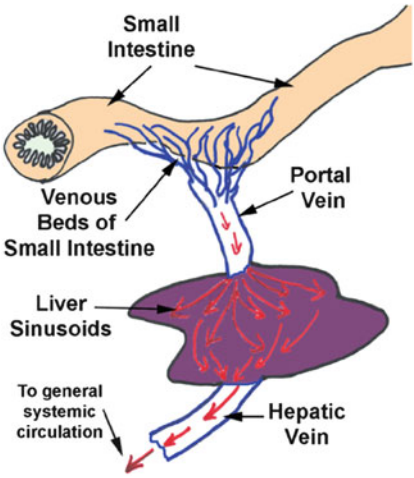

Fig. 2.2 The liver's structure and anatomical placement

Within the liver sinusoids, the oxygen-poor but nutrient rich blood from the portal vein mixes with the oxygenated blood from the hepatic artery. From the sinusoids, the blood enters a system of veins which converge to form the hepatic veins. The hepatic veins follow a course independent of the portal vessels and enter the inferior vena cava. Liver cells, or hepatocytes, are large polygonal cells, usually tetraploid and often binucleate in the adult (Sen and Jalan 2005). Each nucleus has two or more nucleoli. The average life span of liver cells is 5 months. They contain abundant rough endoplasmic reticulum and mitochondria, large deposits of glycogen and lipid droplets of various sizes, and several small elaborate Golgi complexes. They also contain many peroxisomes, a variable amount of smooth endoplasmic reticulum and lysosomes (Vega 2004). In standard histological preparations, liver cells usually appear vacuolated because the glycogen and lipids are removed during processing. Liver cells are capable of considerable regeneration when liver substance is lost (Harada et al. 2009).

2.3.2 The Function of the Liver

The liver is extremely versatile, having both endocrine and exocrine functions. Liver is involved in numerous metabolic activities and acts as a storage depot. The list below is an attempt to categorize its numerous and diverse activities.

2.3.2.1 Blood Supply

As mentioned above, the liver receives a dual blood supply. The hepatic portal vein provides the majority of the incoming blood, the rest coming from the hepatic artery. The blood from the portal vein has already supplied the small intestine, pancreas, and spleen, and is largely deoxygenated. It contains nutrients and noxious

materials absorbed in the intestine. The blood from the hepatic artery supplies the liver with oxygen. Because the blood from the two sources intermingles as it perfuses the liver cells, these cells must carry out their many activities under low oxygen conditions that most cells would not be able to tolerate.

2.3.2.2 The Protein and Lipid Synthesis

The liver synthesizes a number of proteins, as suggested by its abundant rER and Golgi. One of the most important of these is albumins (responsible for several functions including colloid osmotic pressure), the protein parts of several kinds of lipoproteins, nonimmune alpha and beta globulins, prothrombin, and numerous glycoproteins, including fibronectin. These proteins are considered as endocrine secretions because they are released directly from the liver cells into the blood supply. The liver also synthesizes cholesterol and the lipid portion of lipoproteins. The enzymes for these activities are found in the sER.

2.3.2.3 Metabolic Functions

The liver is involved in a wide range of metabolic activities. Among its major metabolic functions are gluconeogenesis (the formation of glucose from non-carbohydrate precursors), and the deamination of amino acids. In addition to the proteins listed above, the liver also releases a number of other products directly into the blood as endocrine secretions. These include products of carbohydrate metabolism, e.g., glucose released from stored glycogen, and modified secretions from other organs, e.g., triiodothyronine (T3).

2.3.2.4 Storage of Metabolites

Liver cells store large deposits of glucose in the form of glycogen, which can be stained by the periodic acid-Schiff or PAS procedure. They also store lipids in the form of droplets of varying sizes. The latter can be revealed with Sudan staining after tissue fixation. The removal of these materials in routine preparations gives liver cells a foamy or vacuolated appearance. Lysosomes within hepatocytes may be a normal storage site for iron, in a form of a ferritin complex. Excessive amounts of iron may accumulate in cells as an unusable yellowish-brown pigment called hemosiderin, a partly denatured form of ferritin. Liver lysosomes also contain pigment granules (lipofuscin) and partially digested cytoplasmic organelles. The liver also stores vitamins, especially vitamin A, which is transported from the liver to the retina to be used in the formation of visual pigments. Most of the vitamin A is not stored in the hepatocytes, however, but in special fat-storing cells, called Ito cells, within the sinusoids. Ito cells also store the other three fat-soluble vitamins, D, E, and K, as well as vitamin B12.

2.3.2.5 Detoxification and Inactivation of Noxious Substances

The liver is, after the gastrointestinal tract, the first organ to receive metabolic substrates and nutrients from the intestine, but it is also the first to receive any toxic substances, for instance carcinogens or drugs that have been ingested. The sER of liver cells contains enzymes involved in the degradation and conjugation of toxins (in addition to those responsible for synthesizing cholesterol and the lipid portion of lipoproteins). Under conditions of challenge by drugs, toxins or metabolic stimulants, the sER becomes the predominant organelle. Stimulation by one drug, e.g., alcohol, enhances its ability to detoxify some other compounds. On the other hand, some toxins can get metabolized by the liver to even more damaging compounds.

2.4 The Structure and Function of the Gallbladder in Pigs

The gallbladder is one of the most dispensable organs in mammal. The gallbladder is a small sac and is the storage depot for bile. Bile is only concentrated in the gallbladder. The gallbladder is located at the back part surface of the liver (Fig. 2.1). It is connected with the liver via the cystic duct and the hepatic bile duct. Together they form the common bile duct, which leads into the small intestine.

2.4.1 The Structure of the Gallbladder in Pigs

The gallbladder is a tubular organ. Its tunica mucosa is a very regular simple columnar epithelium, with no goblet cells or glands. The deep folds in the bottom of the mucosa, which are often cut in cross section, sometimes look like "glands," but they have no secretory activity. There is a scanty lamina propria, and a thin tunica muscularis. There is no muscularis mucosae, and this is one of the "landmarks" for this organ. The gallbladder is only partially covered by a fold of peritoneum; its opposite side is seated in a pocket in the surface of the liver. Bile ducts carry bile to the gall bladder for temporary storage.

The low magnification image above shows the walls of the gallbladder (Fig. 2.3). They vary in height depending on how full the bladder is. At higher magnification, the nature of the gallbladder epithelium is revealed: a simple columnar form, containing no goblet cells. Though these cells do have a few microvilli on their free surfaces, they cannot be considered as real brush borders. There is no muscularis mucosae in these structures, and the tunica muscularis is stringy and scanty with collagen fibers. Since it has only the function of squirting bile into the duct that carries it to the duodenum, it appears little developed.

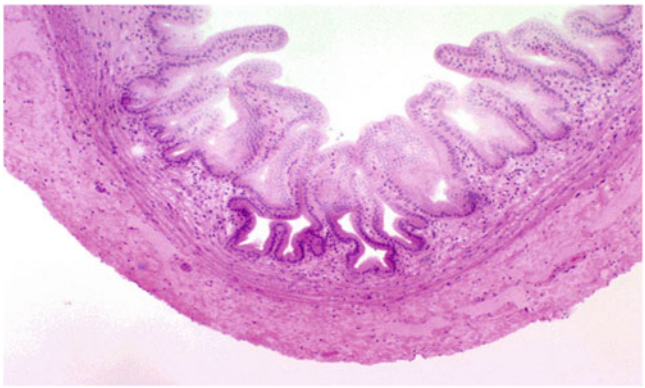

Fig. 2.3 The wall of the gallbladder at the low magnification image

2.4.2 The Function of the Gallbladder in Pigs

Bile is continuously formed in bile canaliculi in the liver hepatocytes, were it is collected and secreted into the hepatic bile duct. Bile is a complex fluid containing water, electrolytes, and a battery of organic molecules including bile acids (water-soluble derivatives of cholesterol), cholesterol, phospholipids, and bilirubin (Mcmaster and Elman 1926). The rate of bile fluid secretion is dependent on the circadian rhythm of the animal.

Bile is the exocrine secretion of the liver, released into ducts which eventually form the common hepatic duct. It, in turn, unites with the cystic duct from the gallbladder to form the common bile duct which enters the duodenum.

Bile contains bile salts, also called bile acids, which are important in emulsifying the lipids of the digestive tract, thereby promoting easier digestion by lipases and absorption. Bile salts are largely recycled between the liver and gut.

Bile also contains cholesterol and phospholipids, and another function of bile salts is to keep them in solution. Cholesterol and phospholipids serve as metabolic substrates for other cells and as precursors for components of cell membranes and steroid hormones. They are largely reabsorbed in the gut and recycled.

Bile also contains pigments, which detoxify bilirubin, the breakdown product of hemoglobin from aging and of abnormal red blood cells destroyed in the spleen. The detoxified bilirubin, mainly in the form of bilirubin glucuronide, is eliminated through the feces (and gives them their color). Failure of the liver to absorb bilirubin, or to conjugate it to its glucuronide, or to secrete the bilirubin glucuronide, may result in jaundice. Finally, bile contains electrolytes (largely reabsorbed in the gut). The latter maintain bile as an isotonic fluid.

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Chapter 3
Development and Renewal of Intestinal Villi in Pigs

Huansheng Yang, Xia Xiong, and Yulong Yin

Contents

Abbreviations

HNF-1α	Hepatocyte nuclear factor-1α
Cdx1	Caudal-related homeobox protein 1
Cdx2	Caudal-related homeobox protein 2
VFE	Vacuolated fetal-type enterocytes
SEM	Scanning electron microscopy
CVA	Crypt–villus axis
TCF	T-cell factor

H. Yang • X. Xiong • Y. Yin (✉)
Chinese Academy of Sciences, Institute of Subtropical Agriculture, Research Center for Healthy Breeding Livestock & Poultry, Hunan Engineering & Research Center of Animal & Poultry Science, Key Laboratory of Agro-ecological Processes in Subtropical Region, Scientific Observing and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Changsha, Hunan 410125, People's Republic of China
e-mail: yinyulong@isa.ac.cn

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_3,

BMP	Bone morphogenetic protein
TGF-β	Transforming growth factor beta
GATA 4	GATA-binding transcription factor 4
GATA 5	GATA-binding transcription factor 5
GATA 6	GATA-binding transcription factor 6
HES1	Hairy/enhancer of split homolog 1
Atoh1	Atonal homolog 1
EAAC1	Excitatory amino acid carrier 1
SGLT1	Sodium/glucose cotransporter 1
SLC6A19	Solute carrier family 6, member 19
ASCT2	Alanine-serine-cysteine transporter 2
PP	Polish landrace/Pietrain
DHW	Duroc/Hampshire/wild boar
GLUT5	Glucose transporter member 5
ENS	Enteric nervous systems
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
EGF	Epidermal growth factor

3.1 Introduction

The gastrointestinal mucosa of pig not only acts as an organ for diet digestion and absorption but also plays an important role in fighting against pathogenic bacteria and toxic substances presented in intestinal lumen. The development of gastrointestinal mucosa begins in the early embryo and self-renews during the whole life. Its homeostasis is preserved via regulating the proliferation, growth arrest, differentiation, and apoptosis of epithelial cell. Moreover, the developmental and renewal processes of gastrointestinal mucosa are regulated by various factors, such as genetic, neural, hormonal and dietary influences and disturbed by weaning stress. Pig gastrointestinal mucosa is not a very common experimental model, and therefore, some information discussed in this chapter is obtained from other species.

3.2 Structural Development

3.2.1 Ontogeny of the Intestinal Mucosa

The growth and ontogenetic development of the mammalian small intestine is a topologically and temporally highly organized process, which decides about the formation of specialized intestinal epithelium that is defined by a set of genes expressed in different epithelial cells. These specialized cells fulfill various important physiological roles, including digestive and absorptive functions, certain

endocrine and immunological roles, and secretion of water, electrolytes, and mucus (Pácha 2000). The early ontogenetic development starts during gestation but the most dynamic changes occur before and after birth. This developmental process can be divided into five phases: (1) morphogenesis, (2) cytodifferentiation and fetal development including preparation of the epithelium for processing colostrum and milk, (3) birth and colostrum suckling period, i.e., the shift from an intrauterine to an extrauterine environment, (4) suckling period, and (5) weaning transition of the offspring from mother's milk to a solid diet (Henning 1981; Menard and Calvert 1991; Pácha 2000). The first two phases occur during prenatal life and prepare the intestine for the postnatal life.

3.2.1.1 Morphogenesis

The mammalian small intestine develops from a simple tubular structure in the early embryo to a mature, highly specialized and organized organ system. In the early embryonic development, the anterior and posterior invaginations of visceral endoderm form a primitive gut tube which is lined by an undifferentiated, stratified epithelium. The primitive gut tube can be divided into three parts: foregut, midgut, and hindgut. Among the three parts, the midgut is destined to become the small intestine. During the developmental process, the midgut endoderm undergoes rapid remodeling converting the stratified epithelium into a simple columnar epithelium. At the same time, the underlining mesenchyme grows upward and forms finger-like projections (nascent villi). The formation of nascent villi and microvilli occurs simultaneously. Then the cellular proliferation along the nascent villi and in the intervillus region forms a proliferating compartment known as the intervillus epithelium. Then, this intervillus epithelium goes through reshaping to form crypts by penetrating into the underlying mesenchyme. In addition to crypts and villus, the proximal colon of the human fetus or neonatal rodents has transient villus-like structures (Ménard et al. 1994). Although they are not true villi, these villus-like transient structures are characterized by properties similar to enterocytes of the small intestine. They are not characterized only by their capability of endocytosis (Ono 1977), but also by the fact that they are equipped with brush-border enzymes (Lacroix et al. 1984; Zweibaum et al. 1984; Foltzer-Jourdainne et al. 1989) and transporters. Moreover, results from chimeric mice revealed that neonatal crypts and adult crypts do not have similar cellular organization. In the neonatal period, the crypts contain cells of a mixed genotype. In contrast, the crypts of mature intestine are composed with cells of one genotype which are derived from a single progenitor (Schmidt et al. 1988).

3.2.1.2 Cytodifferentiation and Fetal Development

Cellular phenotypes are defined by the expression of specific sets of genes in individual cells. The conversion of the stratified epithelium into a simple columnar epithelium in a proximal-to-distal wave is called cytodifferentiation, and the

expression of certain intestine-specific genes is first detected during this period (Davis et al. 2004). The expression of individual genes in the intestine is regulated by specific transcription factors, such as HNF-1α, Cdx1, and Cdx2 (Boudreau et al. 2002). The cytodifferentiation involves cytoplasmic membranes which belong to two domains: the apical and basolateral membranes with biochemical, morphological, and functional differences (Amerongen et al. 1989). This process determines the polarity of epithelial cells because only the basolateral membrane seems to adhere to the basement membrane of the extracellular matrix and the apical membrane is exposed to the luminal gastrointestinal content. The apical and basolateral membranes are separated by different complex protein systems including tight junctions which form the paracellular apical junctions of intestinal epithelia. Tight junctions are composed of specific proteins such as occludin (Furuse et al. 1993), claudins (Itoh et al. 1999), and zonula occludens (Stevenson et al. 1986), which interact with the actin cytoskeleton to stabilize the tight junctions at the apical end of the cell. This conjunctive intestinal epithelium provides the largest mucosal barrier between the internal host and the external environment.

At the same time than the formation of crypts and villi, four epithelial lineages differentiate from the immature primitive cells: absorptive enterocytes, goblet cells, endocrine cells, and Paneth cells (Cheng and Leblond 1974a, b). Absorptive enterocytes, constituting up to 90 % of epithelial cells in the crypt and more than 95 % of villus cells, are absorptive cells with an apical microvillus membrane that contains transporters, receptors, and membrane-anchored hydrolases (Cheng and Leblond 1974a, b; Traber et al. 1991). Goblet cells secrete protective mucus which is essential for the maintenance of the gut epithelial integrity (Forstner 1978). Endocrine cells are involved in local and systemic metabolism by secreting various growth factors and hormones (Xu and Zhang 2003). The Paneth cells are involved in the modulation of the whole body metabolism through the secretion of cytokines (Shanahan 1997). The developing intestine has the capacity to establish and maintain the functional differences of each cell lineage in the differentiation programs, and this capacity of the intestinal epithelium is already apparent in the late fetal life (Roth et al. 1991; Simon and Gordon 1995). It needs to be emphasized that enterocytes in fetal and newborn mammals are not the same as those of adult mammals. Indeed, enterocytes of fetal and newborn mammals have an apical canalicular system leading to production of large vacuoles which are important for colostrum macromolecules uptake. The vacuolated fetal-type enterocytes (VFE), located at the upper part of villi, are firstly observed in the small intestine proximal region of the pig and lamb fetuses, then in the second trimester of pregnancy, and subsequently in the middle and distal regions. After birth this fetal-type enterocytes are gradually replaced by enterocytes lacking apical canalicular system (Skrzypek et al. 2007a).

3.2.1.3 Birth and Colostrum Suckling Period

The pig gut is exposed to various stress factors particularly in the early development period. In response to the stress, the gut goes through profound changes resulting in

accelerated tissue growth and functional maturation (Le Dividich and Seve 2000; Trahair and Sangild 2004; Xu et al. 2000). After birth, changes are induced by dramatic shift from parenteral to enteral nutrition and a vast number of bioactive substances present in the colostrum and milk, and the morphology of the small intestine of piglets experiences great changes during the first 3 days after birth. Xu et al. (1992) reported a 72 % increase in small intestinal weight, a 115 % increase in mucosa weight, a 24 % increase in small intestinal length, a 15 % increase in small intestinal diameter, a 33–90 % increase in villus height and a 14–51 % increase in villus diameter during the first day of piglets. It was also demonstrated that the cellular population in the small intestinal mucosa, as indicated by the DNA content, increased progressively with age, and increased from 84 to 154 % during the first 3 days. Such dramatic changes result from the increase of local intestine blood flow in parallel with the reduction of the basal vascular resistance, the accumulation of proteins of the colostrum in enterocytes, and changes in epithelial cell turnover with increasing mitosis and inhibition of apoptosis (Xu et al. 1992). The types of enterocytes also changed rapidly during this period, especially in the duodenum and in the proximal jejunum. The VFE in the duodenum are only seen at birth. In the proximal jejunum, the VFE are present in the upper part of the villi, and are replaced by mature enterocytes until the day 3 of life. VFE in the mid and distal jejunum and in ileum diminishes gradually after birth and disappears until the day 21 of life (Skrzypek et al. 2007a).

Recently, scanning electron microscopy (SEM) has been used as a powerful tool to observe the surface of the intestine in three dimensions, enabling observations up to single microvillus. At birth, the surface of the mucosa of duodenum is folded and most of it is covered by finger-like villi. Only few villi are branched or incompletely divided (Skrzypek et al. 2005). The villi surface is not smooth, and many transversal furrows are apparent. Transverse incisions on the villi surface, with at least one quarter of villi circumference, can be observed. The enterocyte brush border is well developed, and the microvilli at the edge of enterocyte are shorter than in the central apical area. On the third day of life, the villi of duodenum are finger-like, and the surface of the villi corpus is smoother and the transversal furrows are thinner than at the day of birth (Skrzypek et al. 2005, 2010; Fig. 3.1). In jejunum, the villi of unsuckling neonatal piglets are thin with finger-like shape, but are not uniform in length, with shorter villi in between the taller ones. Because of numerous deep transversal furrows, the villi surface is rough and the apical enterocyte membrane is increased. Compared to the duodenum, the jejunum has relatively more goblet cells. On the third day after birth, there are abundant villi of various lengths in jejunum. The transversal furrows on villi surface are shallower than at birth but still numerous, and no well-defined extrusion zone can be observed (Skrzypek et al. 2005, 2007b, 2010; Fig. 3.2). In ileum, the mucosa is covered by the villi of various sizes at birth. The villi are of flat finger-like shape, as if they are not completely divided. In contrast to duodenum and jejunum, the enterocyte in ileum are of various sizes and the enterocyte seems to be with large lysosomal vacuoles. The goblet cells are more abundant than upper parts of the gut. At the day 3 after birth, the ileal villi are still of flat finger-like shape, but many of them are partly-divided.

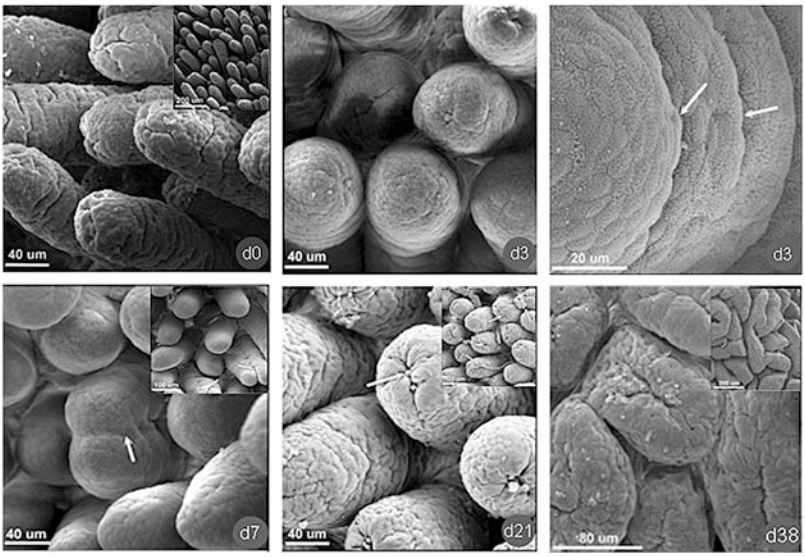

Fig. 3.1 SEM micrograph of duodenal mucosa on day 0 (at birth), day 3, day 7, day 21, and day 38 after birth in neonatal piglets (original images from J Physiol Pharmacol. 2005, 56 Suppl 3:71–87)

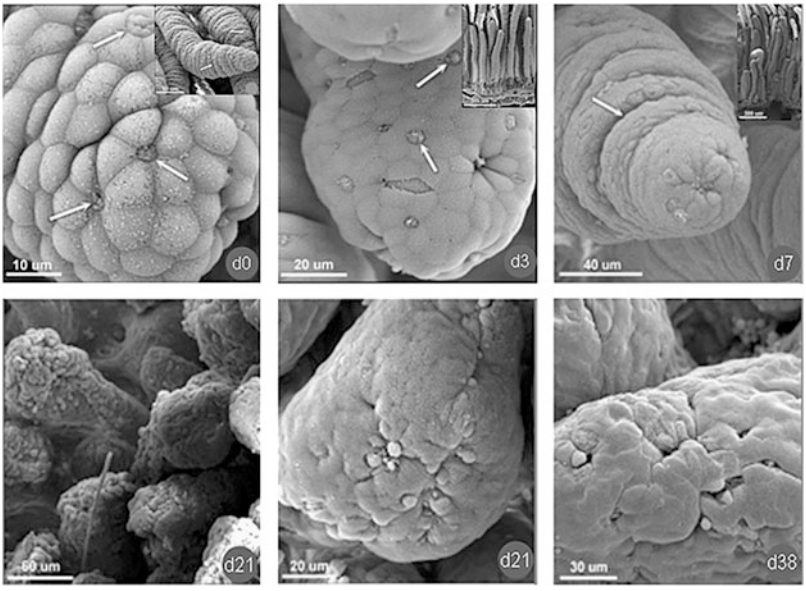

Fig. 3.2 SEM micrograph of jejunum mucosa on day 0 (at birth), day 3, day 7, day 21, and day 38 after birth in neonatal piglets (original images from J Physiol Pharmacol. 2005, 56 Suppl 3:71–87)

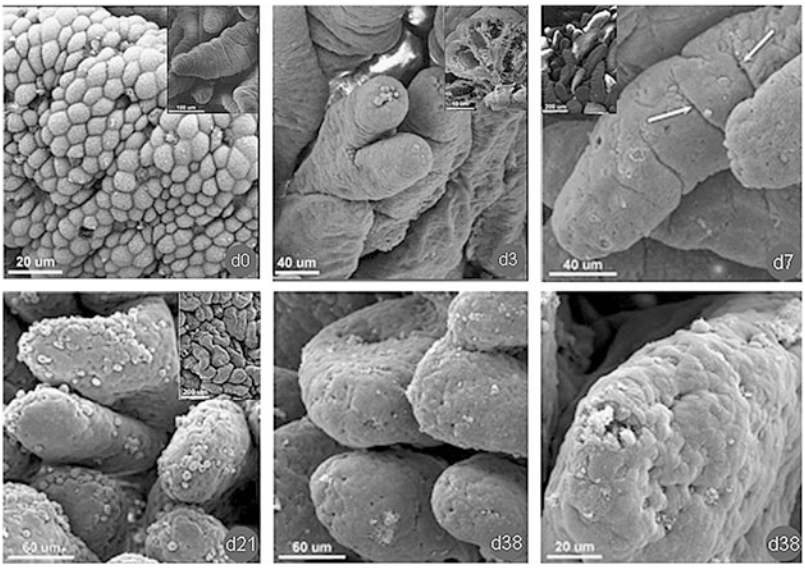

Fig. 3.3 SEM micrograph of ileum mucosa on day 0 (at birth), day 3, day 7, day 21, and day 38 after birth in neonatal piglets (original images from J Physiol Pharmacol. 2005, 56 Suppl 3:71–87)

Like duodenum, the surface of villi is relatively smoother, but some large enterocytes still exist on the top of villi (Skrzypek et al. 2005, 2007b, 2010; Fig. 3.3).

3.2.1.4 Suckling Period

The weaning of piglets usually takes place between 3 and 4 weeks of life (Boudry et al. 2004; Zabielski et al. 2008; Zhang et al. 2012a). During the sucking period, there is an intense rebuilding of small intestine, and the most intensive processes are noted in the epithelium (Boudry et al. 2004; Zabielski et al. 2008). In duodenum, although the architecture of villi on day 7 after birth is similar to that at birth, the transversal furrows are much shallower and less numerous than at day 0. Moreover, the density of villi decreases by 58 % and 8 % compared with birth and day 3 of life, respectively. At day 14, villi are of irregular shape and the villi density is significantly decreased (Skrzypek et al. 2005, 2010). At the day 21 after birth, although most of villi are finger-shaped, a few twin-shaped villi or villi with longitudinal indention are present. Corresponding to the increase in villi length, the number of transversal furrows is further reduced on the surface of villi. In addition, the apex of duodenal villi is not smooth any more since the presence of deep, knife incision-like, furrows and "cell packets" are apparent. These "cell packets" are made of groups of enterocytes that underwent apoptosis (Skrzypek et al. 2005, 2007b, 2010; Boudry et al. 2004; Fig. 3.1). In the jejunum, the shape of villi at day 7 change from finger-like to flat and tongue-like with the transversal furrows being located on the

entire villi. Moreover, the thickness of mucosa significantly decreased and the crypt depth increased by 81 % in comparison with that measured at birth. At day 14, the depth of crypt further increased and achieved the highest value (Skrzypek et al. 2010; Fig. 3.2). On the 21st day of life, the shape of most of the jejunal villi changed to wide tongue-like; and the depth and total numbers of transversal furrows is further reduced. The villus tips are still containing numerous goblet cells. In ileum, the villi on the day 7 are of finger-like, leaf-like and tongue-like shapes, and the villi surface is relatively smooth but still with some transversal furrows. On day 21 after birth, the mucosa is covered by villi with a great diversity in shape, and the villi surface is shed by numerous cells including a large number of goblet cells (Skrzypek et al. 2005, 2007b, 2010; Fig. 3.3).

3.2.1.5 Weaning Transition

During weaning transition, piglets undergo nutritional, environmental, and psychological stresses, with concomitant marked changes in the structure of the small intestine. The most marked structural changes of the small intestine during weaning transition are a reduction in villus height and an increase in crypt depth (Pluske et al. 1997; Fan et al. 2004). Following weaning at day 21, the villus height of piglets reduces to 75 % of the preweaning value at 1 day after weaning, and further declines to approximately 50 % of the preweaning value at 5 days after weaning (Hampson 1986). Crypt depth of duodenum, jejunum, and ileum also increases with age after weaning (Gu et al. 2002; Kelly et al. 1991; van Beers-Schreurs et al. 1998). Associated with the reduction in villus height and the increase in crypt depth, the morphology of the villi are also changed. In duodenum of weaned piglets (38 day old), the villi change from finger-like to leaf-like, with a wide irregular base with no transversal furrows. At that time, few micrometer spaces, on the top of villi, between the epithelial cells as well as partly broken cells are observed (Skrzypek et al. 2005; Fig. 3.1). In jejunum, following weaning on day 38 after birth, the mucosa become thinner, and the villi are of various shapes: tongue-like and fold-like with predominant incomplete division projections. In addition, no transversal furrows are observed, and there are numerous epithelial cells shed along the villi surface (Fig. 3.2). In the ileum of weaned piglets, the villi are dominated by tongue-like shapes. However, single finger-like and leaf-like villi as well as incompletely divided villi are still present. On the surface of the villi, the transversal furrows are not observed, and numerous shed cells are observed in the apical region (Skrzypek et al. 2005; Fig. 3.3). In addition, the structural changes in the small intestine are affected by the age of weaning. The changes in the piglets weaned at 14 days of age are more conspicuous than those weaned at 21 days of age (Pluske et al. 1997).

3.2.2 Epithelial Cells Differentiation Along the Crypt–Villus Axis

The maturation of the intestinal epithelium is completed at the beginning of adulthood, which forms a complex equilibrium system with multiple cell types

(Cheng and Leblond 1974a, b; Mutoh et al. 2002). This equilibrium system undergoes continual renewal that involves highly coordinated processes of cellular proliferation, lineage-specific differentiation, migration, and apoptosis along the crypt–villus axis (CVA) (Gordon and Hermiston 1994). The epithelial cells differentiation along the CVA is observed during gestation. In rodent intestine, it is firstly apparent between day 17 and day 18 of gestation, when the endoderm is converted to an epithelial monolayer overlying nascent villi (Clatworthy and Subramanian 2001; Mathan et al. 1976). Stem cells located near the base of crypts give rise to progenitor cells, which expand through rapid proliferation before undergoing cell cycle arrest and ultimately differentiation into four principal cell lineages: absorptive enterocytes, goblet cells, endocrine cells, and Paneth cells (Clatworthy and Subramanian 2001). The fate of epithelial cells depends on the direction of their migration. After division, some cells go down to the bottom of the crypts and are transformed into Paneth cells with a life-time averaging 20 days. Most of cells migrate up the villus and differentiate into enterocytes, goblet cells, and endocrine cells. These cells are ultimately shed into the intestinal lumen every 3–5 days (Karam 1999). Microarrays results also establish that markers of enterocyte and goblet cell differentiation are maximally expressed in villus cells, whereas Paneth cell markers are up-regulated in crypt cells (Mariadason et al. 2005). Many signaling pathways and transcription factors with regulating functions upon small intestine cell maturation are identified. The known signaling pathways that are implicated in the regulation of cell fate determination and lineage specification in the intestine include Wnt–beta-catenin–TCF (Korinek et al. 1998; Mariadason et al. 2001; van de Wetering et al. 2002), BMP-TGF-beta-SMAD (Batts et al. 2006; He et al. 2004), Notch and its downstream factors HES1 and Atoh1 (Yang et al. 2001), and hedgehog signaling (van den Brink et al. 2004). Many transcription factors, including cdx-1 and cdx-2, kruppel-like factor 4, GATA4, 5, and 6 (Gao et al. 1998), and several forkhead family members (Burgess 1998; Clatworthy and Subramanian 2001; Shie et al. 2000), have also been suggested to be involved in the regulation of the intestinal cell maturation. Some of these transcription factors represent downstream targets of the different signaling pathways. Moreover, integrin-mediated cell-substratum and E-cadherin-mediated cell–cell adhesion, chemotactic gradients, as well as a lot of cytokines, hormones, and growth factors, have also been involved in the regulation of intestinal cell maturation (Burgess 1998; Kedinger et al. 1998). Lastly, microarrays and proteomic analysis suggest that intestinal cell maturation is primarily regulated at the transcriptional level according to the significant correlation between proteomic changes and corresponding gene expression changes along CVA (Chang et al. 2008).

3.3 Functional Development

The ontogeny of the intestinal mucosa function is discussed in Chap. 1 (Development of digestive glands in pigs) and Chap. 4 (Terminal digestion of polypeptides and amino acid absorption by the pig intestine epithelial cells during development).

This section mainly focuses on the functional development of mucosa along CVA. Epithelial cells differentiation CVA is accompanied by its functional specialization. There are four approaches in studying the differentiation-dependent expression of enterocyte function in vivo, including serial sectioning technique, quantitative immunohistochemical analysis, quantitative cytochemical analysis, and sequential cell isolation in combination with biochemical and biomolecular analysis (Smith 1985). Among the four approaches, the first three approaches are difficult to quantify and limited by availability of specific antibodies. Sequential cell isolation in combination with biochemical and biomolecular assays is a useful approach to analyze digestive enzymes and nutrient transporters activities in differentiating enterocytes under various conditions. With this approach, Raul et al. (1977) analyzed the activities of alkaline phosphatase, enterokinase, aminopeptidase, sucrase, amylase in villus and crypt cells of normal rats from 5 days after birth until 8 weeks. These authors found out that the activities of enterokinase and alkaline phosphatase were always located in the upper villus during postnatal development; whereas aminopeptidase and sucrase activities appeared in the crypt cells after birth and then rose to villus during the fourth week of life. The activity of amylase was located along the entire CVA during the first 5 days of life, and then reached its maximum activity in crypt. However, after the fourth week, the maximum activity was detected in the upper villus. Furthermore, Rowling and Sepúlveda (1984), by using sequential cell isolation in combination with biochemical analysis, found a 2–3-fold increase in the number of Na^+-pumping sites accompanying cell differentiation in rabbit jejunal epithelium. In neonatal pigs, Fan et al. (2001) examined the activities of alkaline phosphatase, aminopeptidase N, sucrase, lactase, and Na^+/K^+-ATPase along the crypt–villus using sequential cell isolation in combination with biochemical analysis. The activity of alkaline phosphatase increased quadratically during the enterocyte differentiation along the CVA in both the proximal and the distal small intestine. In addition, aminopeptidase N and sucrase activities showed a linear pattern of increase accompanying enterocyte differentiation along the CVA in both the proximal and the distal segments. Moreover, lactase activity increased cubically during the enterocyte differentiation along the CVA. In enterocytes, total Na^+-ATPase activity includes two components: a ouabain-sensitive Na^+/K^+-ATPase activity and a ouabain-insensitive Na^+-ATPase activity. Both ouabain-sensitive and ouabain-insensitive Na^+-ATPase activities are increased when enterocytes differentiated along the CVA in the small intestine. The ability of nutrient absorption is also altered when enterocytes differentiate along the CVA in the small intestine. The maximal transport activity of L-glutamate was increased during the enterocyte differentiation along the CVA of neonatal porcine small intestine, but the transporter affinity of L-glutamate was decreased during enterocyte differentiation (Fan et al. 2004). At the same time than the L-glutamate uptake alteration, the expression of EAAC-1, the major glutamate transporter, was increased with neonatal porcine enterocyte differentiation along the CVA; and its expression was regulated both at the transcription and translation levels (Fan et al. 2004). As opposed to EAAC1, there is a high level of maximal

SGLT1 uptake activity along the CVA of neonatal porcine small intestine. Although the mRNA abundance of SGLT1 is increased during enterocyte differentiation, there is no significant difference in SGLT1 protein abundance between crypt and villus (Yang et al. 2011). Similar to SGLT1, the SLC6A19 mRNA abundance is increased during enterocyte differentiation along the CVA. However, the B^0AT1 protein is evenly expressed in the epithelium along the CVA. In addition, apical maximal Na^+-Gln uptake activity, which is largely modulated by B^0AT1, is expressed along the entire jejunal CVA (Yang 2011). In contrast, the mRNA abundance of ASCT2, an intestinal AA exchanger, is decreased with neonatal porcine enterocyte differentiation along the CVA, but no difference in ASCT2 protein expression is observed (Buddington 1992).

3.4 Factors Influencing Intestinal Mucosa Development

The survival of animals and humans require physiological regulation of the intestinal mucosa operating as a functional unit. This contributes to the maintenance of epithelial homeostasis by forming a selective barrier to the harsh environment of intestinal lumen. The formation of this functional unit begins in the early embryo and completes at the beginning of adulthood. Moreover, the intestinal epithelium undergoes continual renewal all along life. These developmental and renewal processes are influenced by various factors, such as genetic, neural, hormonal, and dietary factors and weaning stress.

3.4.1 Genetic Influence

Intensive growth of the piglet small intestine is faster than growth of the whole organism due to intensive remodeling of the epithelium. This remodeling is regulated by genetic influence. Skrzypek et al. (2007b) compared the postnatal development of small intestinal mucosa architecture in Polish landrace/Pietrain (PP) and Duroc/Hampshire/wild boar (DHW) crossbreed piglets by scanning electron microscopy. They found differences in villi shape modification, in transversal furrows disappearance, in extrusion zone formation and in the presence of apoptotic cell packets, reflecting differences of PP and DHW piglets in mucosa structure development and renewal. With the age-related alteration in mucosa architecture, marked changes in nutrient transporters and enzymes were also observed during the suckling and weaning periods. Detailed studies of digestive enzymes and nutrient transport showed that the age-related changes in enzymes and nutrient transporters are genetically programmed and little affected by diet or hormones (Henning 1980; Leeper and Henning 1990; Nanthakumar and Henning 1993; Toloza and Diamond 1992). Moreover, molecular biology analysis showed that the ontogenic mechanisms involved in intestine apical fructose transporter GLUT5 expression and function are independent of dietary signals (Davidson et al. 1992; Shu et al. 1997). Although the development and renewal of mucosa can be reprogrammed by

interactions of genetic determinants with other factors, it is ultimately controlled by transcription regulation via multiple transcriptional elements with activatory or repressive roles (Traber and Silberg 1996).

3.4.2 Neural Influence

The enteric nervous systems (ENS), a large network of neurons and glial cells located along the gastrointestinal tract, provide an intricate network for the reflex control of intestinal mucosa (Pácha 2000). In prenatal animals and humans, the nutrients and biologically active substances involved in intestine development are mainly transferred from the mother via placenta. The contribution of ENS is presumably small but increases with fetal development (Zabielski 2007). However, in adults, almost all main gut processes such as secretion, absorption, immune responses, blood flow and complex motility patterns (such as mixing, peristalsis and migrating motor complexes) are regulated or controlled by the ENS (Burzynski et al. 2009). In comparison with adults, virtually nothing is known about the possible involvement of the nervous system in mediating mucosa structure and function development. Nevertheless, it has been suggested that the ENS function was different between early postnatal development and adulthood. Evidences suggest that ENS is involved in the regulation of the development of intestinal motility, and maybe also other function of mucosa (Zabielski 2007; Burns et al. 2009).

3.4.3 Hormonal Influence

The role of hormones in intestinal mucosa development was studied more intensively than that of ENS. A large number of hormones and cytokines have been shown to affect intestinal mucosa development and nutrients transport (Zabielski 2007). The hormones of the IGF family, including insulin, insulin-like growth factor I (IGF-I) and insulin-like growth factor II (IGF-II), showed positive effects on intestinal mucosa development and intestinal adaptation (Ben et al. 2010; Lund 1998; Lemmey et al. 1991). Treatment with oral insulin significantly increased enterocyte proliferation, and decreased cell apoptosis, in rats (Ben et al. 2010). IGF-I was also reported as able to enhance crypt cell migration and cell proliferation (Liao and Lönnerdal 2010; Chen et al. 1999). Moreover, transforming growth factor alpha, epidermal growth factor, and hepatocyte growth factor are also able to promote crypt cell proliferation as measured by the ^{3}H-thymidine incorporation assay (Nishimura et al. 1998; Sheng et al. 2006). In recent years, many newly discovered hormones or regulatory peptides have been also reported as able to regulate intestinal mucosa development. Leptin, whose receptor is widely distributed in the small intestine mucosa, has been shown to enhance small intestinal length and mitotic index, and to reduce the percentage of vacuolated enterocytes in the small intestine of neonatal piglets (Słupecka et al. 2005). Ghrelin, a growth-hormone-releasing acylated peptide, is able to delay piglet intestinal mucosa

development by reducing the length of intestinal villi and increasing crypt depth (Kotunia et al. 2006). Glucagon-like peptide-2 also involves in the stimulation of stem cell proliferation and in the simultaneous reduction of the programmed cell death in neonatal piglets, thus acting as an important regulator of growth and maturation of the small intestinal mucosa (Burrin et al. 2005).

3.4.4 Dietary Influence

The compounds in diet not only play a nutritional role but also directly stimulate the growth and development of the intestinal mucosa (Buts et al. 1993; Yao et al. 2011; Kong et al. 2012b). The alterations in dietary input (amniotic fluid, maternal milk, postweaning diet) during the ontogeny of the intestinal mucosa impose different functional demands in relationship with its structure (Buddington 1994). Similarly, amniotic fluid, maternal colostrum and milk contain many biologically active substances that stimulate the development of the intestinal mucosa (Weaver et al. 1988; Xu 1996). Although the family of biologically active substances in milk is continuously increasing, EGF, IGF-I, glucocorticoids, and insulin were confirmed as compounds with important roles in stimulating epithelial cells proliferation and differentiation (Burrin et al. 1996; Buts et al. 1997; Houle et al. 1997; Xu 1996; Yeh et al. 1991). The importance of nutrients in amniotic fluid for mucosa development, and the ability of the fetal intestine to absorb exogenous nutrients were confirmed by infusing galactose into amniotic fluid of rabbits. This infusion increases fetal mucosal weights and total intestinal capacities to transport aldohexoses (Buchmiller et al. 1992). Although milk is the principal source of nutrients for most suckling mammals, the composition of milk is different between species (Jenness and Sloan 1970). In addition, the regulatory role of maternal milk in intestinal mucosa development is not only dependent on the content of nutrients and non-nutrient components, but also depends on the gradual alteration of the milk composition (Keen et al. 1981). The milk for rat pups exceeds the demand during the first week of life, whereas it becomes the limiting factor during the second week. The transition from milk to a solid diet results in significantly shorter villi and deeper crypts in the small intestinal mucosa of swine. A variety of nutrient deficiencies can impair animal growth after weaning and some of these deficiencies preferentially target intestinal mucosa development (Williams et al. 1996). The villi heights of rat pups are smaller in all segments of the intestine when there dams were subjected to protein deficiency (Subramoniam 1979).

3.4.5 Weaning Stress

Weaning is one of the most significant event in the pig life, as it corresponds to a transition from milk, which contains highly digestible protein, fat, and lactose, to dry and less-digestible starch-based diet. This transition results in reduced energy intake

for the maintenance of the epithelial structure (Kim et al. 2012). Moreover, weaning of piglets coincides with, or is preceded by, the appearance of adults hydrolytic and transport characteristic of adults, and is accompanied by increases in the circulating concentrations of hormones and cytokines which are involved in the intestinal development (Buddington 1994). In response to weaning, the small intestinal mucosa of piglets undergoes major changes in structure and function (Pluske et al. 1997; Xu et al. 1996). The most obvious structural changes are the reduction in villus height and increase in crypt depth, changes which are suspected to result from the increased rate of cell loss and the increase in crypt cell proliferation (Pluske et al. 1997). It has been shown that villus height is reduced to 75 % of the preweaning value one day after weaning; and then further declines to approximately 50 % of the preweaning value 5 days after weaning (Hampson 1986). Along with the reduction in villus height and increase in crypt depth, the villi morphology also changes from long finger-like projections to leaf- or tongue-like structures (Cera et al. 1988).

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Chapter 4
Terminal Digestion of Polypeptides and Amino Acid Absorption by the Pig Intestine Epithelial Cells During Development

François Blachier, Yulong Yin, Guoyao Wu, and Mireille Andriamihaja

Contents

4.1 Introduction

Terminal digestion of alimentary and endogenous polypeptides followed by absorption of oligopeptides and amino acids are performed by the absorptive intestinal epithelial cells of the small intestine mucosa. The intestinal transport of amino acids is critical for the supply of amino acids to all tissues (Bröer 2008). Although intestinal amino acid absorption in the pig has been the subject of numerous reports, there is a paucity of available data regarding the terminal digestion of peptides in pig enterocytes.

F. Blachier (✉) • M. Andriamihaja
UMR 914 Nutrition Physiology and Ingestive Behavior, INRA-CRNH-IdF-AgroParisTech, Paris, France
e-mail: francois.blachier@agroparistech.fr

Y. Yin
Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, China

G. Wu
Department of Animal Science and Faculty of Nutrition, Texas A&M University, College Station, TX, USA

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_4,

4.2 Neonatal Intestinal Growth in Piglets, Terminal Digestion, and Intestinal Absorption of Amino Acid and Oligopeptides

The major site for the digestion and absorption of N-containing compounds in diets containing wheat and milk protein concentrates in young pigs is in the first 25–50 % of the total small intestine from the pylorus, while for other dietary sources (that are a mixture of wheat and protein concentrates from soya bean and lupins), the major site of digestion and absorption is in the 50–75 % of the small intestine (Leibholz 1985). However, for instance, all villus enterocytes from the small intestine of newborn pigs have been found to be able to transport alanine (Smith 1981). Using neonatal piglets between the ages of 14 and 18 days, it was found that in the small intestine, the activity of aminopeptidase N is higher in the distal than in the proximal segment (Fan et al. 2001a, b). From histological observations, it was concluded that the dipeptidases are predominantly localized in the cytoplasm of enterocytes, and as expected, in the villus enterocytes but not in the crypts (Noren et al. 1977). Dipeptides taken up by villus enterocytes from the lumen of the small intestine are rapidly hydrolyzed to release the constituent amino acids.

In pigs, the very rapid postnatal growth of the small intestine and its mucosal protein as observed by Widdowson et al. (1976) has been attributed to mucosal hyperplasia, mucosal protein biosynthesis, and endocytosis of ingested immunoglobulins (Burrin et al. 1992; Simmen et al. 1990; Werhahn et al. 1981). These rapid changes after birth coincide with changes in intestinal morphology (Xu et al. 1992) and enterocyte ultrastructure (Komuves et al. 1993). In the intestine of neonatal pigs, it has been found that the speed of epithelial cell migration increases from birth until 3 weeks (Klein 1989). Indeed, in piglets, one day after birth, the enterocyte replacement is estimated to take between 9 and 10 days in the proximal region of the small intestine and between 7 and 10 days in the distal region. However, 3 weeks after birth, the cell turnover is considerably shortened to 2–4 days (Moon 1971). From more recent work (Zhang et al. 1997), it has been demonstrated that most of the spectacular changes in intestinal structure and functions occur within the first 6 h of suckling and that the rapid neonatal intestinal growth is due to both hyperplasia and hypertrophy. Measurement of intestinal brush border membrane-associated aminooligopeptidases in neonatal pigs reveals an increase in their enzymatic activities after onset of suckling and suggest that these enzymes are important for hydrolyzing the peptides recovered from milk proteins during the suckling period (Zhang et al. 1997).

The absorption of amino acids and oligopeptides from the intestinal lumen to the portal bloodstream in pigs has been the subject of several studies. In order to move from the luminal content to the bloodstream, amino acids have to firstly enter the intestinal absorptive epithelial cells through transporters present in the brush border membranes at the apical side, and then to be exported from the enterocyte to the bloodstream through transporters present in the baso-lateral membranes. Amino acid absorption has been studied during development in pigs with rates of absorption being measured for five amino acids that are substrates for the acidic

(aspartate), basic (lysine), neutral (leucine and methionine), and imino (proline) amino acid carriers (Buddington et al. 2001). The rates of absorption (which were calculated as the sum of carrier-mediated and apparent diffusion) were highest at birth for all the amino acids considered (except for proline), and then declined after 24 h of suckling. Thereafter, there were declines for leucine, methionine, and proline (but not for aspartate and lysine) in rates of absorption up to 42 days after birth (that is the longest period of time tested, Buddington et al. 2001). Using isolated loops of the proximal, middle, and terminal small intestine of pigs, it has been determined that the absorption capacity of nitrogen from a mixture of proteins, peptides, and amino acids is lower in the proximal small intestine when compared with other regions of the small intestine (Buraczewska 1981). Using the same experimental model, it has been shown that the peptide fraction of the mixture disappeared more rapidly from the intestinal lumen than did the free amino acids, leading the authors to propose that peptidases in the intestinal mucosa play an important role in the process of amino acid absorption (Buraczewska 1981). By measuring the net portal amino acid absorption in pigs (calculated by multiplying the porto-arterial plasma amino acid concentration difference by the portal vein flow rate) after ingestion of different mixtures of proteins and amino acids, it has been determined that free amino acids like lysine and threonine are more rapidly absorbed than protein-bound lysine and threonine (Yen et al. 2004). By measuring amino acid influx across the brush border membrane of the pig ileal epithelium, it has been demonstrated that bipolar amino acids have similar affinities for transport by systems B and ASC (Munck et al. 2000). Using pig jejunal brush border membrane vesicles, Maenz and Patience (Maenz and Patience 1992) have shown that the transport system for L-threonine (system B) is functionally distinct from other amino acid transport systems. In Tibetan suckling piglets, it has been reported that the expression pattern of the amino acid transporter system b(0,+), which mediates apical uptake of cationic amino acids and cysteine, is similar in duodenum and proximal jejunum and the abundance of the transporter mRNA is increased during the suckling period (Wang et al. 2009). Lastly, the Na^+-dependent high affinity X_{AG}^- system and/or the low affinity B° system have been shown to be involved in the transport of L-glutamate from the lumen into the intestinal epithelial cells (Fan et al. 2004).

In a recent work with multi-catheterized minipigs, Remond et al. (2011) have demonstrated that small intestine has the capacity to sequester large amounts of dietary cysteine in the process of absorption of this amino acid, thus limiting its release into the bloodstream. The cysteine may also be utilized for the synthesis of glutathione in enterocytes.

Dipeptide transport in the pig jejunum has been documented (Winckler et al. 1999) as well as the presence of a PepT1-like transporter which is likely to contribute to the overall amino acid absorption from the small intestine. The functional characterization of the pig intestinal peptide transporter PepT1 was studied, and the results obtained have demonstrated that this peptide transporter is able to transport a variety of di- and tripeptides but not tetrapeptides (Klang et al. 2005). Figure 4.1 is shown to illustrate how L-glutamate (taken as an example of amino acid uptake) is transported from the intestinal lumen to the bloodstream.

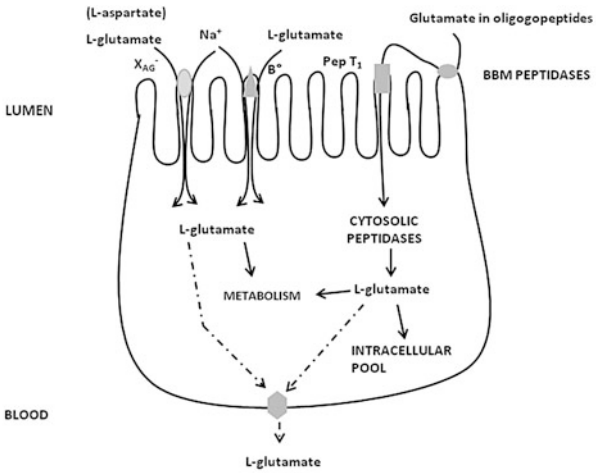

Fig. 4.1 Schematic view of the absorption of amino acids (L-glutamate being taken as an example) from the lumen of the small intestine to the portal bloodstream. L-glutamate can be transported inside enterocytes through the Na^+-dependent high affinity X_{AG}^- system and/or the low affinity B° system present in the brush border membranes (BBM). Oligopeptides (containing, for instance, glutamate) has to be degraded to dipeptides and tripeptides by brush border membranes before being transported inside enterocytes through PepT1 transporters. Then, L-glutamate is released from di and tripeptides by cytosolic peptidases. Intracellular glutamate which is not used for metabolism is released into the portal bloodstream through baso-lateral membranes using transport systems

Amino acid absorption by pig small intestine can be modified by different factors. For instance, the oral administration of insulin-like growth factor-I (IGF-I) to piglets is able to stimulate the Na^+-dependent glutamine absorption by the jejunum without modifying the jejunal mucosal mass (Alexander and Carey 2002). Conversely, phytate, in addition to interfering with protein digestion, may inhibit amino acid absorption (Selle et al. 2012).

Supplementing the diet with alpha-ketoglutarate has been suggested as a mean to spare dispensable amino acids in pigs (Kristensen et al. 2002). Although transporters for di- and tricarboxylic acids have been detected in the pig small intestine, only a small percentage of alpha-ketoglutarate administered lumenally to pigs appears in the portal circulation, a result that is attributed to mucosal metabolism, and possibly to limited absorption by the small intestine (Lambert et al. 2002). Results of experiments involving pigs have suggested that small intestine absorption is probably also affected by low basolateral transfer from the enterocytes to the bloodstream as proposed by Buddington et al. (2004).

4.3 Amino Acid Absorption by the Piglet Colon

There is some evidence which suggest that the colonic epithelial cells of the piglets possess the capacity for the transport of some amino acid from the lumen. For instance, Smith and James (1980) have shown that it is possible to measure unidirectional fluxes of different amino acids across the newborn pig colon. Long-chain amino acids appeared to enter the proximal colon of newborn pigs more readily than did short- chain amino acids (Sepulveda and Smith 1979). The proximal colon of newborn pigs is able to transport methionine from the luminal side, but this capacity markedly decreases in 4-day-old piglets and remains undetectable in 10-day-old piglets (James and Smith 1976). The evolution of the capacity of colonocytes for amino acid transport in the piglets after birth may be related to marked ultrastructural changes of absorptive cells like the reduction of the height of the microvilli within few days following birth (Wooding et al. 1978). However, the nutritionally relevance of amino acid transport by the piglet colon has been questioned (Darragh et al. 1994).

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Chapter 5
Developmental Amino Acid Metabolism in the Pig Small and Large Intestine Epithelial Cells

François Blachier, Guoyao Wu, Yulong Yin, Yongqing Hou, and Mireille Andriamihaja

Contents

5.1 Introduction

From experimental works performed on pigs, it appears clearly that a significant part of several dispensable and indispensable amino acids present in the small intestine content are metabolized during their transcellular journey through the small intestine epithelial cells (enterocytes) when moving from the luminal side of the intestine to

F. Blachier (✉) • M. Andriamihaja
UMR 914 Nutrition Physiology and Ingestive Behavior, INRA-CRNH-IdF-AgroParisTech, Paris, France
e-mail: francois.blachier@agroparistech.fr

G. Wu
Department of Animal Science and Faculty of Nutrition, Texas A&M University, College Station, TX, USA

Y. Yin
Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, China

Y. Hou
Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, Wuhan, China

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_5,

the portal bloodstream. Importantly, this metabolism in enterocytes is different according to the pig developmental stages, and we will see in this chapter that this has important metabolic and physiological consequences. It has been determined that some amino acids are catabolized in enterocytes and some other amino acids are generated by the small intestine epithelium. These results are mostly derived from both in vivo and in vitro experiments. A major in vivo experimental design used to estimate the apparent amino acid intestinal absorption is the porto-arterial balance sheet which consists of measuring the amino acid concentrations at different time following a meal in both the arterial and portal blood as well as measuring continuously the blood flow in the portal vein. This allows to have an estimation of the apparent intestinal absorption of amino acids as well as to presume if a given amino acid is globally degraded or generated during its transport through the intestinal mucosa. Although very informative, these in vivo methods are limited by the fact that the portal vein does not exclusively drain amino acids from the intestine but also from other visceral tissues (notably stomach, pancreas, and spleen). Numerous teams in the world have also used alimentary proteins labelled with stable isotope to estimate the metabolic fate of amino acids during their intestinal absorption and further on in the body.

The situation is further complicated by the fact that a part of both endogenous and alimentary proteins present in the small intestine luminal content can be transferred to the large intestine through the ileocaecal junction (Darragh and Hodgkinson 2000). In the large intestine, microbiota proteases intensively degrade these proteins in peptides and amino acids. Amino acids are not believed to be absorbed through the large intestine epithelium of pigs except in a short period following birth (Darragh et al. 1994). In contrast, amino acids are further converted to numerous bacterial metabolites, some of them being then absorbed through the colonic mucosa or used further by the microbiota (Blachier et al. 2007). Some of these metabolites can also be absorbed through the colonic epithelium and interfere with host metabolism.

The in vitro study of amino acid metabolism in the small and large intestine epithelial cells generally uses isolated living enterocytes and colonocytes for measuring their metabolic capacity towards different amino acids. Although this in vitro design has proven much useful in understanding the metabolic fate of amino acids in intestinal cells, it is only fair to say that the results obtained do not necessarily reflect what is happening in vivo in the intestinal mucosa. Indeed, the intestinal mucosa does not exclusively contain epithelial cells but also numerous other cell types including smooth muscular and immune cells. As a matter of fact, it is tempting to say that the best way to see clearer in this complicated situation of amino acid metabolism in the intestinal mucosa is to compare the data obtained from in vivo and in vitro studies.

In this chapter, we examine the metabolic fate of several amino acids in the intestine epithelium according to the stage of pig development, and we will see that this intestinal metabolism is related to energy supply to enterocytes and colonocytes, to the synthesis of metabolites with important local and peripheral physiological functions, and to the synthesis of other amino acids.

5.2 Metabolic Fate of Glutamate, Glutamine (and Related Amino Acids: Aspartate, Alanine, Proline, Citrulline, and Ornithine) in Pig Enterocytes and Colonocytes

The Na^+-dependent high-affinity X_{AG}^- system and/or the low-affinity B° system have been shown to be involved in the transport of L-glutamate from the lumen into the pig small intestine epithelial cells (Fan et al. 2004). Although Na^+-independent carriers (system L and B° transporters) are present in the brush border membranes of porcine jejunal enterocytes (Fan et al. 1998), the Na^+-dependent B° transport system appears to represent the major route of luminal glutamine absorption (Fan et al. 1998). In piglet small intestine, the oral administration of insulin-like growth factor-I (IGF-I) stimulates Na^+-dependent glutamine absorption (Alexander and Carey 2002).

L-glutamate and L-glutamine are extensively metabolized in pig enterocytes. L-glutamate can be used in enterocytes for protein synthesis (Bos et al. 2005), can enter an intracellular pool (Blachier et al. 2009), or can be extensively metabolized in other pathways including those involved in enterocyte ATP production (Fig. 5.1).

A number of studies have shown that a large proportion of L-glutamate is metabolized during its transcellular journey through pig enterocytes. Indeed, in pigs, virtually all the enteral L-glutamate is metabolized by the gut during absorption (Reeds et al. 1996). In a 7-kg piglet, an increase in L-glutamate concentrations was observed in portal and arterial blood plasma when the basal milk formula, administered enterally at a rate of 510 μmol kg/h, was supplemented with a relatively high dose of monosodium glutamate, i.e., 1,250 μmol/kg/h (Janeczko et al. 2007). Similarly, in larger (60 kg) pigs, transient portal and arterial hyperglutamatemia was observed when the diet (i.e., 800 g meal containing 142 g casein) was supplemented with 10 g monosodium glutamate (Blachier et al. 1999), indicating that only very large doses of L-glutamate may exceed the intestinal capacity to catabolize this amino acid.

It has been demonstrated that dietary L-glutamate is the most important contributor among substrates for mucosal oxidative energy metabolism in pigs (Stoll et al. 1999). ATP production and utilization are very active in pig enterocytes. Indeed, although the gastrointestinal tract represents only approximately 5 % of body weight, it is responsible for around 20 % of whole-body oxygen consumption (Vaugelade et al. 1994). The pig intestinal epithelial cells have notably high energy demand (Madej et al. 2002; Wu and Knabe 1995) due to the rapid renewal of the epithelium within few days (Wiese et al. 2003), thus requiring intense anabolic metabolism. Sodium extrusion at the basolateral membranes through the activity of Na/K ATPase following nutrient absorption may also represent a major ATP-consuming process in enterocytes (Buttgereit and Brand 1995).

The metabolic steps involved in L-glutamate utilization in pig enterocytes involve firstly transamination with oxaloacetate to produce alpha-ketoglutarate and L-aspartate (Blachier et al. 2009) (Fig. 5.1). L-glutamate can also be transaminated in the presence of pyruvate to produce L-alanine and alpha-ketoglutarate. Transamination

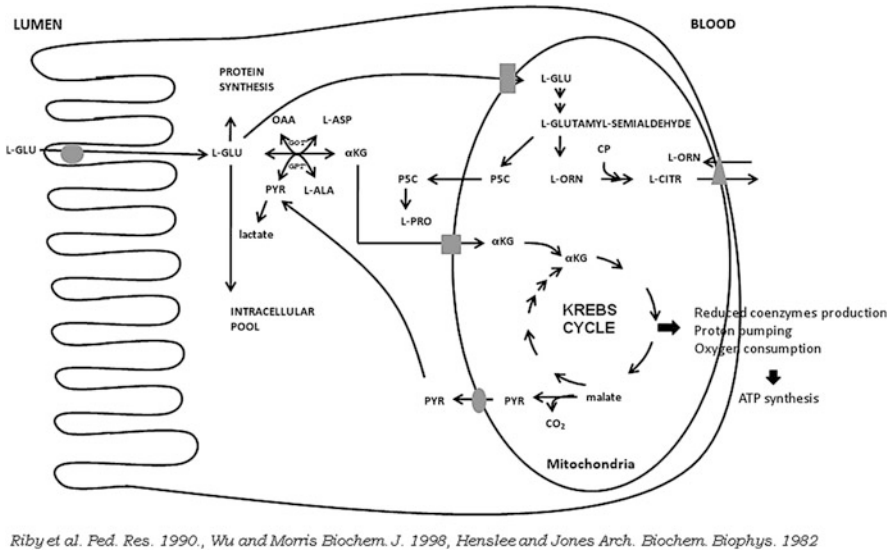

Fig. 5.1 Schematic view of the metabolism of L-glutamate (L-GLU) in small intestine absorptive epithelial cells. This schema is related to the involvement of L-glutamate in the production of other amino acids as well as conversion of L-glutamate into α-ketoglutarate (α-KG) for entry into the Krebs cycle. OAA is oxaloacetate, L-ASP is L-aspartate, PYR is pyruvate, L-ALA is L-alanine, L-ORN is L-ornithine, L-CITR is L-citrulline, CP is carbamoylphosphate, P5C is pyrroline-5-carboxylate, PRO is L-proline

appears to be the principal route by which L-glutamate is converted to alpha-ketoglutarate in pig enterocytes, because these cells have little capacity for the conversion of L-glutamate into alpha-ketoglutarate (and ammonia) through glutamate dehydrogenase (Madej et al. 2002). L-glutamate and L-glutamine are similarly oxidized by pig enterocytes (Blachier et al. 1999). However, for L-glutamine, this amino acid has to firstly enter the mitochondria in order to be degraded to L-glutamate and ammonia by the phosphate-dependent glutaminase activity (Wu et al. 1994, 1995a; Fig. 5.2).

L-glutamate that arises within the mitochondria can be either metabolized locally or exported into the cytosol, where the amino acid is metabolized into alpha-ketoglutarate by transamination before reentering the mitochondria and the tricarboxylic acid cycle (Duée et al. 1995). When both L-glutamate and L-glutamine are simultaneously presented to isolated pig enterocytes, L-glutamate can inhibit L-glutamine utilization and oxidation by inhibiting mitochondrial glutaminase activity (Blachier et al. 1999). Presumably, the sparing effect of L-glutamate over L-glutamine is dependent on the relative concentration of both amino acids in mitochondria.

L-glutamate and/or L-glutamine can be used by pig enterocytes to produce other amino acids including L-aspartate (Blachier et al. 1999), L-alanine (Wu et al. 1995a), L-proline (Wu and Knabe 1994), L-ornithine (Blachier et al. 1992), and L-citrulline (Wu et al. 1994) (Fig. 5.1). Note that neither ornithine nor citrulline is present in

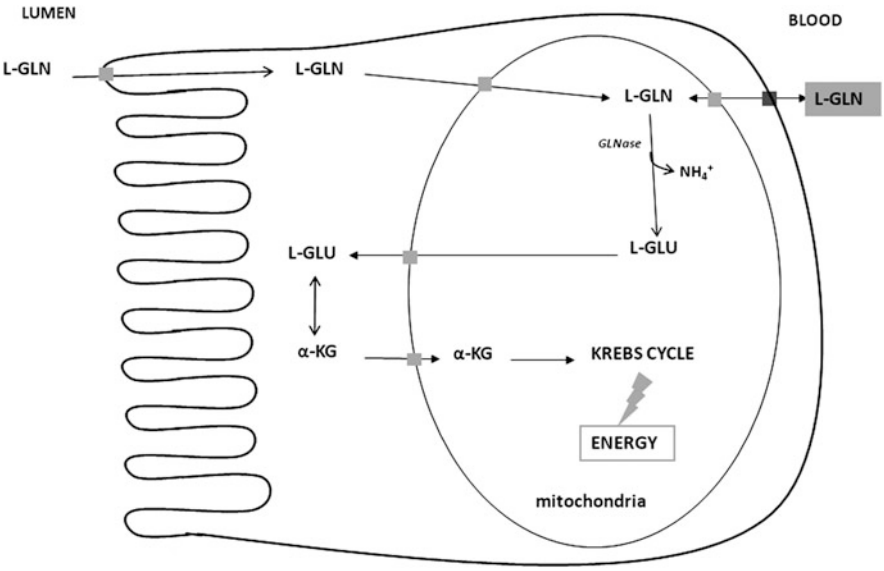

Fig. 5.2 Schematic view of the metabolism of L-glutamine (L-GLN) in small intestine absorptive epithelial cells. This schema is related to the metabolism of L-GLN to α-ketoglutarate (α-KG) for entry into the Krebs cycle. GLNase is glutaminase

proteins. L-aspartate and L-alanine are thus produced in the course of glutamate catabolism in pig enterocytes. L-aspartate, either produced endogenously from glutamate/glutamine or originating from the exogenous luminal content, can enter mitochondria and is then oxidized in the TCA cycle (Windmueller and Spaeth 1976), thus representing another oxidative fuel for enterocytes. The stepwise conversion of L-glutamate to L-ornithine is performed in mitochondria. Then, L-ornithine can serve as a precursor for citrulline production (Blachier et al. 1991b). L-citrulline is then released into the portal vein and passes through the liver without major uptake and is then presumably used for de novo synthesis of L-arginine in kidneys (Flynn and Wu 1996).

Together with L-cysteine and glycine, L-glutamate is the precursor for the synthesis of glutathione in pig enterocytes (Fig. 5.3).

Studies in pigs suggest extensive utilization of dietary cysteine by the intestine (Stoll et al. 1998). It has been reported that in fed piglets, mucosal glutathione is derived largely from the direct metabolism of enteral L-glutamate (Reeds et al. 1997). The ratio of reduced to oxidized glutathione is an important measure for both the determination of the intracellular redox status and for the cell's capacity to control intracellular concentrations of both oxygen-reactive and nitrogen-reactive species (Chakravarthi et al. 2006; Kemp et al. 2008).

Metabolism of L-glutamate and L-glutamine in enterocytes has been studied in growing piglets. Enterocytes isolated from piglet small intestine at birth display a high capacity for L-glutamine oxidation (Darcy-Vrillon et al. 1994), indicating that this amino acid can be used as a fuel at this stage of development when the growth

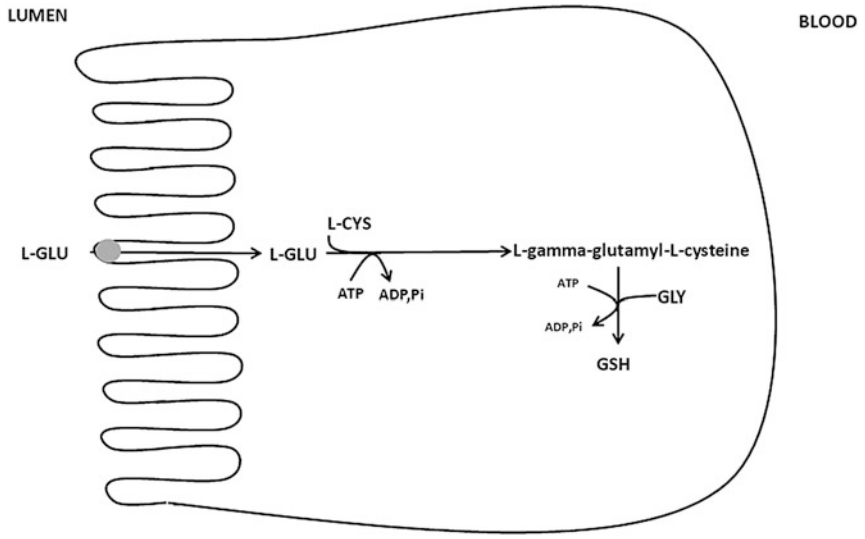

Fig. 5.3 Schematic view of the conversion of L-glutamate (L-GLU) into glutathione (GSH) in small intestine absorptive epithelial cells. L-CYS is L-cysteine, GLY is glycine

of the intestine mucosa is very intense (Widdowson et al. 1976; Klein and McKenzie 1983a, b). Furthermore, enterocytes isolated from newborn pigs are able to convert L-glutamine into L-citrulline and then L-arginine (Blachier et al. 1993; Wu et al. 1994). This metabolic capacity of enterocytes may reflect the high content in sow's milk proteins of glutamate and glutamine and the relatively low content of arginine (Wu and Knabe 1994). In suckling piglets, it has been observed that, soon after birth, the net enterocyte's capacity to convert L-glutamine to L-citrulline is severely decreased compared with piglets at birth (Wu et al. 1995b). In contrast, L-glutamine still represents a major oxidative energy substrate for enterocytes in these animals. Indeed, in suckling piglet enterocytes, L-glutamine is approximately eight times more rapidly oxidized than in enterocytes isolated from weaned pigs (Darcy-Vrillon et al. 1994). In addition, conversion of enteral glutamate to proline in neonatal piglet gastrointestinal tract has been measured by Murphy (1996). Some interactions have been reported between glutamine and carbohydrate metabolism in piglet enterocytes. For instance, in these cells, the metabolism of D-glucose in the pentose cycle which allows NADPH synthesis appears to increase the net conversion of L-glutamine into L-proline (Wu 1996).

Colonic epithelial cells are also able to metabolize glutamate and glutamine. An important characteristic of the colonic epithelium, as said above, is that except for a short period after birth, there is little or no transfer of amino acids from the lumen to the portal blood (Darragh et al. 1994). Then, amino acids (including L-glutamate and L-glutamine) must be taken from arterial blood. Pig colonocytes can use L-glutamine as oxidative substrates (Darcy-Vrillon et al. 1994). Like in enterocytes, L-glutamine

is firstly converted into L-glutamate and ammonia by the mitochondrial glutaminase, and then into alpha-ketoglutarate, mainly by transamination, followed by entry into the tricarboxylic cycle.

Colonocytes can also use luminal organic acids generated from the microbial activity, including short-chain fatty acids, as oxidative substrates (Darcy-Vrillon et al. 1993). Dietary substrates for short-chain fatty acid production are mainly dietary fibers, resistant starch, and some amino acids including L-glutamate (Bindelle et al. 2011; Htoo et al. 2007). Regarding amino acid metabolism by the microbiota, although alimentary protein digestion followed by amino acid and oligopeptide absorption by the small intestine is efficient in pigs (Stein et al. 1990), substantial amounts of nitrogenous compounds of both exogenous (alimentary) and endogenous origin may enter the pig large intestine through the ileocaecal junction. The first event in the large intestine protein degradation is hydrolysis of proteins and polypeptides by microbiota and residual pancreatic proteases and peptidases, which results in peptide and amino acid release, followed by the production of numerous bacterial metabolites. In the large intestinal lumen, L-glutamate released from proteins and peptides is the precursor for acetate and butyrate production (Blachier et al. 2007), but the relative contributions of L-glutamate and alimentary indigestible polysaccharide to acetate and butyrate production have not been determined. Glutamine synthetase, in terms of protein expression and catalytic activity, has been measured in pig colonocytes (Eklou-Lawson et al. 2009). Because the L-glutamine-degrading enzyme glutaminase is also highly expressed in colonocytes, this raises the open question of the physiologic meaning of the expression within the same cells of L-glutamine-synthesizing and -degrading enzymes. Because ammonia at concentrations that can be found in the pig colonic lumen inhibits short-chain fatty acid oxidation in colonocytes (Darcy-Vrillon et al. 1996), it has been proposed that cytosolic glutamine synthetase activity, which converts L-glutamate and ammonia into L-glutamine, may represent a way to reduce the intracellular concentration of ammonia during its transfer from the lumen to the bloodstream (Eklou-Lawson et al. 2009), thus avoiding metabolic disturbances.

Utilization of L-glutamate in colonocytes is not restricted to energy metabolism. Indeed, L-glutamine metabolism in pig isolated colonocytes leads to a net production of L-aspartate, L-alanine, and lactate (Darcy-Vrillon et al. 1993). However, unlike what is observed in enterocytes, pig colonocytes produce more L-aspartate than L-alanine from L-glutamine.

5.3 Metabolic Fate of Arginine (and Related Amino Acids, i.e., Ornithine, Citrulline, and Proline) in Pig Enterocytes and Colonocytes

In pig enterocytes isolated from postweaning animals, arginine is degraded mainly into urea, ornithine, and citrulline and to a much lesser extent to nitric oxide (NO) (Wu et al. 1995a; Blachier et al. 1993; M'Rabet-Touil et al. 1993, 1995).

The synthesis of ornithine and urea from arginine is made from the catalytic activity of arginase. Although urea represents a metabolic end-product, a part of ornithine derived from arginine can be used as a substrate together with carbamoylphosphate to allow citrulline synthesis through the activity of carbamoylphosphate synthetase isoform I (CPS I). Citrulline can also be produced together with NO through the activity of nitric oxide synthase (NOS) which catalyzes the conversion of arginine into these two metabolites. However, the amount of citrulline produced through the NOS pathway appears lower than the amount of citrulline produced through the arginase/CPS I pathways (Blachier et al. 1991a).

Metabolism of arginine in pig enterocytes has been studied in growing piglets and has revealed that the metabolism of arginine is orientated towards synthesis in neonatal and suckling piglets, but then is orientated towards degradation up to the weaning period (Wu and Knabe 1995; Blachier et al. 1993). Indeed, at birth, several amino acids are used by enterocytes as precursors for arginine synthesis. Thus glutamine, proline, and citrulline can be used as precursors for the net synthesis of arginine in neonatal enterocytes (Wu et al. 1994; Wu 1997; Blachier et al. 1993; Flynn and Wu 1996; Wu and Knabe 1995). Interestingly, the arginine content in sow's colostrum and milk proteins (and as free arginine) is relatively low when compared with the other amino acids, with glutamine and proline being very abundant in porcine milk (Wu and Knabe 1994; Davis et al. 1994). Thus, it has been proposed that the metabolic capacity of the absorptive epithelial intestinal cells to synthesize arginine would compensate for the relatively low content of this amino acid in milk protein (Wu and Knabe 1995), and would thus contribute to the endogenous supply of this amino acid in a context of piglet rapid growth.

Accordingly, the arginase activity in enterocytes is very low but increases slightly in the suckling period to a relatively high level in weaned animals (M'Rabet-Touil et al. 1996; Wu 1995). Also, the NOS activity is very low in enterocytes of neonatal piglets, but increases progressively up to the period of weanling (M'Rabet-Touil et al. 1993). The production of NO from arginine in enterocytes isolated from weaned pigs can be increased by the production of NADPH (a cofactor used for NOS catalytic activity) from D-glucose in the pentose phosphate pathway (Blachier et al. 1991a). In addition, although the activity of ornithine decarboxylase, the enzyme which allows the synthesis of putrescine and CO_2 from ornithine, is present at a low level in enterocytes at birth, this activity falls down to a value close to the limit of detection in suckling and weaned animals (Blachier et al. 1992).

What can be the physiological consequences of these metabolic changes during piglet development? Firstly, this low arginine catabolism in enterocytes will spare a conditionally essential amino acids in a context of limited alimentary supply and de novo synthesis. Then, the increase in NO biosynthesis capacity by enterocytes from birth to weaning, although representing a low arginine utilization, is likely related to the emergence in the suckling period of different NO-dependent physiological processes including the protection of the gastrointestinal mucosa (Stark and Szurszewski 1992; Miller et al. 1993; Quintero and Guth 1992; Konturek et al. 1992; MacKendrick et al. 1993); the regulation of the intestinal motility (Calignano et al. 1992; Hata et al. 1990); and the modulation of the intestinal epithelial

permeability (Kubes 1992, 1993). In newborn piglets, the low ornithine decarboxylase activity would contribute to the endogenous pool of putrescine produced from ornithine in enterocytes. However, from measurement of the capacity of enterocytes isolated from neonatal piglets for polyamine (putrescine, spermidine, and spermine) uptake, it appears that most of the intracellular polyamines likely originate from extracellular sources (Blachier et al. 1992). These sources include the polyamines present in the intestinal luminal content. Interestingly, since sow's milk contains relatively high concentration of spermidine (Kelly et al. 1991) and spermine (Motyl et al. 1995), these polyamines may represent a significant source for piglet's enterocyte intracellular content. In the piglets, growth of the intestinal mucosa is very intense during the first days after birth (Widdowson et al. 1976; Klein and McKenzie 1983b) and several experimental works have established that polyamines are central for the intestinal mucosal growth (Wang et al. 1991; Ginty et al. 1989). Finally, the synthesis of arginine from ornithine and the synthesis of ornithine from arginine demonstrate the existence of an intracellular arginine–ornithine cycle in suckling piglets (Blachier et al. 1993; Wu 1995).

The results obtained in vitro using enterocytes isolated from neonatal piglets which showed that these cells are orientated towards arginine production from various precursors including glutamine were complemented by in vivo experiments using suckling piglets treated with gabaculine, i.e., an inhibitor of ornithine aminotransferase. This inhibitor decreases the sequential intestinal conversion of glutamine to arginine. Using this experimental design, it was possible to demonstrate that the intestinal production of citrulline plays an important role in the endogenous synthesis of arginine and thus in its homeostasis in neonatal piglets (Flynn and Wu 1996). In these animals, proline is able to ameliorate arginine deficiency during enteral but not parenteral feeding, pointing out that the gut likely acts as an important player for the whole-body arginine supply in piglets by using proline as a substrate for arginine biosynthesis (Brunton et al. 1999; Wu 1997). The synthesis of arginine from proline may be mitigated by the plasma concentration of lactate (Dillon et al. 1999). Indeed, in enterocytes isolated from suckling piglets, lactate is able to inhibit citrulline and arginine synthesis from proline via an inhibition of proline oxidase, i.e., the enzyme responsible for the conversion of proline into delta1-pyrroline-5-carboxylate (Dillon et al. 1999). Although the factors responsible for the developmental changes in arginine metabolism in enterocytes remain unclear, it has been demonstrated that glucocorticoids play an important role in mediating the enhanced catabolism of arginine during weaning (Flynn and Wu 1997).

In pig colonic epithelial cells, much less studies have been performed regarding arginine metabolism. Amino acids from the luminal origin are not believed to be absorbed to any significant extent through the pig colonic epithelium except for a short period after birth (Smith and James 1976). Then, in contrast to the situation found in the small intestine enterocytes, which import amino acids from both luminal and blood origins, colonic epithelial cells import amino acids almost exclusively from arterial blood for metabolic and physiological purposes. L-arginine metabolism in pig colonocytes has not received much attention. Nonetheless, absorptive colonic epithelial cells isolated from the proximal colon of weaned pigs

are characterized by a higher NOS activity when compared with the activity measured in enterocytes isolated from the jejunum. In contrast, arginase activity was found to be similar between pig colonocytes and enterocytes (M'Rabet-Touil et al. 1993). In the large intestine lumen, arginine released from proteins and peptides which are transferred from the ileon to the caecum may presumably serve as a precursor for the synthesis of agmatine, putrescine, and nitric oxide by the microbiota (Blachier et al. 2007). However, to the best of our knowledge, there is no published data on the production of these bacterial metabolites in the pig colonic lumen.

5.4 Metabolic Fate of Branched-Chain Amino Acids (Leucine, Isoleucine, and Valine) in Pig Enterocytes

In milk-fed piglets, 32 % of leucine in the diet are extracted by the portal-drained viscera in the first pass, with 21 % of the extracted leucine being utilized for the intestinal mucosal protein synthesis (Stoll et al. 1998). Overall, it has been estimated that 44 % of total branched-chain amino acids (BCAAs) are extracted by first-pass splanchnic metabolism in neonatal piglets (Elango et al. 2002). The catabolism of BCAAs in enterocytes isolated from developing piglets has been studied. In these cells, BCAAs are extensively transaminated and between 15 and 50 % of decarboxylated branched-chain alpha-ketoacids are oxidized depending on the age of piglets (Chen et al. 2002). Enterocytes isolated from postweaning pigs also actively degrade BCAAs (Chen et al. 2002). Other essential amino acids (i.e., histidine, lysine, methionine, phenylalanine, threonine, and tryptophan) are apparently less catabolized in developing piglet enterocytes (Chen et al. 2002, 2009) and in weaned pigs (Chen et al. 2002, 2007). Metabolism of essential amino acids by colonic epithelial cells remains to be determined.

5.5 Metabolic Fate of Sulfur-Containing Amino Acids in Pig Enterocytes

In piglets, the net portal balance of methionine represents 48 % of intake suggesting that a relatively large part of the dietary methionine is consumed by the intestine (Stoll et al. 1999). Accordingly, parenteral methionine requirement is approximately 69 % of the enteral requirement in neonatal piglets (Shoveller et al. 2003). In addition, the parenteral methionine requirement is approximately 70 % of the enteral requirement when measured in the presence of an excess of dietary cysteine suggesting that cysteine is equally effective in sparing dietary methionine whether fed enterally or parenterally (Shoveller et al. 2003). A recent study has shown that, in

neonatal piglets, sulfur-containing amino acid deficiency results in small intestine atrophy with lower goblet cells and lower glutathione content (Bauchart-Thevret et al. 2009). These effects were associated with upregulation of the intestinal methionine cycle activity. The piglet gastrointestinal tract was reported to consume approximately 20 % of the dietary methionine (Riedijk et al. 2007). Furthermore, the piglet gastrointestinal tract appears to be a site for net homocysteine production and to be a significant site for whole-body transmethylation and transsulfuration, these two metabolic pathways being responsible for a majority of methionine utilization by the gastrointestinal tract (Riedijk et al. 2007). However, as previously said, methionine is less catabolized in pig enterocytes raising the view that methionine may be substantially consumed in other cells of the portal-drained viscera and/or of the intestinal mucosa and/or may be degraded by the small intestine microbiota (Blachier et al. 2007).

5.6 Metabolic Fate of Threonine in Pig Enterocytes

As noted above, the metabolic capacity of pig enterocyte for threonine catabolism is virtually absent (Chen et al. 2009). The pig intestinal mucins are glycoproteins very rich in threonine, serine, and proline (Fogg et al. 1996). In a model of minipigs with ileitis, it has been demonstrated that intestinal inflammation increases gastrointestinal threonine uptake and mucin synthesis (Rémond et al. 2009). In neonatal piglets, the threonine requirement during total parenteral nutrition is approximately 45 % of the mean enteral requirement (Bertolo et al. 1998). In growing pigs, it has been shown that the catabolism through the threonine dehydrogenase pathway does not account for the relatively high first-pass extraction rate of dietary threonine by the portal-drained viscera (Le Floc'h and Seve 2005). Dietary threonine extraction by the small intestine is likely to reduce threonine availability for other tissues when young piglets are fed a diet marginally deficient in threonine (Hamard et al. 2009). Lastly, a moderate threonine deficiency was responsible for an alteration of the intestinal functionality in terms of paracellular permeability (Hamard et al; 2010). In piglets, although the absolute amount of systemic and dietary threonine utilized by the portal-drained viscera was reduced in protein-restricted diet, the percentage of dietary threonine intake used by the portal-drained viscera did not differ between groups, with the value being above 85 % (Schaart et al. 2005). As expected, dietary rather than systemic threonine is preferentially utilized for protein synthesis in the piglet small intestine mucosa. Thus, the portal-drained viscera requirement for threonine is high and the high rate of utilization by the piglet intestinal mucosa is largely due to the incorporation of this amino acid in the proteins of the mucosa (Schaart et al. 2005).

5.7 Metabolic Fate of Lysine and Phenylalanine in Pig Enterocytes

In enterocytes isolated from the small intestine, lysine is less catabolized (Chen et al. 2009). In milk protein-fed piglets, when expressed as a percentage of the enteral tracer input, it was calculated that there is a substantial first-pass metabolism of lysine (35 %) (Stoll et al. 1998). However, only 18 % of what is used in the first-pass metabolism is recovered in intestinal mucosal protein. These experiments suggest that lysine catabolism in the intestinal mucosa is quantitatively greater than amino acid incorporation into mucosal proteins. However, from the low capacity of absorptive intestinal cells for lysine catabolism, it is likely that other cell types are able to use lysine. The in vivo catabolism of lysine by the intestinal microbiota may indeed be substantial. However, evidences have been produced in favor of de novo synthesis of lysine by the pig microbiota, a part of it being available for the host (Torrallardona et al. 1996; Backes et al. 2002). The net result of lysine utilization and production by the intestinal microbiota remains to be determined. Interestingly, it has been demonstrated that dietary lysine used by the portal-drained viscera is driven by its luminal bioavailability; and this utilization is markedly stimulated immediately after the meal ingestion (Bos et al. 2003). Additionally, this study has shown that not only the quantity of portal-drained viscera utilization is increased, but there is also a preferential first-pass use of dietary lysine rather than systemic arterial lysine during bolus when compared with continuous feeding. In a situation of protein restriction, neonatal piglets use a large amount of the dietary supply of lysine (van Gaudoever et al. 2000). In milk-fed piglets, when expressed as a percentage of the enteral tracer, it has been determined that there is a marked first-pass metabolism of phenylalanine (35 %) with 18 % of what is used in the total first-pass metabolism of this amino acid being recovered in mucosal proteins. Once again, since phenylalanine is virtually not catabolized by piglet enterocytes, it is likely that the intestinal microbiota represents a significant contributor for the catabolism of this amino acid.

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Chapter 6
Development of Micro-ecological System in Small and Large Intestine of Piglets

Shengping Wang, Gang Liu, Lichuang Cai, Jiannan Ding, and Yulong Yin

Contents

The piglet gastrointestinal (GI) tract can be considered as a big "warehouse" since it is populated with as many as 100 trillion of microorganism cells (1×10^{14}) belonging to 500–1,000 species. Bacteria make up most of the gut microbiota, which include 300–1,000 different species (Sears 2005). However, about 99 % bacteria belong to 30 or 40 species. Fungi and protozoa also make up a part of the piglet gut microbiota, but little is known about their activities. The gross weight of bacteria in piglet GI tract represents as much as 1.5 kg.

S. Wang • G. Liu • Y. Yin (✉)
Chinese Academy of Sciences, Institute of Subtropical Agriculture, Research Center for Healthy Breeding Livestock & Poultry, Hunan Engineering & Research Center of Animal & Poultry Science, Key Laboratory of Agro-ecological Processes in Subtropical Region, Scientific Observing and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Changsha, Hunan 410125, People's Republic of China
e-mail: yinyulong@isa.ac.cn

L. Cai • J. Ding
Biological Resources Institute, Jiangxi Academy of Sciences, Nanchang 330029, P.R. China

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_6,

Most of the gut bacteria benefit the piglet, for example Bifidobacterium and Lactobacillus. However, some other gut bacteria such as *Clostridium perfringens*, *Pseudomonas*, *Wales* bacteria, and *Staphylococcus aureus* are potentially pathogenic. The normal gut microbiota maintains a stable gut micro-ecological environment (Hou et al. 2011; Wang et al. 2011a, b; Kong et al. 2009). Microbiota activity can impact piglet metabolism and immunity. However, the normal gut microbiota is not static, and the microbiota composition can change according to diet, age, environment, and physiology. The gut microbiota can move from one stable state into another stable state in order to adapt to the changes of internal and external environment (Björkstén et al. 2001).

Recent studies suggest that the gut microbiota plays an important role in piglet health and nutrition by maintaining a stable gut environment, by providing a barrier for colonization of pathogens, and by exerting important metabolic functions (fermentation of non-digestible fibers and amino acids originating from undigested proteins; salvage of energy as short-chain fatty acids; and production of folic acid, vitamin K, vitamin H, B vitamins, essential amino acids, and antibiotic peptides). The microbiota is also able to stimulate the development of the immune system and to participate in the maintenance of piglet energy homeostasis. Disturbances of the gut microbiota can result in GI tract malfunction, resulting in phenomena such as diarrhea, enteritis, and indigestion. So, the relationship between the gut microbiota and piglet is not merely commensal, but rather a symbiotic relationship (Sears 2005).

Because the gut microbiota is involved in many aspects of piglet health and nutrition, it is important to understand how the composition of this microbiota is established. The gut microbiota forms an essential part of a complex ecosystem that plays an important role in piglet health, nutrition, and metabolism. Research related to the study of the gut microbiota has involved an increasing number of laboratories all around the world in recent years. The major objective of this present chapter is to provide insights into new development in piglet gut microbiota research, including the composition, development, and factors influencing the gut microbiota.

6.1 The Composition and Diversity of the Gut Microbiota in Piglet

The piglet gut hosts a dynamically evolving microbial ecosystem that consists of various bacterial populations. These bacteria are either permanent intestinal residents such as *Lactobacillus* and *Streptococcus* or transient inhabitants, for example allochthonous members, introduced from the environment. It is believed that intensive selection and coevolution result in the genetic diversity of the intestine. Horizontal gene transfer (HGT) is assumed to function as the principal evolutionary force in shaping the host microbiome (Palmer et al. 2007).

The piglet gut microbiota is characterized by a relatively small number of bacterial and archaeal species, compared to other environments such as soil or stromatolite. The 16S rRNA gene sequence analysis revealed that the piglet gut

microbiota is made of only nine major bacterial divisions, including Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Verrucomicrobia, Cyanobacteria, Spirochaetes, VadinBE97, and Actinobacteria (Rajilić-Stojanović et al. 2007; Palmer et al. 2007). However, there are different bacterial cell density and bacterial species in different sites along the digestive tract (Figs. 6.1 and 6.2). Generally, the number and diversity of bacteria flora are increasing from stomach to foregut and then to hindgut. The spatial distribution of the piglet gut microbiota is also different, not at random, but rather organized. There are noticeable difference for the gut microbiota distribution between the mucus layers and the luminal content (Fig. 6.2). For example, *Lactobacillus* is the major bacteria adhering to the mucus layer or to the epithelial cells, while *Streptococcus* mainly distributes within the gut lumen. The diversity of the piglet gut microbiota in the gut wall is lower than that in the gut lumen. In addition, the piglet gut microbiota maintains a dynamic equilibrium with both temporary variation and continuous stability. For example, some *Lactobacillus* species such as *Lactobacillus reuteri*, *Lactobacillus amylovorus*, *Lactobacillus acidophilus*, and *Lactobacillus mucosae* and some Streptococcus species such as *Streptococcus hyointestinalis, Streptococcus alactolyticus*, and *Streptococcus gallolyticus* can live in the piglet gut for the whole life, but the number of these bacteria may change with age, diet, or environment (Wang et al. 2009). However, Clostridium has a high temporal variation. Indeed, some *Clostridium* species rapidly emerge, and then rapidly disappear. The possible reason is that *Clostridium* may be susceptible to the change of internal and external environment.

6.2 The Development of the Gut Microbiota in Piglet

The piglet is axenic in the sow uterus, and is suddenly plunged at birth into a complex bacterial environment. During its passage through the maternal vagina, the piglet is in contact with bacteria for the first time, and then meets the enormous bacterial population from the sows' feces and rearing environment. Thus, the newborn piglet is very likely to meet potentially pathogens, and accordingly, the survival of the piglet depends on the very early establishment of defense systems. Therefore a fast and stable establishment of the gut microbiota will play a very important role in keeping the health and growth of the piglets.

6.2.1 The Nature of the First Established Strains

The establishment of the microbiota is very rapid in the piglet digestive cavities. Only few bacteria are enumerated at the third hour of life. However, after 10–12 h, the population density reaches 10^8–10^9 bacterial cells per gram in feces. After 24 h, the piglet already possesses a dominant microbiota established at a level which will not change until weaning (Ducluzeau 1983). There are differences in the order of implantation of the main bacterial groups according to the animal species. In the

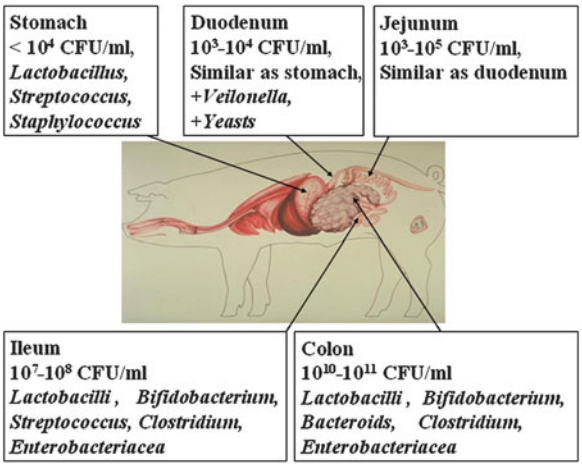


Fig. 6.1 Bacterial cells density and major bacterial species for digesta in the different site along the digestive tract of pig

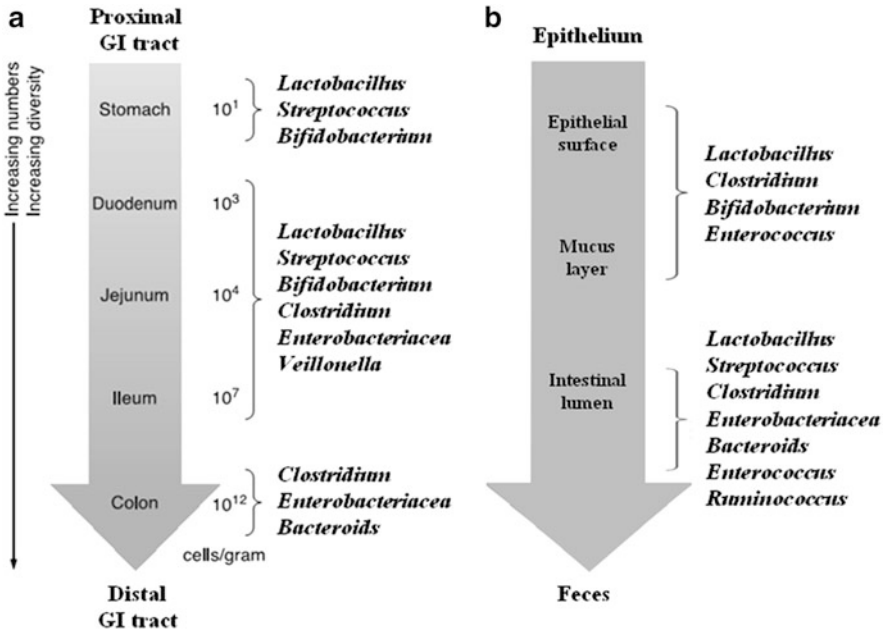


Fig. 6.2 (**a**) Bacterial cells density and major bacterial species for digesta in the different site along the digestive tract of pig; (**b**) Bacterial cells density and major bacterial species for epithelium in the different site along the digestive tract of pig

young mouse, facultatively anaerobic bacteria appear very early; and strictly anaerobic bacteria, which will eventually become dominant, will appear later at the third week after birth (Ducluzeau 1983). The opposite is observed in the young hare and rabbit (Ducluzeau et al. 1975). The piglet represents an intermediate case, with a simultaneous establishment from the first day of both facultatively anaerobic bacteria (mainly *Lactobacillus*, *Streptococcus*, and *Escherichia*) and strictly anaerobic bacteria belonging to *Clostridium*, *Bifidobacterium*, *Fusobacterium*, *Peptostreptococcus*, and sometimes Bacteroides genera (Wilbur et al. 1960; Pesti 1962).

6.2.2 Origin of the Piglet Gut Microbiota

During the process of birth, the newborn piglet meets successively several different microbial ecosystems: the sow vagina, the sow feces, and the rearing environment. It may be considered a priori that each of these ecosystems is liable to contribute to the creation of the piglet gut microbiota. In fact, the newborn piglet is provided with powerful selective systems allowing only some bacteria species to develop among all the bacteria. The first bacteria which become established in the digestive tract actually come from the sow or from the environment, but they are not the most abundant in the ecosystems met by the piglet.

6.2.3 Mechanisms Controlling the Establishment of the Gut Microbiota in Piglet

One of the main factors liable to account for the selection exerted by the newborn piglet among all the bacteria met is its dietary regime. Within 7 days after birth the piglet receives the mother's milk as feed, which is favorable to the growth of some bacteria and unfavorable to others in the piglet gut. For example, it is observed in the piglet born to gnotobiotic sow harboring only *Escherichia coli* and *Shigella flexneri* that these bacteria are implanted in a high number after 12 h (Hayashi et al. 2002). Conversely, when the sow harbors strictly anaerobic bacteria such as bacteroides and Clostridium, these bacteria are detected in the piglet between 10 and 15 days of age, i.e., when the piglet begins to consume solid food in addition to milk. To take another example, many of *Lactobacillus sp*. and *Lactobacillus salivarius* are implanted in the piglet ileum when the piglet receives liquid milk. However, after weaning these bacteria gradually decrease, and *Streptococcus alactolyticus* as well as *Streptococcus hyointestinalis* increase if the piglets receive solid food (Wang et al. 2009).

The immune status of the sow, which affects the composition of the colostrum given to the piglet, might also influence the order of the establishment of the bacteria in the piglet intestine. Colostrum, undoubtedly, plays a role in the fight against microbial infection of digestive origin, but it has never been demonstrated that it has an effect on the microflora in the gut lumen. Immunization of the sow against different bacterial species did not prevent the further establishment of these

bacteria in the piglet (Ducluzeau 1983; Hooper and Macpherson 2010). Ducluzeau studied the establishment of the fecal flora between birth and 48 h of age in a litter of conventional piglets divided into two groups: in the first one the animals were left with their dam and suckled colostrums and milk, while in the other, animals were taken off at birth, were housed in a cage inside the dam's pen, and received sterile cow's milk. Despite these experimental differences, no difference was observed in the systematic order of implantation of the first bacteria between both groups of piglets (Hooper et al. 2002).

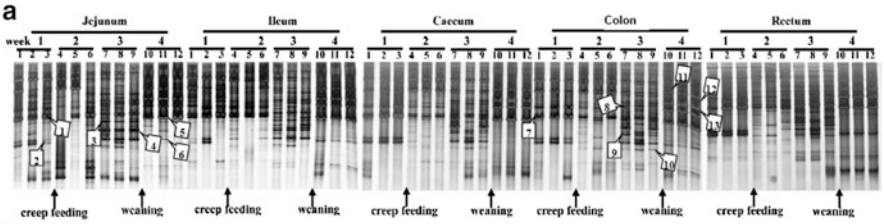
Bacterial interactions certainly play an important role in the order of establishment of the piglet gut microbiota. The early development of some bacteria in the digestive tract prevents proliferation of other bacteria. A strain of *E. coli* is capable to eliminate from the digestive tract a strain of *Lactobacillus casei* derived from a commercial preparation and previously established in the axenic newborn (Dethlefsen et al. 2006). In human newborn it was shown that the inoculation of a strain of *E. coli* without plasmid within 2 h following birth led to the elimination or to a marked decrease of the *E. coli* population carrying plasmids of resistance to antibiotics, while these strains were frequently dominant in the control.

In the piglet, many studies indicate that single supplement with Lactobacillus in diet decreases the number of *E. coli* in the gut lumen, and concomitantly decreases the diarrhea rate among animals (Konstantinov et al. 2008).

The order of establishment of the bacteria in the piglet intestine is probably important for its survival. Many studies have shown that the supplement with antibiotics in the diet enables to increase the rate of survival in piglets and to decrease diarrhea episodes due to toxicogenic bacteria such as *Clostridium* and *E. coli* (Rohde et al. 2009). However, the use of antibiotics may result in the formation of resistant bacteria. In addition, the disturbance by the supplement of antibiotics of the initial establishment of the gut microbiota may lead to some negative effect in the piglets like an impairment of the piglet immune response, thus producing cross infection. The practical application of the bacterial interaction concept was found to be more useful in the above case than the use of antibiotics (Rohde et al. 2009). If the young hare are inoculated within the hour following birth with a complex flora derived from a healthy young hare, the animal is totally protected from neonatal diarrhea without further pathological manifestation at weaning. This protective flora, preserved in gnotobiotic mice, has never been simplified up to now to obtain a mixture of pure strains liable to be cultured in vitro (Ducluzeau et al. 1981a, 1981b). A protective effect was also demonstrated in the axenic newborn piglet: the early inoculation of a selected strain of *E. coli* from pig origin was found to protect the animals from the further development of toxinogenic strains of *E. coli* provided that the latter were not capable of adhering to the gut mucosa (Duval-Iflah et al. 1983).

6.2.4 Lactobacilli Community Development

There are several hundred bacterial species in the piglet GI tract. All the members of the piglet gut microbiota are needed for the gut to develop its specific intestinal functions (Hooper and Gordon 2001). However, Lactobacilli are the most prevalent



b

N	Closest Relative	Identity %	Clone ID	Accession	N	Closest Relative	Identity %	Clone ID	Accession
1	*Lactobacillus delbrueckii subsp. lactis*	98	YRJ9	AY662527	7	*Lactobacillus delbrueckii subsp. lactis*	96	YRC4	AY662519
2	*Lactobacillus sp. oral clone* CX036	95	YRJ28	AY662528	8	*Lactobacillus delbrueckii subsp bulgaricus*	97	YRC89	AY662523
3	*Lactobacillus crispatus* strain TSK V38-1	99	YRJ39	AY662530	9	*Lactobacillus delbrueckii*	92	YRC69	AY662521
4	*Lactobacillus delbrueckii*	94	YRJ58	AY662531	10	*Lactobacillus amylovorus*	98	YRC86	AY662522
5	*Lactobacillus reuteri*(DSM20016T)	97	YRJ100	AY662532	11	*Lactobacillus acidophilus johnsonii*	95	YRC100	AY662525
6	*Lactobacillus reuteri*(DSM20016T)	98	YRJ37	AY662529	12	*Lactobacillus sp.* G35	88	YRC95	AY662524
					13	*Lactobacillus reuteri*(DSM20016T)	97	YRC37	AY662520

Fig. 6.3 (**a**) Succession of the Lactobacillus community of digesta during the first month in the piglet intestinal tract. Changes of feeding strategy are indicated by *arrows*. The bands identified from the 16S rDNA clone libraries are numbered and are indicated by *arrowheads*. (**b**) Closest relative as determined by comparative sequence analysis, identity with this relative, clone ID, and accession number for each band identified in (**a**)

bacteria, and are often considered beneficial because they are important for maintaining a healthy and stable microbiota in the GI tract. It has been postulated that Lactobacilli has several health-promoting effects, including immuno-stimulation, alleviation of food intolerance and allergy, and prevention of diarrhea and intestinal infection (Servin 2004; Bengmark 2000; Salminen et al. 1998). Consequently, a normal Lactobacilli community development in time and space in the gut is significantly important for the health and growth of the piglet.

The experiments conducted by Yao et al. analyzed *Lactobacillus* diversity and development of neonatal piglet gut microbiota during the first 4 weeks of life by a combination of denaturing gradient gel electrophoresis (DGGE) and 16S ribosomal DNA sequence (Fig. 6.3a). The bands in DGGE profile represent the majority of the dominant *Lactobacillus* populations in the community, and their appearance and disappearance reflect approximate changes in the *Lactobacillus* community composition. The intensity of a band provides a rough estimate of the proportion of the corresponding population in a sample. The *Lactobacillus* communities in each compartment changed from simple to complex and returned to simple during this 4-week period. The most complex banding pattern appeared at week 3. There were several new bands (or new lactobacilli) only observed at this time, and disappeared again at week 4 (Yao et al. 2011).

Yao et al. also compared the mobility of the cloned fragments with the mobility of fragments amplified from the original samples by DGGE, and the DNA sequences of clones that corresponded to dominant bands were determined (Fig. 6.3b). Six dominant DGGE bands generated from jejunal digesta were matched with sequences that show 94–98 % similarity to those derived from *L. reuteri*, *L. delbrueckii*, and *L. crispatus*. Seven dominant DGGE bands generated from colon digesta were matched with sequences that show 88–99 % similarity to those derived from *L. reuteri*, *L. delbrueckii*, *L. amylovorus*, and *L. acidophilus*.

Amplicons related to *L. reuteri* were found in all DGGE fingerprints from jejunal digesta from 1-, 3-, and 4-week-old animals. Amplicons related to *L. amylovorus* were present in all DGGE fingerprints from colon digesta from 1-, 3-, and 4-week-old animals. Amplicons related to *L. delbrueckii* were found before weaning, *L. crispatus* in the stage of creep feeding, and *L. acidophilus* after weaning (Yao et al. 2011). In the experiments conducted by Wang et al., *L. reuteri* and *L. amylovorus* were the dominant Lactobacilli species in the ileum in both nursing and weaned piglet (Wang et al. 2009).

In conclusion, *Lactobacillus* communities follow successional changes associated with piglet age and diet shifting. Creep feeding stabilizes the *Lactobacillus* community of weaning piglets. Within the *Lactobacillus* community, some members like *L. reuteri* and *L. amylovorus*/*L. sobrius* might be permanent colonizers, while *L. delbrueckii*, *L. acidophilus*, *L. salivarius*, *L. mucosae*, and *L. crispatus* might be transient members of the *Lactobacillus* communities in the piglet's GI tract.

6.3 Factors Influencing the Composition of Piglet GI Tract Microbiota

As said above, the piglet is born without microorganisms. Starting at birth, the gut microbiota must develop from a simple unstable community into a complex community, which comprises microorganisms (bacteria, archaea, fungi, viruses, and protozoa) that represent superior competitors for a complex intestinal ecosystem (Palmer et al. 2007). It is generally considered that the genotype of host and the diet are the major factors defining a microbial intestinal niche. However, many other factors also influence mammal intestinal microbiota such as interactions between individual colonizers, age, weaning, as well as transient microorganisms (pathogens and probiotics) (Deng et al. 2007; Yin and Tan 2010).

6.3.1 The Genotype of the Piglet Influences Gut Microbiota

The genotype of the piglet is considered as a factor influencing variation of gut microbiota between individuals. Differential action of genes that control the immune system is involved in such an effect (Ley et al. 2006). For instance, the piglet immune system modulates the intestinal microbiota composition by restricting microbial penetration through the host mucosal barrier, and by secreting different antimicrobial products such as peptides, as well as antimicrobial enzymes (Cobb et al. 2004; Macdonald and Monteleone 2005). The genotype of the piglet determines the availability of specific attachment sites and host-derived resources, which influences the intestinal microbiota. So, monozygotic twins exhibit fewer differences in the intestinal microbiota than their unrelated marital partners. However, there are small differences between the intestinal microbiota of the monozygotic twins, suggesting that maternal transmission (Ley et al. 2005) is another determining factor. The initial colonizing microbiota influences the eventual microbial composition of the intestine.

6.3.2 Diet Influences Piglet Gut Microbiota

It was demonstrated that diet can have an important influence on the composition of intestinal microbiota. Some studies have shown a clear correlation between diet and the presence of specific bacterial groups. For example, a diet rich in inulin and related fibers promote an increase in *Bifidobacter*ia (Roberfroid 1998). Supplementation with astragalus polysaccharide and related polysaccharide in diet increases the number of *Lactobacillus* and *Bifidobacteria*. When nursing piglet is provided breast milk, there are many *Lactobacillus salivarius* and *Lactobacillus mucosae* in intestine. However, when the diet is evolving from liquid breast milk to solid foods, the two species of *Lactobacillus* are largely replaced by *Lactobacillus amylovorus* (Wang et al. 2009). In addition, mucin and other host-derived products, as well as the competition and cooperation between bacteria, are thought to be important influencing factors of intestinal microbiota (Dethlefsen et al. 2006).

6.3.3 Weaning Influences Piglet Gut Microbiota

Recent cultivation-independent studies provided a more comprehensive understanding of the development of the microbiota in pigs from birth to adulthood. Previously, such studies were hampered by the fact that a large proportion of the intestinal microbiota is inaccessible to cultivation approaches (Zoetendal et al. 2004 and Zoetendal et al. 2006). Cultivation-based studies have shown that lactic acid bacteria, enterobacteria, and streptococci were the most important first colonizers of the piglet gut (Stewart et al. 1997). Analysis of 16S rRNA gene clone libraries indicated that ileal samples of 2-day-old piglets harbored a consortium of *E. coli*-, *Shigella flexneri*-, *L. sobrius*-, *L. reuteri*-, and *L. acidophilus*-related sequences (Konstantinov et al. 2006). While populations of *L. sobrius*, *L. reuteri*, and *L. acidophilus* remained stable and abundant before weaning, their numbers dropped significantly after weaning. The experiment conducted by Wang et al. also led to similar results which are shown in Table 6.1 (Wang et al. 2009). Remarkable shifts in the microbiota composition and metabolic activities are found immediately after weaning (PW) for both ileal and colonic microbiota. Similar sequential changes in fecal microbiota were also found in other studies (Konstantinov et al. 2003 and Inoue et al. 2005). Hence, the early PW period is characterized by compositional and functional instability of the predominant piglet gut microbiota. In addition, several potentially beneficial lactobacilli are significantly suppressed during the weaning transition. Possibly, lactobacilli, having complex nutritional requirements, are affected by the restricted feed intake after weaning. This microbiota instability followed by the rapid decline of previously predominant lactobacilli may have greater contributions to PW intestinal disturbances than previously appreciated. Hence, the microbiota can be seen as an important potential target in dietary interventions targeting piglet GI tract

Table 6.1 Major microbiota in ileal digesta of piglets

Groups	Species	Rate of clones/%		
		Nursing piglets aged 21 days	Nursing piglets aged 28 days	Weaned piglets aged 28 days
Lactobacillus	*L. amylovorus*	41.5 (71/171)	25.8 (50/194)	31.6 (54/171)
	L. mucosae	4.7 (8/171)	3.1 (6/194)	0.6 (1/171)
	L. reuteri	12.3 (21/171)	18.6 (36/194)	16.4 (28/171)
	L. salivarius	9.4 (16/171)	6.2 (12/194)	1.2 (2/171)
	Lactobacillus sp.	12.9 (22/171)	10.8 (50/194)	
	Uncultured Lactobacillus sp.	6.4 (11/171)	4.6 (9/194)	0.6 (1/171)
	L. acidophilus	0.6 (1/171)	1.5 (3/194)	1.8 (3/171)
	L. coleohominis		0.5 (1/194)	
Streptococcus	*S. alactolyticus*		5.2 (10/194)	7.0 (12/171)
	S. gallolyticus	0.1 (2/171)	0.1 (2/194)	
	S. hyointestinalis	0.1 (2/171)	10.8 (21/194)	13.5 (23/171)
	Streptococcus sp.		0.5 (1/194)	0.6 (1/171)
	S. suis	0.1 (2/171)	3.6 (7/194)	5.3 (9/171)

disturbances, specifically aiming at supporting the predominant microbiota stability while maintaining indigenous lactobacilli communities.

Finally, age, antibiotics, and probiotics have crucial influences on the mammal intestinal microbiota. It is noted that the effect differs between antibiotics or probiotics. The composition of intestinal microbiota in adults has generally been considered to be stable as long as the host is not subjected to stressful circumstances. Some studies reported, however, that the elderly subjects have fewer bifidobacteria and more fungi and enterobacteria in their intestinal luminal content when compared with younger subjects (Hopkins et al. 2001). Lastly, *Clostridium difficile* is isolated more frequently in elderly mammals than in young subjects (Hopkins et al. 2001).

6.4 Conclusion and Perspectives

Piglet gut microbiota displays remarkable metabolic and immune versatility. Microbiota is involved in the synthesis of a variety of molecules with enormous importance, and also regulates key metabolic pathways and processes that are vital to the growth, development, health, and homeostasis of piglet. These findings exemplified the power of basic research on gut microbiota, nutrition, and immunity for the discovery of new knowledge and for solving significant practical problems in piglet production. However, in order to enlarge the available knowledge on this topic in the context of piglet production, it appears necessary to clarify the composition and development of piglet gut microbiota.

Studies of piglet gut microbiota have been largely based on traditional approaches (e.g., culture-dependent method), polymerase chain reaction-denaturing

gradient gel electrophoresis (PCR-DGGE), 16S rRNA gene sequencing, as well as terminal-restriction fragment length polymorphism (T-RLFP). These techniques have played historically significant roles in the development of the field. However, recent advances of meta-genome sequencing, transcriptomics, metabolomics, and proteomics have provided new insights into the complex interactions between the piglet and gut microbiota. Using these approaches, the composition and development of the piglet will be gradually discovered. Furthermore, the relationship between the gut microbiota and the piglet both at the intestinal mucosa level and at the peripheral level needs further investigations. It is becoming increasingly evident that the gut microbiota may shape the host metabolic and immune network activity. It would be important to capitalize on these revolutionary methods in future endeavors in order to rapidly and extensively expand our knowledge on the composition and metabolic capacities of the intestinal microbiota as well as the consequences of the modifications of such parameters for development, nutrition, immunity, and health in piglet and other species.

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Chapter 7
Structure, Metabolism and Functions of Amino Acids: An Overview

Wenkai Ren, Yinghui Li, Yulong Yin, and François Blachier

Contents

W. Ren • Y. Li • Y. Yin (✉)
Institute of Subtropical Agriculture, The Chinese Academy of Sciences, 410125 Hunan, People's Republic of China
e-mail: yinyulong@isa.ac.cn

F. Blachier
UMR 914 INRA/AgroParisTech, Nutrition Physiology and Alimentary Behavior, 16 rue Claude Bernard, 75005 Paris, France

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_7,

7.1 Introduction

Amino acids are classically considered as the building blocks for the synthesis of proteins. However, amino acids are also used as precursors for the biosynthesis of numerous compounds with various physiological functions (Wu et al. 2007; Kim et al. 2007; Li et al. 2007). For instance, compounds like glutathione, creatine, carnitine, taurine, and heme of hemoglobin are synthesized in the body from amino acids in different metabolic pathways. Some amino acids like glutamine, aspartate, and lysine are nitrogen and carbon precursors for the synthesis of ribo- and deoxyribonucleotides which are precursors for DNA and RNA synthesis. Furthermore, some amino acids like arginine and glutamine are precursors for amino acids which are not present in proteins like ornithine and citrulline and which play important roles in detoxification metabolism (ammonia metabolism in the urea cycle, e.g.) and in interorgan metabolism. In addition, some amino acids like glutamine, glutamate, and aspartate are precursors of the tricarboxylic acid cycle intermediates and represent oxidative substrates in numerous cell types which use them as major energy substrates (Yao et al. 2012). Amino acids can also be used indirectly as energy source, notably in the postprandial phase, as precursors for neoglucogenesis and ketogenesis.

In addition, and more recently, amino acids by themselves or after conversion in secondary metabolites have been associated with "signal functions" associated with metabolic and physiologic modifications. Just to take an example, L-arginine, after its enzymatic conversion by one isoform of nitric oxide synthase into nitric oxide (NO) and L-citrulline in endothelial cells, allows the action of NO which acts as a regulator of the arterial pressure. It is also feasible to consider that some amino acids, which are acting as neurotransmitter (like glutamate) or are precursor of neurotransmitter (like tryptophan which is a precursor of serotonine) or of biogenic amines (like histidine which is a precursor for histamine production), are amino acids with signaling functions.

Furthermore, an amino acid-like L-arginine is able to exert both metabolic regulatory (by activating the first step of ammonia detoxification in the urea cycle) and secretagogue functions (by stimulating for instance insulin and growth hormone secretion). Last but not least, the role of leucine as a stimulator of the protein synthesis through its action on the intracellular signaling pathways represents a very interesting example of the signaling functions of the amino acids (Yin et al. 2010; Li et al. 2011).

Proteins of all biological organisms, from the virus and bacteria to humans, are made up from the same set of 20 amino acids. Nineteen of these are α-amino acids with a primary amino group ($-NH_3^+$) and a carboxyl group ($-COO^-$) attached to a central carbon atom, aspartate ammonia-lyase, which is called the α-carbon atom (C_α) because it is adjacent to the carboxyl group (Fig. 7.1). The remaining groups attached to the C_α atom are a hydrogen atom (H) and a variable side chain or R' group. The one exception to this general structure is proline, a cyclic amino acid, which has a secondary amino group and is thus an α-imino acid. The only difference between any two amino acids is in their different side chains. Each side chain has

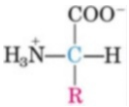


Fig. 7.1 General structure of an amino acid. Chemical structure of an amino acid showing the four different groups around the central α-carbon atom (with the exception of proline). The *R* group or side chain (*red*) attached to the α-carbon (*blue*) is different in each amino acid

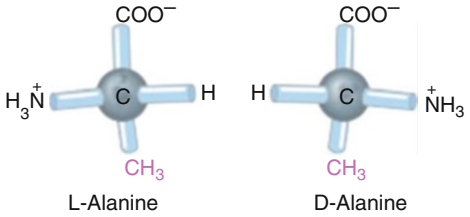


Fig. 7.2 Stereoisomerism in amino acids. The two stereoisomers of alanine, L- and D-alanine, are nonsuperimposable mirror images of each other (enantiomers)

distinct properties, including charge, hydrophobicity, and polarity. It is the arrangement of amino acids, with their distinct side chains, that is mainly responsible for protein structure and related functions.

All the 20 amino acids, except glycine (Gly or G), have four different groups arranged in tetrahedra around the central C_α atom which is thus known as an asymmetric center or chiral center and has the property of chirality. We note in Fig. 7.1 that the α-carbon is asymmetric, bonded to four different substituent groups: a carboxyl group, an amino group, an *R* group, and a hydrogen atom. Because of the tetrahedral arrangement of the bonding orbitals around the α-carbon atom of amino acids, the four different substituent groups can occupy two different arrangements in space, which are nonsuperimposable mirror images of each other. These two forms are called enantiomers or stereoisomers. Enantiomers are physically and chemically indistinguishable by most techniques, but can be distinguished on the basis of their different optical rotation of plane-polarized light. Molecules are classified as dextrorotatory (D; Greek *dextro* = right) or levorotatory (L; Greek *levo* = left) depending on whether they rotate the plane of plane-polarized light in a clockwise or an anticlockwise manner (Fig. 7.2). Surprisingly, only the L-amino acids are found in proteins, while D-isomers have been found only in small peptides of bacterial cell walls and in some peptide antibiotics.

Amino acids joined together by peptide bonds form the primary structure of a protein. The amino group of one molecule reacts with the carboxyl group of the other in a condensation reaction resulting in the elimination of water and the formation of a dipeptide (Fig. 7.3). A short sequence of amino acids is called a peptide, with the term polypeptide applied to longer chains of amino acids, usually of known sequence and length. When joined in a series of peptide bonds, amino acids are called "residues" to distinguish between the free form and the form found in protein. The peptide bond has a partial double-bond character.

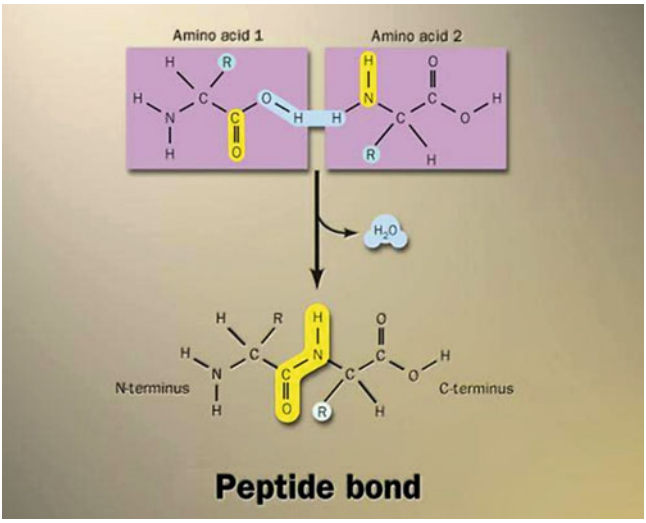


Fig. 7.3 Formation of a peptide bond. The peptide bond is a chemical, covalent bond formed between the α-amino group of one amino acid and the α-carboxyl group of another. Once two amino acids are joined together via a peptide bond to form a dipeptide, there is still a free amino group at one end and a free carboxyl group at the other, each of which can in turn be linked to further amino acids

7.2 Classification of Amino Acids

There are reasons to believe that amino acids are the oldest nutrients that have existed on earth. They have been used as the source of life and are sometimes called as the building blocks of life. There are as many as hundred thousand kinds of proteins that constitute the body, and these proteins are made with only 20 amino acids arranged in various combinations. The combination of the amino acids in proteins depends on the genetic code. In addition to the amino acids found in proteins, some amino acids not involved in the proteosynthesis are found in living organisms.

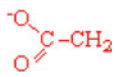
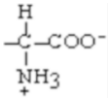
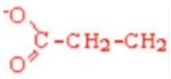
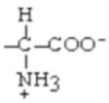
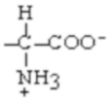
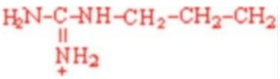
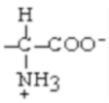
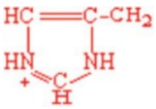
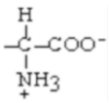
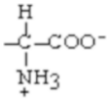
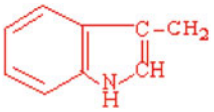
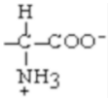
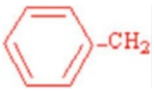
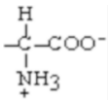
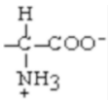
The names of the amino acids are often abbreviated, either to three letters or to a single letter. Thus, for example, alanine is abbreviated to Ala or A (Table. 7.1).

The hydrophobicity index informs on the relative hydrophobicity of amino acids. Higher positive value indicates stronger hydrophobicity. Hydrophilic amino acids have negative values. In a protein, hydrophobic amino acids are more likely to be located in the protein interior, whereas hydrophilic amino acids are more likely to face the aqueous environment.

7.2.1 Common Proteinogenic Amino Acids

Some of the 20 standard amino acids are called essential amino acids, because they cannot be synthesized by the body from other compounds through available metabolic pathways in the body, but instead must be provided by dietary sources.

Table. 7.1 Names, symbols, chemical structures, and hydrophobicity indices of the 20 amino acids found in proteins

Name	Symbol		*R* group		Hydrophobicity
	3 Letter	1 Letter			
Aspartate	Asp	D			−3.5
Glutamate	Glu	E			−3.5
Lysine	Lys	K			−3.9
Arginine	Arg	R			−4.5
Histidine	His	H			−3.2
Tyrosine	Tyr	Y			−1.3
Tryptophan	Trp	W			−0.9
Phenylalanine	Phe	F			2.8
Cysteine	Cys	C			2.5

(continued)

Table. 7.1 (continued)

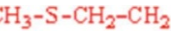
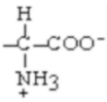
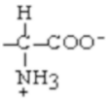
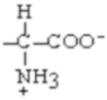
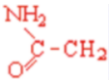
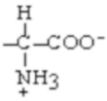
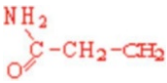
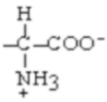
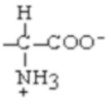
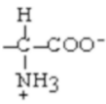
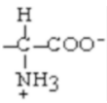
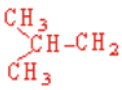
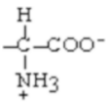
Name	Symbol		*R* group		Hydrophobicity
	3 Letter	1 Letter			
Methionine	Met	M			1.9
Serine	Ser	S			−0.8
Threonine	Thr	T			−0.7
Asparagine	Asn	N			−3.5
Glutamine	Gln	Q			−3.5
Glycine	Gly	G			−0.4
Alanine	Ala	A			1.8
Valine	Val	V			4.2
Leucine	Leu	L			3.8

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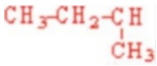
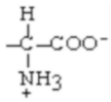
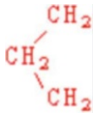
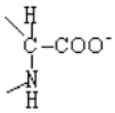
Table. 7.1 (continued)

Name	Symbol		*R* group		Hydrophobicity
	3 Letter	1 Letter			
Isoleucine	Ile	I			4.5
Proline	Pro	P			−1.6

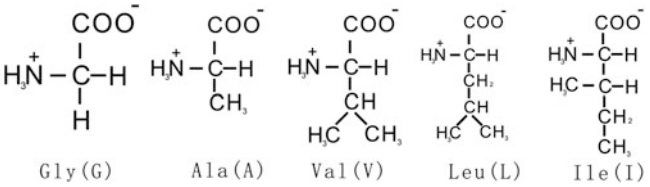


Fig. 7.4 Neutral aliphatic amino acids

In humans, the nine essential amino acids are lysine, leucine, isoleucine, methionine, phenylalanine, threonine, tryptophan, valine, and (in children) histidine. It is thus necessary to provide these essential amino acids from proteins in food in well-balanced, appropriate amounts and, in some cases, to provide some individual amino acids which are limiting for protein biosynthesis. Some amino acids are considered as conditionally indispensable since in some physiological situation (for instance in neonates) and in some pathological situations, the needs for some amino acids are increased to such an extent that endogenous synthesis capacity is not able to provide enough amino acids related to these needs.

Based on the chemical structure of the *R* groups, the 20 amino acids of proteins can be divided into aliphatic amino acids, aromatic amino acids, and heterocyclic amino acids, and among them, aliphatic amino acids are the most.

7.2.1.1 Aliphatic Amino acids

1. Neutral amino acids (Fig. 7.4):

   (a) Glycine (Gly or G): The smallest amino acid with the simplest structure, has an hydrogen atom in the side-chain position, and thus does not exist as a pair of stereoisomers since there are two identical groups (hydrogen atoms) attached to the C_α atom. In addition, Gly has minimal steric hindrance and in protein structure Gly offers the most flexibility.

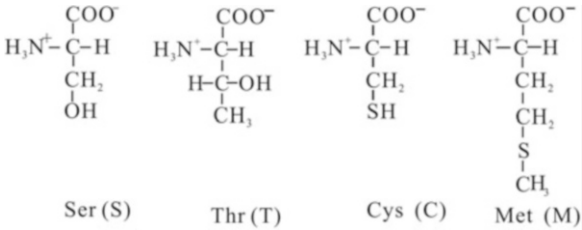


Fig. 7.5 Hydroxyl-containing or sulfur-containing amino acids

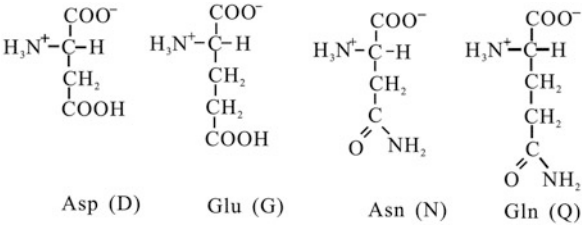


Fig. 7.6 Acidic amino acids and their amide derivatives

(b) Alanine (Ala or A).
(c) Valine (Val or V).
(d) Leucine (Leu or L).
(e) Isoleucine (Ile or I).

2. Hydroxyl-containing or sulfur-containing amino acids (Fig. 7.5):

(a) Serine (Ser or S).
(b) Threonine (Thr or T).
(c) Cysteine (Cys or C): Capable of reacting with another cysteine to form a disulfide bond.
(d) Methionine (Met or M).

3. Acidic amino acids and their amide derivatives (Fig. 7.6):

(a) Aspartate (Asp or D).
(b) Glutamate (Glu or E).
Asp and Glu have carboxyl in their *R* groups. They have net negative charge at pH 7.0, thus usually named as aspartate and glutamate (conjugate base names, instead of aspartic acid and glutamic acid, unionized form).
(c) Asparagine (Asn or N).
(d) Glutamine (Gln or Q).

4. Basic amino acids (Fig. 7.7):

(a) Lysine (Lys or K).
(b) Arginine (Arg or R).

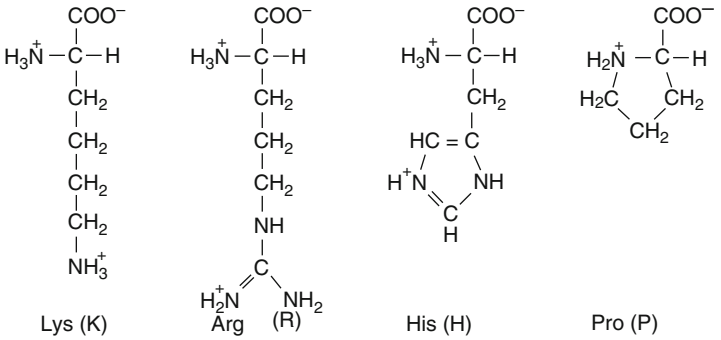
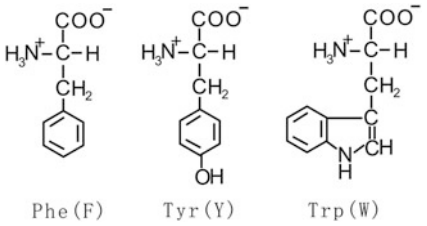


Fig. 7.7 Basic amino acids and heterocyclic amino acids

Fig. 7.8 Aromatic amino acids



7.2.1.2 Aromatic Amino Acids (Fig. 7.8)

1. Phenylalanine (Phe or F): Which contains a benzene ring.
2. Tyrosine (Tyr or Y): Which contains a benzene ring.
3. Tryptophan (Trp or W): Which contains an indole ring.

They are jointly responsible for the light absorption of proteins at 280 nm, a property used as a measure of the concentration of proteins.

7.2.1.3 Heterocyclic Amino Acids (Fig. 7.7)

1. Histidine (His or H).
2. Proline (Pro or P): Has an imino group, instead of an amino group, forming a ring structure, being rigid in conformation. It is often found in the bends of folded protein chains and often present on the surface of proteins. It offers the least flexibility.

Based on the polarity of *R* groups, the 20 amino acids of proteins can be classified as follows: (1) nonpolar, (2) polar but uncharged, (3) positively charged, and (4) negatively charged (Table. 7.2).

Table 7.2 The 20 standard amino acids of proteins

Nonpolar amino acids	*Polar-uncharged amino acids*
Gly	Ser
Ala	Cys
Pro	Thr
Val	Asn
Leu	Gln
Ile	Tyr
Met	
Phe	
Trp	
Positively charged amino acids	*Negatively charged amino acids*
Lys	Asp
Arg	Glu
His	
Nonpolar amino acids	*Polar-uncharged amino acids*
Gly	Ser
Ala	Cys
Pro	Thr
Val	Asn
Leu	Gln
Ile	Tyr
Met	
Phe	
Trp	
Positively charged amino acids	*Charged amino acids*
Lys	Asp
Arg	Glu
His	

1. Nonpolar amino acids
   The *R* groups in this class of amino acids are nonpolar and hydrophobic. The bulky side chains of alanine, valine, leucine, and isoleucine, with their distinctive shapes, are important in promoting hydrophobic interactions within protein structures. Glycine has the simplest amino acid structure. Where it is present in a protein, the minimal steric hindrance of the glycine side chain allows much more structural flexibility than the other amino acids. Proline represents the opposite structural extreme. The secondary amino (imino) group is held in a rigid conformation that reduces the structural flexibility of the protein at that point. Methionine, one of the two sulfur-containing amino acids, has a nonpolar thioether group in its side chain. Phenylalanine and tryptophan, with their aromatic side chains, are relatively nonpolar (hydrophobic).

2. Polar-uncharged amino acids
   The *R* groups of these amino acids are more water soluble, or hydrophilic, than those of the nonpolar amino acids, because they contain functional groups that form hydrogen bonds with water. This class of amino acids includes serine, threonine, cysteine, asparagine, glutamine, and tyrosine. The polarity of serine, threonine, and tyrosine is contributed by their hydroxyl groups; that of cysteine and methionine by their sulfur atom; and that of asparagine and glutamine by their amide groups.
3. Positively charged amino acids
   The amino acids in which the *R* groups have significant positive charge at pH 7.0 are lysine, which has a second amino group at the e position on its aliphatic chain; arginine, which has a positively charged guanidino group; and histidine, containing an imidazole group. Histidine is the only standard amino acid having a side chain with a pK_a near neutrality. This group of amino acids is sometimes refereed as cationic amino acids.
4. Negatively charged amino acids
   The two amino acids having *R* groups with a net negative charge at pH 7.0 are aspartate and glutamate, each with a second carboxyl group. These amino acids are the parent compounds of asparagine and glutamine, respectively. This group of amino acids is sometimes referred as anionic amino acids.

7.2.2 Uncommon Proteinogenic Amino Acids

In addition to the 20 usual amino acids that are common in all proteins, other amino acids have been found as components of only certain types of proteins (Fig. 7.9). Each of these is derived from one of the 20 usual amino acids, in a modification reaction that occurs after the standard amino acid has been inserted into a protein. For example, 4-hydroxyproline, a derivative of proline, is found in the fibrous protein collagen of connective tissues and 4-hydroxylysine is found in plant cell-wall proteins. *N*-methyllysine is found in myosin, a contractile protein of muscle. Another important unusual amino acid is γ-carboxyglutamate, found in the blood-clotting protein prothrombin as well as in certain other proteins that bind Ca^{2+} for their biological function. More complicated is the nonstandard amino acid desmosine, a derivative of lysine residues, found in the fibrous protein elastin. Selenocysteine contains selenium rather than the oxygen of serine, and is found in glutathione peroxidase and a few other proteins.

7.2.3 Nonprotein Amino Acids

Some 300 additional amino acids have been found in cells and have a variety of functions but are not substituents of proteins (Fig. 7.10). Ornithine and citrulline which are also present in proteins deserve special attention because they are key intermediates in the biosynthesis of arginine and in the urea cycle.

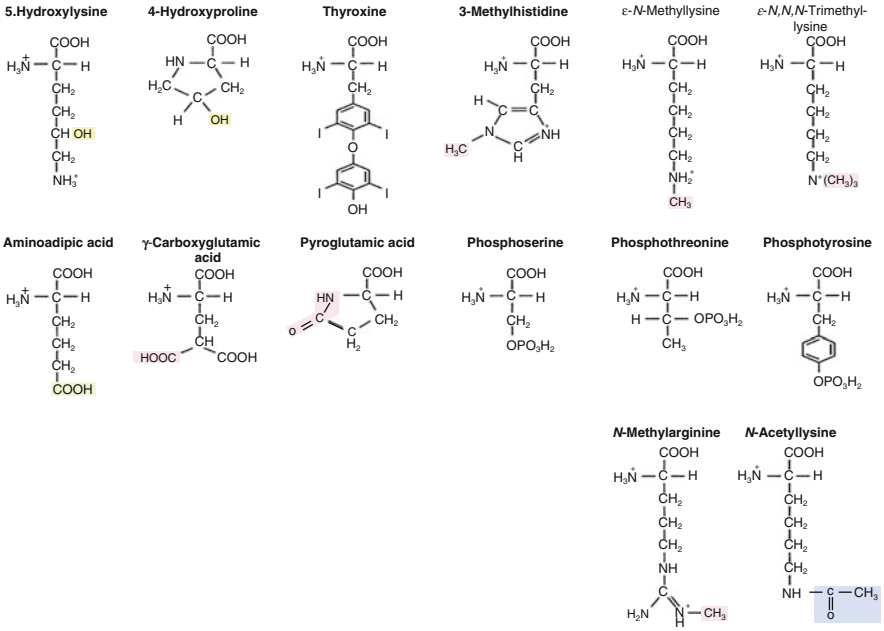


Fig. 7.9 Some of the uncommon proteinogenic amino acids

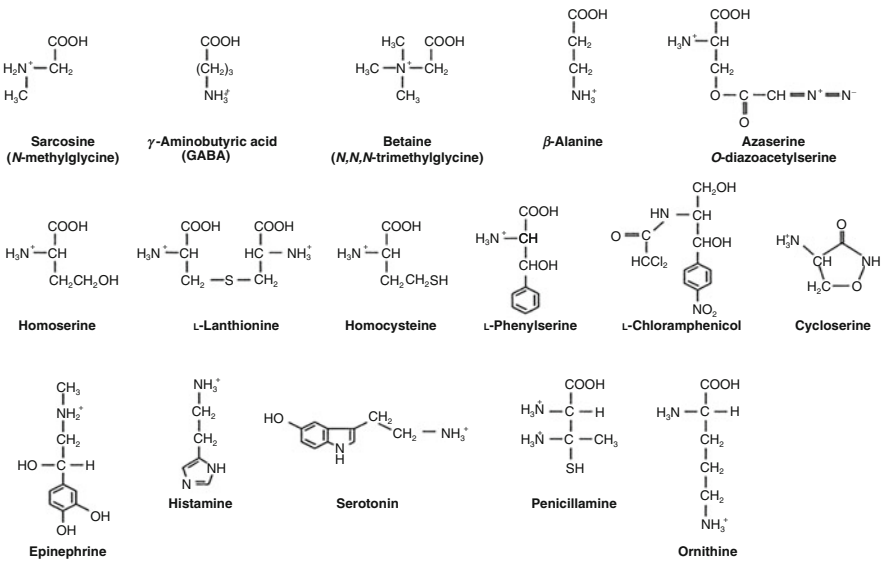


Fig. 7.10 Some of the amino acids and nitrogenous metabolites not found in proteins

7.3 Function of Amino Acids

The aim of this paragraph is to briefly recapitulate the metabolism of each amino acid together with their main biological functions.

7.3.1 Alanine

Alanine transaminase catalyzes the reversible conversion of L-alanine and alpha-ketoglutarate into pyruvate and glutamate. Alanine is a substrate for the neoglucogenesis.

7.3.2 Arginine

L-arginine is a precursor for biosynthesis of other amino acids present in proteins (glutamate and proline) or not present in proteins (ornithine, citrulline). Arginine is a precursor of urea, polyamines, creatine, and nitric oxide. Polyamines are involved in cell mitosis. Creatine is synthesized from arginine, glycine, and methionine but can also be provided by the diet. Creatine and creatine phosphate are mainly found in skeletal muscles. Phosphocreatine plays a central role in muscle contraction. Nitric oxide has been implicated in numerous functions, but has been initially shown to be produced in vascular endothelial cells and to be implicated in vasorelaxation. Arginine is used in urea cycle for ammonium detoxification.

7.3.3 Asparagine

L-asparagine can be converted into aspartate and ammonium.

7.3.4 Aspartic Acid

Aspartate transaminase is responsible for the reversible conversion of aspartate and alpha-ketoglutarate into oxaloacetate and glutamate. Aspartate is involved in the urea cycle for the conversion of citrulline into argininosuccinate. Aspartate is a precursor for pyrimidine synthesis.

7.3.5 Cysteine

Cysteine can be produced from the metabolic conversion of methionine and serine. Cysteine is a precursor for taurine biosynthesis and is one of the amino acids which are precursors for glutathione and hydrogen sulfide (H_2S). Cysteine is a precursor for pyruvate synthesis and for the synthesis of the nucleotidic coenzyme CoA. Taurine is a beta-amino sulfonic acid which is not incorporated into proteins. The circulating concentrations of taurine are much dependent on the dietary intake (Laidlaw et al. 1998). Taurine functions are numerous. This amino acid is involved in the synthesis of conjugated biliary acids. Taurine has been implicated as a regulator of the cellular volume, and as such, as an agent with cytoprotective effect. A protective role of taurine against deleterious effects of the "oxidative stress" has been proposed and may be due to the reaction between taurine and hypochlorous acid (Schuller-Levis and Park, 2004). Glutathione is a tripeptide which is synthesized in cells from glutamate, cysteine, and glycine. Circulating glutathione is usually micromolar (Jones et al. 2000). This compound from alimentary origin can be absorbed and the jejunum represents the main site of absorption (Hagen et al. 1990). Glutathione can exist in cells both in reduced (GSH) and oxidized form (GSSG) with reduced glutathione being much prominent. The GSH/GSSG couple represents the main component fixing the intracellular redox state. In mammals, the amount of cysteine used for glutathione synthesis is very high since it represents as much as 30–50% of the total body utilization (Fukagawa et al. 1996). Glutathione is involved in several physiological functions including the elimination of electrophilic compounds, control of intracellular reactive oxygen species, etc.

7.3.6 Glutamic Acid

Glutamic acid is metabolized in transamination pathways. This amino acid can be converted in the presence of pyruvate into alpha-ketoglutarate and alanine, and in the presence of oxaloacetate into aspartate and alpha-ketoglutarate. Glutamic acid can also undergo conversion to alpha-ketoglutarate and ammonium. As such, glutamate is an oxidative substrate in several cell types. Glutamic acid can be synthesized from other amino acids like arginine, proline, and histidine. Glutamine is a precursor of glutamate and ammonium. Inversely, glutamate is a precursor for glutamine synthesis. Lastly, glutamate is one of the amino acid precursors for glutathione synthesis (Wang et al. 2008).

7.3.7 Glutamine

Glutamine is metabolized into glutamate, ammonium, alanine, aspartate, citrulline and proline. Glutamine is a major oxidative substrate in numerous cell types. Glutamine is a nitrogen donor for the biosynthesis of nucleotides.

7.3.8 Glycine

Glycine is a precursor for the biosynthesis of serine and for the synthesis of glutathione, creatine, purines, and heme in hemoglobin.

7.3.9 Histidine

Histidine is abundant in hemoglobin. Histidine is a precursor of glutamate and histamine. This latter compound is involved in allergy and in inflammatory reactions.

7.3.10 Isoleucine, Leucine, and Valine

The amino acids with branched chain undergo the transamination in the presence of alpha-ketoglutarate allowing the synthesis of glutamate and of the corresponding alpha-ketoacids (alpha-ketoisocaproate from leucine, alpha-keto-beta-methyl-valerate from isoleucine, and alpha-ketoisovalerate from valine). Each of these ketoacids undergoes then an oxidative decarboxylation by a ketoacid dehydrogenase (BCKAD). The products of decarboxylation undergo then several metabolic steps leading to the synthesis of acetyl-CoA, acetoacetic acid, propionyl-CoA, and succinyl CoA. It is well known that branched-chain amino acids can be in competition with other amino acids (notably tryptophan and tyrosine) for transport through cellular membranes (Anderson and Johnston 1983). Although branched-chain amino acids are not direct precursors for neurotransmitter synthesis, they can affect the transport of other amino acids across the blood–brain barrier, and then the concentrations of some neurotransmitters in the central nervous system (Fernstrom, 1973).

7.3.11 Lysine

Lysine is a precursor for acetoacetyl-CoA, carnitine, and cadaverine. Carnitine is a metabolite with important functions in the intermediary metabolism. Carnitine is involved in the transport of fatty acids with long chain from the cytosol to the

mitochondrial matrix where the beta-oxidation occurs. In the body, carnitine is originating mainly from dietary source, with endogenous synthesis playing a minor role (Vaz and Wanders, 2002). Carnitine is mainly synthesized in the liver from lysine and methionine. Cadaverine is a polyamine produced by the intestinal microbiota.

7.3.12 Methionine

Methionine is a precursor of succinyl-CoA, homocysteine, cysteine, creatine, and carnitine. In addition, methionine is a precursor for S-adenosyl-methionine synthesis which is involved in polyamine, creatine, and phosphatidylcholine (one of the membrane phospholipids) metabolism.

7.3.13 Phenylalanine

This amino acid is a precursor for the synthesis of tyrosine and acetoacetyl-CoA.

7.3.14 Proline

Proline is a precursor for glutamate synthesis in several cell types. Proline can undergo hydroxylation leading to the production of hydroxyproline. Proline and hydroxyproline are abundant in collagens.

7.3.15 Serine

Serine is one of the precursors for the biosynthesis of cysteine and for the synthesis of the sphingolipid sphingosine. This amino acid can also be metabolized into pyruvate.

7.3.16 Threonine

This amino acid is a precursor for the synthesis of glycine and acetyl-CoA. This amino acid is very abundant in mucins (Wang et al. 2007).

7.3.17 Tryptophan

Tryptophan is a precursor of numerous metabolites including the neurotransmitter serotonine (5-hydroxytryptamine), tryptamine, the hormone melatonin, and niacin (vitamin B3). In addition, tryptophan is a precursor for the synthesis of acetyl-CoA (Yao et al. 2011).

7.3.18 Tyrosine

Tyrosine is a precursor for active substances including the neurotransmitters epinephrine and norepinephrine, the pigment melanin, and the hormone thyroxine. Tyrosine is also a precursor for the production of acetoacetyl-CoA.

It is worth to note that although metabolites derived from amino acids play major role in the body physiology, the metabolic pathways involved in their synthesis represent a very minor utilization of the amino acid precursors when compared with the utilization of the amino acids for protein synthesis.

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Chapter 8
Synthesis and Degradation of Proteins in Pigs

Fugui Yin, Yulong Yin, and Yongqing Hou

Contents

8.1 Introduction

Growth of animals is the complex result of competition between anabolic and catabolic process, which implies constant changes and remodeling through synthesis of new proteins and breakdown of existing proteins (Jobgen et al. 2006; Tan et al. 2009). Together, these processes are called protein turnover and produce muscle growth or hypertrophy when synthesis is greater than breakdown and muscle wasting when synthesis is less than breakdown (Norton and Layman 2006). Protein turnover requires large amounts of ATP. However, this costly metabolic cycle fulfills key obligatory

F. Yin • Y. Yin (✉)
Chinese Academy of Sciences, and Ministry of Agriculture, Observation and Experiment Station of Animal Nutrition and Feed Science in South-Central China; Hunan Provincial Engineering Research Center for Healthy Livestock and Poultry Production; Key Laboratory of Agri-Ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Changsha, Hunan 410125, People's Republic of China
e-mail: yinyulong@isa.ac.cn

Y. Hou
Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, Wuhan 430023, China

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_8,

functions, including protein homeostasis, cell turnover, removal of aged and damaged proteins, synthesis of new proteins like heat-shock and immunological proteins, gluconeogenesis from amino acids, wound healing, tissue repair, adaptation to nutritional and pathological alterations, and immune responses (Wu 2009). In pigs, although the protein syntheses increase in all tissues, the greatest response occurs in skeletal muscle in response to feeding stimulation. The elevated postprandial protein synthesis in skeletal muscle of pigs therefore increases the protein deposition during the post-absorptive period allowing growth and development. A sharp increase of circulating glucose, insulin, and amino acids, especially some nutritional indispensable amino acids, is observed after meal (Yin et al. 2010, 2011; Kong et al. 2012), in accordance with higher protein deposition in pigs (Drew et al. 2012). The mechanism responsible for the stimulation of protein synthesis by feeding was therefore focused on the roles of postprandial circulating glucose, insulin, and amino acids (Kong et al. 2012). The protein degradation is a process where proteins are broken into smaller peptides as well as free amino acids, the latter being either reused for new protein synthesis or further degraded into several metabolites, several of them being able to generate ATP for energy use. In addition, the protein degradation plays important roles in animal physiological process, especially in the cellular signal transduction system as well as in the maintenance of the integrity of the proper folded state of protein.

8.2 Protein Syntheses in Pigs

Protein biosynthesis is the process in which cells build or manufacture proteins. This process which refers to a multistep pathway begins with amino acid synthesis and transcription of nuclear DNA into messenger RNA, which are then used as inputs for translation. In pigs, the neonatal period is characterized by rapid growth and elevated rates of protein synthesis, a phenomenon which decreases sharply with age. The declined efficiency of protein synthesis and deposition with age in the whole animal depends on two factors: (1) the energy cost of protein synthesis and (2) the extent by which the efficiency of protein synthesis in muscle is done (Bates and Millward 1981). The elevated capacity for protein synthesis in the young pig is driven by the high ribosomal content together with the increased efficiency of the translation process, allowing accelerated protein synthesis rates (Davis et al. 2008). In accordance with the elevated protein synthesis rate, the deposition of protein therefore is increased. Protein degradation is modest during early life. Hormonal and some nutritional factors, such as glucose and amino acids, are thought to be the most potent signal molecules that regulate the postnatal muscle protein synthesis. However, the feeding can be considered as a basic and active stimulator of muscle protein synthesis in neonatal pigs even if the response to such a stimulation tends to decrease with age. The feeding-induced stimulation of muscle protein synthesis is modulated by an enhanced sensitivity to the postprandial increase in insulin and amino acids. The developmental decline in response to insulin and amino acids parallels a marked decrease in the feeding-induced activation of the translation initiation factors that regulate the binding of mRNA to the 40S ribosomal complex.

The abundance and activation of many known positive regulators of the nutrient- and insulin-signaling pathways that are involved in translation initiation are high, whereas those of many negative regulators are low in skeletal muscle of younger pigs (Davis et al. 2008).

8.2.1 Signaling Pathways in Response to Protein Synthesis

The mammal target of rapamycin (mTOR) is a major protein kinase that modulates translation initiation components. The phosphorylation of mTOR on Ser^{2448} activates the kinase and is stimulated by both insulin and amino acids (Fig. 8.1) (O'Connor et al. 2003a, b; Davis 2008). The mTOR system consists of (1) rapamycin-sensitive complex 1 (mTOR1, mTOR raptor (regulatory associated protein of TOR), and G protein b-subunit-like protein); and (2) rapamycin-insensitive complex 2 (mTOR2) (mTOR rictor (rapamycin-insensitive companion of TOR), mitogen-activated-protein kinase-associated protein 1, and G protein b-subunit-like protein) (Wu 2009). These two complexes are structurally and functionally distinct in cells. The mTOR1 regulates mRNA translation by phosphorylation of the 70-kDa S6 kinase-1 (S6K1) and eIF4E-binding protein 1 (4E-BP1). S6K1 activation results in phosphorylation of the ribosomal subunit, ribosomal protein S6, which may lead to an increase in the translation of mRNAs that encode proteins found in the protein synthetic machinery (Kimball and Jefferson 2004). Phosphorylation of the repressor protein, 4E-BP1, permits the dissociation of eIF4E, allowing it to bind to eIF4G. This active complex of eIF4E.eIF4G mediates the binding of mRNA to the 40S ribosomal complex in the initiation of mRNA translation (Davis et al. 2000; Kimball et al. 2000; Suryawan et al. 2001). In addition to mRNA binding to the ribosome, translation initiation requires initiator methionyl-tRNA (met-tRNAi) binding to the start codon, a step mediated by eIF2 (Wilson et al. 2009). In its GTP form, eIF2 binds the ribosome and locates the mRNA start site, causing eIF2 hydrolysis and disassociation; eIF2B then recharges eIF2 with a GTP (Wek et al. 2006). The phosphorylation of the α-subunit of eIF2 transforms it into a competitive inhibitor of eIF2B, preventing activation of eIF2 and, consequently, reducing met-tRNAi binding to the ribosome (Bush et al. 2003; O'Connor et al. 2004). The mTOR2 phosphorylates protein kinase B/Akt and may function to regulate cell proliferation, differentiation, migration, and cytoskeletal reorganization (Sarbassov et al. 2005).

8.2.2 Feeding Induces Protein Syntheses in Pig

The feeding causes complex physiological and biochemical responses in animals. The circulating hormones (e.g., insulin) and nutritional metabolites (e.g., glucose and amino acids) are relatively elevated even with no food intake in pigs, and therefore ameliorate the whole-body nutritional metabolic status. Although with

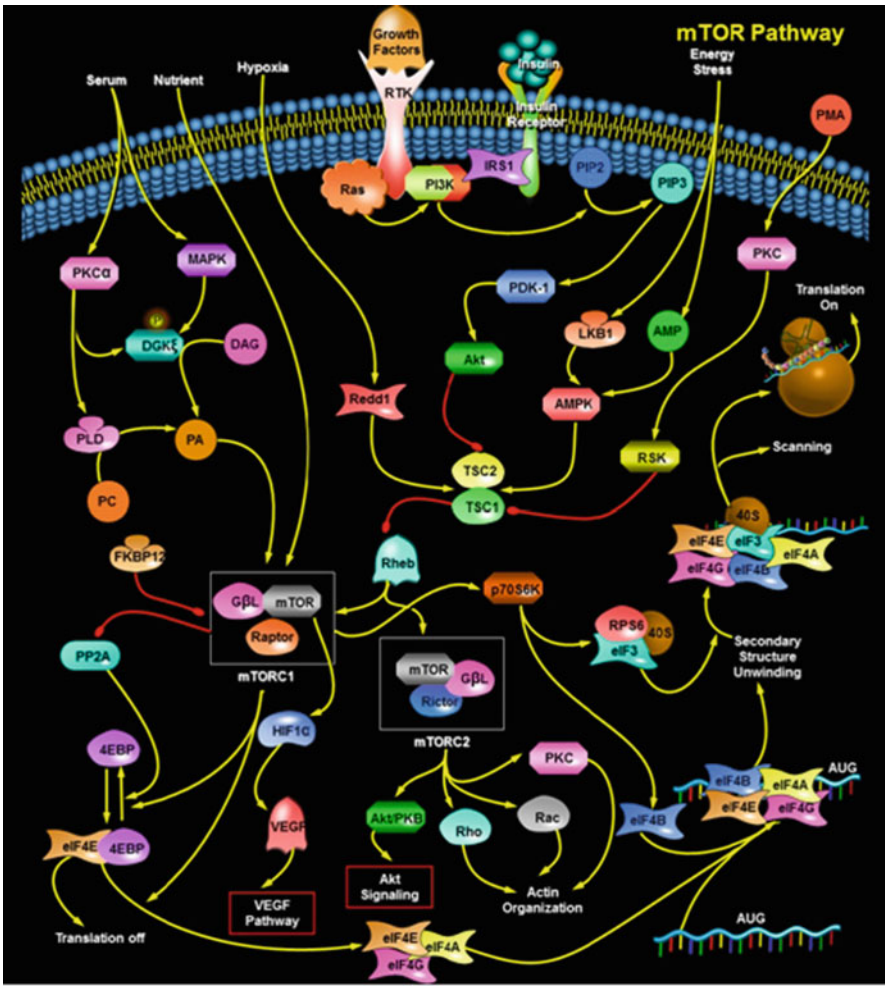

Fig. 8.1 Insulin- and nutrition (main are amino acid and glucose)-signaling pathway leading to the stimulation of translation initiation. Activation of the insulin-signaling pathway is initiated by the binding of insulin to its receptor. This activates the insulin receptor and insulin receptor substrate-1 (IRS-1), followed by the activation of phosphoinositide-3 kinase (PI 3-K). Activated PI 3-K then stimulates the activation of phosphoinositide-dependent kinase 1 (PDK-1) and protein kinase B (PKB). Phosphorylation of PKB inactivates tuberous sclerosis complex 1/2 (TSC1/2), thereby inducing the activation of mammalian target of rapamycin (mTOR). Amino acids as well as insulin can activate mTOR, which exists in a complex with raptor and G protein β-subunit-like protein (GβL). Activated mTOR phosphorylates ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor (eIF) 4E-binding protein-1 (4EBP1). Phosphorylation of S6K1 enhances the activation of ribosomal subunit S6 (rpS6), which increases the translation of specific mRNA. Phosphorylated 4EBP1 releases eIF4E from an inactive eIF4E–4EBP1 complex, allowing the formation of the active eIF4G–eIF4E complex, which mediates the binding of mRNA to the 43S ribosomal complex. Insulin signaling can be attenuated by protein tyrosine phosphatase-1B (PTP-1B), which dephosphorylates the insulin receptor and IRS-1; phosphatase and tensin

some differences in response to different dietary treatments (Deng et al. 2009; Deng et al. 2010), the postprandial circulating concentrations of glucose, amino acids, and insulin raised and are maintained at relatively high levels for 4 h after the meal and then returned to the baseline whatever pigs consumed their daily allowance under the feeding procedures of "six-time intake per day" (Yin et al. 2010; 2011; Deng et al. 2009; Deng et al. 2010), "three-time intake per day" (Liu et al. 2007), or "two-time intake per day" (Regmi et al. 2010) (Tables 8.1 and 8.2, Figs. 8.2 and 8.3). Therefore the whole-body fractional protein syntheses rate may be higher within the postprandial 4 h than during the time from postprandial 4th hour till next meal. In young pigs, the feeding stimulates protein synthesis in most tissues, and the efficiency by which dietary amino acids are used for protein deposition is high (Burrin et al. 1992). Notably, the most profound postprandial increase in protein synthesis occurs in skeletal muscle, which is of high metabolic significance, because skeletal muscle comprises a large proportion of the body mass in pigs. Feeding promotes the protein synthesis largely through glucose-, amino acid-, or insulin-induced formation of the active eukaryotic translation initiation factor (eIF) 4E.eIF4G complex or mTOR pathways (Kimball et al. 2000; Avruch et al. 2006; Jeyapalan et al. 2007; Suryawan et al. 2007). The phosphorylation of mTOR peaked within 30 min after starting the feeding, followed by the phosphorylation of the downstream targets of mTORC1, 4E-BP1, eIF4G, and S6 (Fig. 8.4), and then regulation of the binding of the mRNA with the 43S pre-initiation complex (Wilson et al 2009). These physiological and biochemical processes paralleled the stimulation of skeletal muscle protein synthesis and led to the enhancement of protein synthesis in pigs. In addition, the rate of translation initiation is up-regulated earlier than elongation after feeding. Although the elongation is also increased after meal consumption, this increase is delayed relative to the change in initiation. Interestingly, increased rates of elongation did not further increase muscle protein synthesis in response to feeding, which therefore indicates that initiation is the rate-limiting step in translation (Wilson et al. 2009). Therefore, the increased rates of initiation, but not elongation, are responsible for the increase in muscle protein synthesis in response to feeding in pigs.

8.2.3 Glucose, Amino Acids, and Insulin Stimulate Protein Syntheses in Pig

By using an insulin–amino acid–glucose clamp technique, the protein synthesis in skeletal muscle of young pigs has been found to be stimulated by glucose, insulin,

Fig. 8.1 (continued) homologue deleted on chromosome 10 (PTEN), which inactivates PI 3-K; and protein phosphatase 2A (PP2A), which acts on PKB and S6K1. An increase in adenosine monophosphate (AMP) levels enhances AMP kinase activation, resulting in the activation of TSC1/2 complex and the decreased activation of mTOR (Davis et al. 2008)

Table 8.1 Serum amino acid concentrations after first meal in response to different dietary treatment in pigs (mmol/L)

	Sampling time													
	8:30			9:50			10:50			11:50				
Item	HDRS	MDRS	LDRS	HDRS	MDRS	LDRS	HDRS	MDRS	LDRS	HDRS	MDRS	LDRS	Pooled SEM	Time effect, *P* value
Nutritionally indispensable amino acids														
Arginine	0.089	0.087	0.083	0.179^{a}	0.128^{b}	0.094^{b}	0.163^{a}	0.107^{b}	0.084^{c}	0.070	0.068	0.064	0.004	<0.001
Cysteine	0.055^{a}	0.045^{a,b}	0.038^{b}	0.086^{a}	0.064^{b}	0.068b	0.059	0.045	0.046	0.037	0.034	0.034	0.001	<0.001
Histidine	0.038	0.036	0.032	0.062^{a}	0.041^{b}	0.045^{b}	0.038	0.032	0.044	0.032	0.028	0.040	0.001	0.018
Isoleucine	0.050	0.047	0.048	0.090^{a}	0.055^{b}	0.060^{b}	0.061^{a}	0.050^{a,b}	0.048^{b}	0.049^{a}	0.034^{b}	0.038^{a,b}	0.001	0.001
Leucine	0.205^{a}	0.215^{a}	0.173^{b}	0.310^{a}	0.260^{b}	0.245^{b}	0.271^{a}	0.254^{a,b}	0.204^{b}	0.148	0.154	0.142	0.004	<0.001
Lysine	0.287^{a}	0.261^{a,b}	0.233^{b}	0.583^{a}	0.574^{a}	0.297^{b}	0.320^{a}	0.233^{b}	0.227^{b}	0.242^{a}	0.220^{a}	0.158^{b}	0.009	0.004
Methionine	0.037	0.031	0.031	0.055^{a}	0.038^{b}	0.033^{b}	0.061^{a}	0.034^{b}	0.036^{b}	0.040^{a}	0.029^{b}	0.028^{b}	0.002	0.004
Phenylalanine	0.085	0.089	0.083	0.137^{a}	0.116^{b}	0.102^{b}	0.120^{a}	0.100^{b}	0.103^{b}	0.082	0.071	0.074	0.002	0.001
Threonine	0.452^{a}	0.380^{b}	0.375^{b}	0.525^{a}	0.422^{c}	0.460^{b}	0.384^{a}	0.342^{a,b}	0.308^{b}	0.285^{a}	0.294^{a}	0.247^{b}	0.010	<0.001
Tryptophan	0.235^{a}	0.197^{b}	0.183^{b}	0.288^{a}	0.237^{b}	0.223^{b}	0.246^{a}	0.213^{b}	0.200^{b}	0.200^{a}	0.166^{b}	0.143^{b}	0.007	0.009
Tyrosine	0.104^{a}	0.093^{a,b}	0.084^{b}	0.164^{a}	0.118^{b}	0.117^{b}	0.152^{a}	0.114^{b}	0.093^{b}	0.101^{a}	0.076^{a,b}	0.068^{b}	0.002	<0.001
Valine	0.112	0.094	0.100	0.166^{a}	0.098^{c}	0.129^{b}	0.138^{a}	0.101^{b}	0.100^{b}	0.121^{a}	0.074^{b}	0.087^{b}	0.002	0.011
Nutritionally dispensable amino acids														
Alanine	0.387^{a}	0.334^{b}	0.350^{a,b}	0.450^{a}	0.389^{b}	0.384^{b}	0.476^{a}	0.465^{a,b}	0.414^{b}	0.406^{a}	0.390^{a}	0.366^{b}	0.011	0.001
Aspartate	0.025	0.022	0.028	0.030	0.032	0.030	0.049	0.042	0.042	0.034^{a}	0.026^{b}	0.022^{b}	0.001	0.001
Glutamate	0.241	0.227	0.226	0.248^{b}	0.278^{a,b}	0.298^{a}	0.324^{a}	0.318^{a,b}	0.279^{b}	0.310^{a}	0.205^{c}	0.260^{b}	0.007	0.010
Glycine	0.463^{a}	0.432^{a,b}	0.410^{b}	0.541^{a}	0.520^{a,b}	0.487^{b}	0.549^{a}	0.545^{a,b}	0.522^{b}	0.471^{a}	0.473^{a}	0.427^{b}	0.015	0.014
Proline	0.337^{a}	0.359^{a}	0.256^{b}	0.623^{a}	0.577^{b}	0.555^{b}	0.541^{a}	0.492^{b}	0.435^{c}	0.205	0.232	0.233	0.012	<0.001
Serine	0.105^{a}	0.095^{a,b}	0.084^{b}	0.117	0.124	0.122	0.089^{a,b}	0.095^{a}	0.080^{b}	0.088	0.084	0.071	0.001	<0.001

$^{a–c}$Within the same sampling time, values in a row sharing different superscript letters differ ($P < 0.05$); $n = 6$

HDRS-fed group high digestion rate starch-fed group, *LDRS* low digestion rate starch-fed group, *MDRS* moderate digestion rate starch, *SEM* standard error of the mean

Source: Yin et al. (2010) (with minor modification, the pigs were fed at 8:00 am)

Table 8.2 Serum amino acid concentrations after second feeding in response to different dietary treatment in pigs (mmol/L, continued from Table 8.1)

	Sampling time													
	12:30			13:30			14:30			15:30				
Item	HDRS	MDRS	LDRS	HDRS	MDRS	LDRS	HDRS	MDRS	LDRS	HDRS	MDRS	LDRS	Pooled SEM	Time effect, *P* value
Nutritionally indispensable amino acids														
Arginine	0.112^{a}	0.079^{b}	$0.100^{a,b}$	0.181^{a}	0.132^{b}	0.128^{b}	0.141^{a}	$0.115^{a,b}$	0.080^{b}	0.074	0.065	0.069	0.004	<0.001
Cysteine	0.036^{a}	0.029^{b}	0.036^{a}	0.056^{a}	0.043^{b}	0.041^{b}	0.037	0.034	0.034	0.034	0.033	0.030	0.001	<0.001
Histidine	0.051	0.043	0.046	0.063^{a}	0.041^{b}	0.049^{b}	0.041	0.042	0.050	0.037	0.031	0.040	0.001	0.018
Isoleucine	0.057^{a}	0.037^{b}	0.056^{a}	0.090^{a}	0.055^{b}	0.060^{b}	0.066	0.056	0.047	0.049^{a}	0.036^{b}	$0.041^{a,b}$	0.001	0.001
Leucine	0.192	0.184	0.177	0.271^{a}	0.278^{a}	0.204^{b}	0.213^{b}	0.254^{a}	0.208^{b}	0.159	0.179	0.167	0.004	<0.001
Lysine	0.302^{a}	0.272^{b}	0.234^{b}	0.583^{a}	0.574^{a}	0.297^{b}	0.491^{a}	0.497^{a}	0.222^{b}	0.276	0.263	0.269	0.009	0.004
Methionine	0.044^{a}	0.032^{b}	0.034^{b}	0.055^{a}	0.038^{b}	0.033^{b}	0.046^{a}	$0.037^{a,b}$	0.032^{b}	0.039^{a}	0.030^{b}	0.030^{b}	0.002	0.004
Phenylalanine	0.096	0.087	0.096	0.133^{a}	0.100^{b}	0.103^{b}	0.105^{a}	$0.100^{a,b}$	0.088^{b}	0.087	0.077	0.082	0.002	0.001
Threonine	0.392^{a}	0.316^{b}	$0.345^{a,b}$	0.452^{a}	0.380^{b}	0.375^{b}	0.374^{a}	0.411^{a}	0.126^{b}	$0.276^{a,b}$	0.326^{a}	0.257^{b}	0.010	<0.001
Tryptophan	0.243^{a}	0.192^{b}	0.171^{b}	0.280^{a}	0.226^{b}	0.223^{b}	0.252^{a}	0.217^{b}	0.208^{b}	0.190^{a}	0.156^{b}	0.147^{b}	0.007	0.009
Tyrosine	0.128^{a}	0.090^{b}	0.094^{b}	0.164^{a}	0.114^{b}	0.093^{b}	0.146^{a}	$0.116^{a,b}$	0.092^{b}	0.112^{a}	$0.083^{a,b}$	0.074^{b}	0.002	<0.001
Valine	0.127^{a}	0.082^{b}	0.117^{a}	0.166^{a}	0.098^{c}	0.129^{b}	0.138^{a}	0.100^{b}	0.101^{b}	0.120^{a}	0.074^{b}	$0.089^{a,b}$	0.002	0.011
Nutritionally dispensable amino acids														
Alanine	0.473^{a}	0.434^{b}	$0.452^{a,b}$	0.480^{b}	0.474^{b}	0.569^{a}	0.582^{a}	0.594^{a}	0.486^{b}	0.530^{a}	0.341^{b}	0.374^{b}	0.011	0.001
Aspartate	0.033	0.029	0.036	0.035	0.033	0.044	0.033	0.042	0.041	0.036	0.027	0.029	0.001	0.001
Glutamate	0.273^{a}	0.226^{b}	0.282^{a}	0.291	0.266	0.299	0.305	0.319	0.345	0.319^{a}	0.227^{b}	$0.252^{a,b}$	0.007	0.010
Glycine	$0.491^{a,b}$	0.525^{a}	0.479^{b}	0.551^{b}	0.624^{a}	$0.590^{a,b}$	0.587^{b}	0.684^{a}	0.483^{c}	0.499^{a}	$0.473^{a,b}$	0.444^{b}	0.015	0.014
Proline	0.561^{a}	0.527^{b}	0.537^{b}	0.727^{a}	0.781^{a}	0.512^{b}	0.654^{b}	0.714^{a}	0.330^{c}	0.252^{a}	0.269^{a}	0.218^{b}	0.012	<0.001
Serine	0.100	0.101	0.089	0.126^{a}	$0.115^{a,b}$	0.093^{b}	$0.108^{a,b}$	0.115^{a}	0.084^{b}	0.089	0.088	0.077	0.001	<0.001

[a–c]Within the same sampling time, values in a row sharing different superscript letters differ ($P < 0.05$); $n = 6$

HDRS high digestion rate starch-fed group, *LDRS-fed group* low digestion rate starch-fed group, *MDRS* moderate digestion rate starch-fed group, *SEM* standard error of the mean

Source: Yin et al. (2010) (with minor modification, the pigs were fed at 12:00 am)

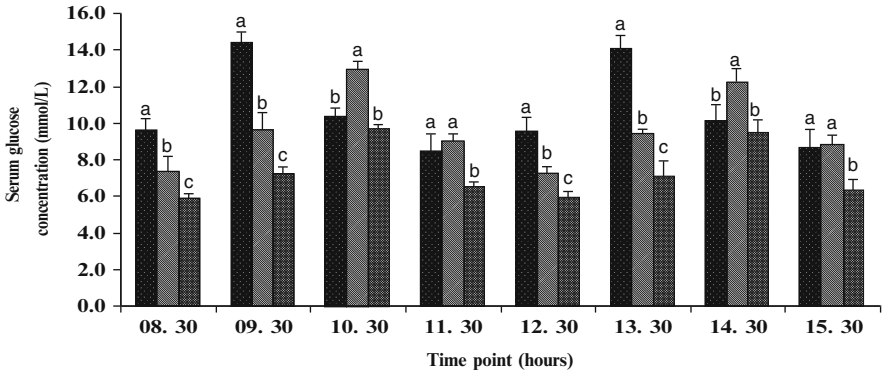

Fig. 8.2 Variation in postprandial serum systemic circulating glucose in two feeding cycles. All pigs were fed at 8:00 and 12:00 pm, respectively. (▦), HDRS-fed group; (▨), MDRS-fed group; (▩), LDRS-fed group. [a–c]mean values (n, 8 per group) within the same sampling time with unlike letters were significantly different ($P < 0.05$). *HDRS* high digestion rate starch, *LDRS* low digestion rate starch, *MDRS* moderate digestion rate starch. *Source*: Yin et al. (2011)

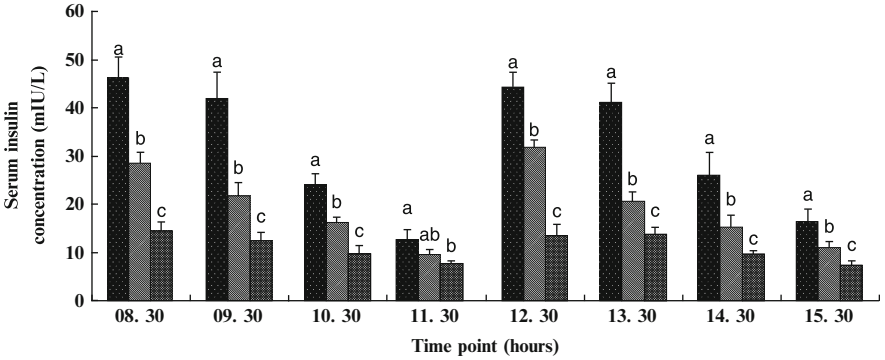

Fig. 8.3 Variation in postprandial serum systemic circulating insulin concentration in two feeding cycles. All pigs were fed at 8:00 am and 12:00 pm, respectively. (▦), HDRS group; (▨), MDRS group; (▩), LDRS group. [a–c]mean values (n, 8 per group) within the same sampling time with unlike letters were significantly different ($P < 0.05$). *HDRS* high digestion rate starch, *LDRS* low digestion rate starch, *MDRS* moderate digestion rate starch. *Source*: Yin et al. (2011)

and amino acids used independently or together (O'Connor et al. 2003a, b; Jeyapalan et al. 2007). Raising glucose alone increased protein synthesis more in fast-twitch glycolytic muscles than in other tissues like the liver, intestine, pancreas, spleen, kidney, and lung (Jeyapalan et al. 2007). Raising either insulin or amino acids alone increases protein synthesis in most tissues, especially in skeletal muscle (Davis et al. 2001; O'Connor et al. 2003a, b). Interestingly, elevating both insulin and amino acids or raising glucose, insulin, and amino acids together to postprandial levels increases protein synthesis in several visceral tissues, such as pancreas, kidney, spleen, and liver, as well as in skeletal muscles (Davis et al. 2000). Since the circulating

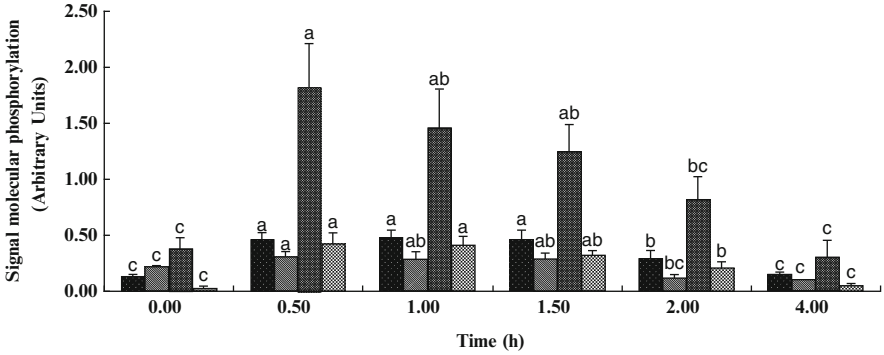

Fig. 8.4 Time course of the changes in phosphorylation of important molecular signal in protein syntheses in skeletal muscle of neonatal pigs after a meal. All pigs were fed at 8:00 am. (▦), mTOR; (▨), 4EBP1; (▩), eIF4G; (▩), S6. [a–c]The multiple comparison was conducted within the same parameter with different sampling time, unlike letters indicated a significantly difference between sampling time ($P < 0.05$). All results are corrected for total protein. *mTOR* the mammal target of rapamycin, *4EBP1* 4E-binding protein-1, *eIF4G* eukaryotic initiation factor (eIF) 4G, *S6* S6 kinase-1. *Source*: Wilson et al. (2009)

concentration of glucose, amino acids, and insulin did not peak simultaneously, the maximal stimulation of protein synthesis after meal may occur during the time when circulating concentrations of these metabolites and hormones raised and are maintained at a moderate level, normally 30–120 min in the postprandial phase, rather than that at their own individual peak point.

Glucose stimulation of protein synthesis in fast-twitch glycolytic is associated with increased phosphorylation of protein kinase B (PKB) and enhanced formation of the active eIF4E.eIF4G complex. This enables mRNA to bind to the 43S pre-initiation complex, thereby enhancing translation initiation (Wang and Proud 2006). However, eIF4E.4E-BP1 and eIF4E.eIF4G complexes gradually return to the baseline by 4 h in the postprandial phase, thus slowly than the decline in protein synthesis. This suggests that assembly of eIF4E.eIF4G is not the rate-limiting step in the feeding-induced stimulation of skeletal muscle protein synthesis (Wilson et al. 2009).

The signaling pathway by which amino acids stimulate protein synthesis is less well understood. Intracellular amino acids may directly or indirectly affect signaling components that stimulate protein synthesis. The amino acid-induced signaling component converts Rheb-guanosine diphosphate (GDP) to Rheb-guanosine triphosphate (GTP) and the association between Rheb and mTOR to promote mTOR activation (Saucedo et al. 2003; Long et al. 2005; Kimball 2007). Some amino acids (e.g., arginine) have been shown to be of particular importance in mediating the increase in translation initiation and protein synthesis (Yao et al. 2008). Indeed, either infusion of leucine or arginine or increased dietary leucine or arginine intake acutely increases protein synthesis in skeletal muscle of neonatal pigs by an mTORC1-mediated process (Suryawan et al. 2008; Yao et al. 2008; Yao et al. 2011; Li et al. 2011). The circulating leucine and arginine levels increased by 30 min after the initiation of eating and are maintained at a relative higher level until 240 min

postprandial, mirroring the increase in rates of protein synthesis (Wilson et al. 2009; Yin et al. 2010). However, little is known about the mechanisms by which amino acids directly or indirectly modulate the activation of signaling components upstream of mTOR.

Insulin initiates the protein synthesis-signaling pathway by binding to insulin receptor (InR), which therefore activates the InR and insulin receptor substrate-1 (IRS-1). This activates the phosphoinositide 3-kinase (PI 3-kinase) (Di Guglielmo et al. 1998). Then activated PI 3-kinase stimulates the activation of phosphoinositide-dependent kinase 1 (PDK-1) and PKB. The PKB then phosphorylates and inactivates the tuberous sclerosis complex 1 and 2 (TSC2), thereby inactivating the function of the TSC1/2 complex, and inducing the activation of mTOR signal pathway (Di Guglielmo et al. 1998). Insulin signaling can be attenuated by the action of a number of phosphatases including protein tyrosine phosphatase-1B (PTP-1B) which dephosphorylates the InR and IRS-1, phosphatase and tensin homologue which inactivates PI 3-kinase, and PP2A which attenuates the action of PKB and S6K1 (Goberdhan and Wilson 2003). In pigs, feeding rapidly increases the activation of the InR, IRS-1, PI 3-kinase, PDK-1, and PKB in skeletal muscle. Activation of PKB is rapid and peaked at submaximal insulin concentrations, returning to baseline by 90 min, prior to the reduction in mTORC1 activation (Tremblay et al. 2005). The discrepancy between PKB and mTOR activation reflects the ability of both amino acids and insulin (Wang et al. 2006) to stimulate mTORC1 rather than PKB (Suryawan et al. 2007). In addition, physiological changes in the levels of amino acids have no effect on the activation of these early steps in the insulin-signaling pathway.

8.3 Protein Degradation in Pigs

Protein degradation is a part of a major mechanism by which cells regulate the concentration of particular proteins and eliminate mis-folded proteins. Some peptides derived from protein degradation could be reused for new protein syntheses while others could be degraded into free amino acids. Generally, a large percentage of free amino acids released from protein by degradation can be further hydrolyzed into small molecules, such as nitric oxidize (NO), ammonia (NH_3), carbon dioxides (CO_2), and water (H_2O).

The protein degradation plays important roles in animal physiological process, especially in the cellular signal transduction as well as in maintaining the integrity of the proper folded state of protein.

8.3.1 Main Signaling Pathway in Protein Degradation

There are two pathways for intracellular protein degradation, the proteasome and lysosome pathways. Intracellular proteins are mainly degraded by the

ubiquitin–proteasome pathway (Lecker et al. 2006). The organelles contain several acid-optimal proteases, including cathepsins B, D, and H and many other acid hydrolases. Some cytosolic proteins are degraded in lysosomes after being engulfed in autophagic vacuoles that fuse with lysosomes (Baehrecke 2005). In most cells, this process is accelerated by the lack of insulin or essential amino acids and in liver by glucagon (Gronostajski et al. 1984). Another cytosolic proteolytic system in mammalian cells is the Ca^{2+}-activated (ATP-independent) proteolytic process, which involves the cysteine proteases termed calpains. These proteases seem to be activated when cells are injured and cytosolic Ca^{+} rises; therefore they may play an important role in tissue injury, necrosis, and autolysis (Goll et al. 2003). In addition, the caspases are also an important family of cytosolic proteases that cleave proteins after aspartic acid residues. These enzymes, which are cysteine proteases, are critical in destruction of cell constituents during apoptosis (Salvesen and Dixit 1997).

The ubiquitin–proteasome pathway consists of concerted actions of enzymes that link chains of the polypeptide cofactor, ubiquitin, onto proteins to mark them for degradation (Glickman and Ciechanover 2002). This tagging process leads to their recognition by the 26S proteasome, a very large multicatalytic protease complex that degrades ubiquitinated proteins to small peptides (Baumeister et al. 1998). Three enzymatic components are required to link chains of ubiquitin onto proteins that are destined for degradation: E1 (ubiquitin-activating enzyme), E2s (ubiquitin-carrier or conjugating proteins) which prepare ubiquitin for conjugation, and E3 (ubiquitin-protein ligase). The E3 is a key enzyme in the process because it recognizes the protein substrate and catalyzes the transfer of activated ubiquitin to it. Till now, more than thousands of proteins have shown to be degraded by this pathway.

8.3.2 Degradation of Amino Acids in Pigs

All amino acids contain at least one nitrogen atom, which forms their α-amino group. Some amino acids also contain additional nitrogen atoms in their side chains. Theoretically, nitrogen is useless in energy metabolism and needs to be eliminated. Recent studies in young pigs indicated that both nutritional essential amino acids and non-nutritional essential amino acids derived from dietary protein sources are degraded extensively by the small intestine in first pass, with less than approximately 20 % of the extracted amino acids being utilized for intestinal mucosal protein synthesis (Stoll and Burrin 2006). Among dietary amino acids, glutamate, glutamine, aspartate, and proline are degraded faster than others in the small intestine (Yao et al. 2012). There are two key processes in the process of amino acid degradation: (1) transamination removes the α-amino group from one amino acid and transfers it to α-ketoglutarate, which leads to the accumulation of glutamate Yao et al. 2012, and (2) release of nitrogen from glutamate and its conversion to urea, which is accomplished by the urea cycle in the liver. Removal of nitrogen is typically an early step in amino acid degradation and leaves behind the carbon skeleton. Particularly, glutamine is the

main energy source for the intestinal epithelium, and the total rate of utilization of glutamine by the gut may be greater than that for others (Wu 2010). Intestinal degradation of amino acids has profound impacts on nutrition and health in pigs. Firstly, degradation of glutamine, glutamate, as well as aspartate provides the necessary ATP to maintain gut integrity and function (Watford 2008). Secondly, the extensive catabolism of amino acids by the small intestine significantly decreases the potentially neurotoxic effects of the excitatory neurotransmitter glutamate. Thirdly, the endogenous synthesis of nonessential amino acids (for example, citrulline, arginine, proline, and alanine) mainly relies on the transformations of amino acids in the intestine and thus modulating the availability of dietary amino acids to extra-intestinal tissues (Wu 1998; Bergen and Wu 2009).

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Chapter 9
Factors That Affect Amino Acid Metabolism in Pigs

Qinghua He, Yulong Yin, Yongqing Hou, Guixin Qin, Hui Sun, Jianhong Liu, Bo Liu, and Zongkun Zheng

Contents

Q. He
Department of Food Science and Engineering, College of Chemistry and Chemical Engineering, Shenzhen University, Shenzhen, Guangdong 518060, People's Republic of China

Y. Yin (✉)
Chinese Academy of Sciences, Institute of Subtropical Agriculture, Research Center for Healthy Breeding Livestock & Poultry, Hunan Engineering & Research Center of Animal & Poultry Science, Key Laboratory of Agro-ecological Processes in Subtropical Region, Scientific Observing and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Changsha, Hunan 410125, People's Republic of China
e-mail: yinyulong@isa.ac.cn

Y. Hou
Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, Wuhan, China

G. Qin • H. Sun
Jilin Agricultural University, No. 2888 Xincheng Street, Changchun, Jilin Province 130118, People's Republic of China

J. Liu • B. Liu • Z. Zheng
Department of Food Science and Engineering, College of Chemistry and Chemical Engineering, Shenzhen University, Shenzhen, Guandong 518060, People's Republic of China

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_9,

9.1 Introduction

Dietary protein is digested and decomposed into free amino acids and peptides in the gastrointestinal lumen, because protein cannot be absorbed as such and needs to be converted into amino acids and peptides (Krehbiel and Matthews 2003). These amino acids and peptides will flow into intestinal venous blood from intestinal lumen, and will be partly used for protein synthesis and deposition through hepatic metabolism. It is a very important issue in pig nutrition research to understand the digestion and metabolism of dietary proteins and amino acids as well as the regulation involved in such process(Yin and Tan 2010). This issue is also important for the accurate calculation and supply in diet formulation, which will be helpful to decrease the nitrogen excretion in pig production (Zhang et al. 2012). In addition, the researches about this issue might be helpful to resolve the other relative problems, including antibiotics and unconventional feedstuff in the feed industry. However, this is a complicated issue, which needs to resolve two problems. One is to know what factors affect the amino acid metabolisms, which need the deep and intensive understanding about the amino acid metabolic processes and its regulations in pigs. Another one is to know how to regulate the amino acid metabolism by nutritional methods according to the above impacting factors. The ultimate goal is to exert economic and environmental values of dietary amino acids in pig production. Since the impacting factors and regulating means of amino acid metabolism are not only various and complicated but also associated with each other, we will elaborate on the progress of these two aspects in this chapter.

9.2 Factors That Affect Amino Acid Metabolisms in Pigs

The extensive catabolism of dietary amino acids by luminal microbes or by the intestine may result in an alteration in nutritional efficiency of amino acids. However, knowledge about digestion, absorption, and metabolism of amino acids and epithelial cell transport of amino acids in the gastrointestinal tract remains incomplete.

9.2.1 Effects of Gut Microbiota on Intestinal Amino Acid Metabolism and Nitrogen Emissions in Pigs

Nutritional value of food nutrients and other functional elements are related to composition and content, but also to the digestion, absorption, and metabolism of these elements in the gastrointestinal tract. Intestinal tract is well known to be the

key place for digestion, absorption, and metabolism of dietary amino acids. Gut microbiota is a complex assortment of microorganisms inhabiting the mammalian gastrointestinal tract (Wang et al. 2011). Because the gut microbiota participates in the metabolic activity of amino acids together with the intestinal mucosa, it represents an important environmental factor that affects the nitrogen harvested from the diet and also nitrogen deposition in host (Kong et al. 2009).

Studies have shown that the gut microbiota plays an important role in the regulation of energy and lipid metabolism in host (Velagapudi et al. 2010) and increases efficiency of energy harvest from diet (Turnbaugh et al. 2006). Recent studies also indicated that human intestinal microbiota from children was sharpened by a modern western diet and a rural diet (De Filippo et al. 2010). It has been hypothesized that the gut microbiota has coevolved with the polysaccharide-rich diet of individuals from rural African village, allowing hosts to maximize energy intake from fibers. Most certainly, the gut microbiota has a tremendous influence on catabolism and de novo synthesis of amino acids and thus can modulate digestion, absorption, and metabolism of amino acids in diet (Libao-Mercado et al. 2009; Fang et al. 2010).

Since gut microbiota can hydrolyze urea and deaminate amino acids in the intestine, these processes usually result in metabolism and loss of amino acids (especially essential amino acids (EAA)), which otherwise should have been used for the synthesis and deposition of body protein. Researches in animal nutrition have indicated that EAA including branched-chain amino acids (leucine, isoleucine, and valine), histidine, lysine, methionine, phenylalanine, threonine, and tryptophan can be degraded by intestinal mucosa in pigs (Dai et al. 2005). However, there is still a lack of direct evidences to validate the effects of porcine small intestinal microbiota on the catabolism of EAA. The key point is to differentiate intestinal cells and gut microbes for their respective capacity to oxidize EAA. Based on this consideration, experiments were carried out to investigate this issue (Yin et al. 2004). The oxidation of amino acids in piglet intestinal mucosal cell was investigated in vitro under the conditions of inhibition or without inhibition. The results obtained indicate that only branched-chain amino acids were exclusively degraded in mucosal cell. In contrast, other EAA, including histidine, lysine, methionine, phenylalanine, threonine, and tryptophan, were not oxidized exclusively in intestinal cells. The oxidation of these amino acids may thus be carried out partly by gut microbes (Chen et al. 2007, 2009).

The balance of intestinal microbiota also has important impacts on the amino acid metabolism in the intestines. The activity of gut microbes affects the intestinal structure and functions, thus affecting the digestion and absorption of amino acids. In addition, it has to be taken into account that when the intestinal mucosa is facing the harmful effect of some bacteria, intestinal mucosa may need more energy resources.

Carbon- and nitrogen-containing substances can be fermented by intestinal microbes and used for body protein synthesis. Some studies have showed that microbial nitrogen is the main component of endogenous nitrogen in ileal digesta

and feces of pig. Indeed, the endogenous nitrogen is about 47–74 % of total ileal nitrogen emission in growing pigs. Microbial nitrogen is about 30–50 % of the amount of ileal endogenous nitrogen excretion. The endogenous nitrogen is about 75–90 % of fecal nitrogen emissions. Microbial nitrogen is about 70–90 % of the amount of fecal endogenous nitrogen excretion (Yin and Tan 2010). Thus, the increase in the amount of endogenous nitrogen is related to the metabolic activities of microbes in large intestines (Yin et al. 2000b). Thus, the main reason of the nonoptimal conversion of dietary protein to body protein and nitrogen pollution in the pig industry is the microbial nitrogen.

9.2.2 Effects of Intestinal Mucosal Metabolism on Utilization of Amino Acids in Pigs

The researches in recent 20 years have shown that the amino acids are involved in the metabolism of intestinal mucosa. For example, some amino acids, including threonine and valine, are likely representing energy sources for the intestinal mucosa (Dudley et al. 1998; Stoll et al. 1998). Threonine is important for mucin synthesis and maintenance of the gut barrier integrity and it has been reported that as much as 60 % of dietary threonine is retained by the intestine (Wang et al. 2009, 2010; Yin et al. 2010a, 2010b). Recently, the metabolic signatures in the jejunal mucosal tissues obtained from piglets on age of 21 days have been characterized in situ directly by HR-MAS NMR spectroscopy (He et al. 2011a). Marked changes were found in jejunal metabolites, including decreased levels of valine, alanine, glutamine, glutamate, trimethylamine-N-oxide, lactate, creatine, glucose, galactose, phenylalanine, tyrosine, and taurine in intrauterine growth restriction (IUGR) piglets compared with normal-weight piglets ($P < 0.05$). These findings suggest that IUGR piglets can be affected in energy supply, utilization of amino acids, and protein metabolism.

Amino acids also participate in the synthesis of secreted protein in intestinal mucosa and can be transferred into other amino acids by deamination and transamination (Hoerr et al. 1993).

Because intestinal tissues are able to utilize amino acids for their own needs, amino acid metabolism in intestinal mucosa will have obvious impacts on the net portal absorption, thus affecting the utilization of dietary amino acids in the body.

9.2.2.1 Effects of Gut Mucosal Metabolism on the Net Absorption and Composition Patterns of Amino Acids in Portal Vein

The amount of amino acids that disappeared in the digestive tract is not equal to the net amount of absorption in portal vein. The latter is significantly lower than the former (MacRae et al. 1997a, b). Studies found that not all absorbed amino acids

entered into the portal vein. Indeed, the net apparent absorption of amino acids in portal vein only account for a part of total intake amino acids. The proportion of threonine is about 40–50 %. The proportion of lysine is about 50–69 %. The proportion of glutamic acid and aspartate is close to zero. Glutamine shows a negative balance. But the proportion of alanine is more than 100 %, which is almost twice higher than that of threonine or lysine (van der Meulen et al. 1997; Deutz et al. 1998).

Yin et al. (2003) found similar results in growing pig study. In this study, pigs were fed two kinds of diets. One contained starch and casein (SC). Another contained low-level starch and sub-flour of wheat (LSW). The plasma samples and intestinal digesta were collected during 8 h after feeding. Studies showed that only 50–60 % of dietary digested and absorbed amino appeared in the portal vein, which can be used for protein synthesis in the peripheral tissues. The remaining amino acids (40–50 %) were used by the portal vein-drained viscera (PDV, gastrointestinal tract, pancreas, spleen, and omental fat).

Thus, the metabolism of intestinal mucosa can affect the net portal absorption and composition of amino acids in the portal vein. The intestinal mucosa and presumably the microbiota can oxidize nonessential amino acids and branched-chain amino acids. The nonessential amino acids can be both degraded and also synthesized in the intestine.

9.2.2.2 Effects of Net Absorption and Composition of Amino Acids in the Portal Vein on Nitrogen Deposition

Protein deposition, which is the main indicator for pig growth, depends on the net portal absorption of the EAA. The regression equations between average daily gain (ADG) and the net portal absorption of amino acids were developed in growing pigs (Huang et al. 2006). In these equations, the net portal absorption of nutrients is independent variables. ADG is the dependent variable. It is showed that there are relatively high correlations ($P = 0.0295$) between the net portal absorption and ADG. The related regression equations are as follows:

$$\mathrm{ADG} = 51.9321\ \mathrm{EAA} + 446.0028\ (R^2 = 0.9419, P = 0.0295)$$
$$\mathrm{ADG} = 305.5017\ \mathrm{Leu} + 491.7915\ (R^2 = 0.9514, P = 0.0246)$$
$$\mathrm{ADG} = 502.1378\ \mathrm{Ile} + 471.1824\ (R^2 = 0.8538, P = 0.0760)$$
$$\mathrm{ADG} = 483.8102\ \mathrm{Val} + 441.0182\ (R^2 = 0.8361, P = 0.0856)$$
$$\mathrm{ADG} = 305.5941\ \mathrm{His} + 568.1355\ (R^2 = 0.8229, P = 0.0929)$$

From these equations, it was deduced that the correlations are relatively high ($P < 0.05$ or 0.1) between ADG and the net portal absorption of total EAA, leucine (Leu), isoleucine (Ile), valine (Val), and histidine (His), while the correlations are low ($P < 0.05$ or 0.1) between ADG and the net portal absorption of glucose and

nonessential amino acids. In addition, several independent variables, such as some essential amino acids, including Met, His, and the hexose glucose can be introduced into equations as independent variables when stepwise regressions were performed in growing pigs. In these regression equations, the correlations can be greatly improved ($P < 0.01$). Relationships are as follows:

$$\text{ADG} = 263.7903\ \text{Leu} + 197.6090\ \text{Met} + 482.7015\ (R^2 = 0.9999, P = 0.0097)$$
$$\text{ADG} = 35.9182\ \text{EAA} + 129.3837\ \text{His} + 471.7131\ (R^2 = 0.9998, P = 0.0121)$$
$$\text{ADG} = 1345.0675\ \text{Ile} - 15.5291\ \text{Glucose} + 514.3573\ (R^2 = 0.9999, P = 0.0078)$$

9.2.3 *Effects of Anti-nutritional Factors on Amino Acid Metabolism*

As mentioned above, a major source of nitrogen pollution in the pig industry is related to fermentation of microorganisms in the digestive tract. The anti-nutritional factors in feed are the major factors that cause the intestinal microbial fermentations.

9.2.3.1 Non-starch Polysaccharides

Generally, non-starch polysaccharides (NSPs) in feed are considered as anti-nutritional factors for pigs. These NSPs not only have a great negative impact on digestion and absorption of nutrients in pigs but also are associated with the intestinal microbial fermentations, which is the energy source of microbial fermentation in gut. Additionally, NSP content in feed can affect the digestibility of dietary protein and amino acid. It was reported that the apparent ileal digestibility of crude protein and most of amino acids decreased by 3–5 % in growing pigs when β-glucan in diet increased from 29.0 to 31.8 g/kg (Yin et al. 2000b, c). It was also reported that the apparent ileal digestibility of crude protein and amino acid is reduced by 12 % and 6 %, respectively, when total NSP in diet increased from 83 to 193 g/kg (Yin et al. 2000a).

NSP not only can affect secretion of endogenous nitrogen but also affect deposition and biological value of feed nitrogen. It was reported that cereal fiber content can significantly increase the excretion of endogenous nitrogen and amino acids in pigs (Low and Rainbird 1984; Yin et al. 2000a). Pectin can reduce the amount of nitrogen deposition and nitrogen microbiological value (Xu et al. 2005). It has been suggested that pectin and other gel of polysaccharides (such as methyl cellulose) can affect the secretion of endogenous protein and amino acids, and increase endogenous excretion of nitrogen, which increased the amount of nitrogen coming from the small intestine to the large intestine, and thus led to the decrease of body nitrogen retention and nitrogen biological value.

The main factor responsible for the intestinal secretion of endogenous nitrogen in roughage feed is not the insoluble part of non-starch polysaccharides (INSP) but the soluble part of non-starch polysaccharides (SNSP) (Yin et al. 2000a, b, c, 2004). Although SNSP do not affect the digestive process of feed nitrogen and amino acids (that is the true digestibility), there is a positive linear relationship between SNSP content and intestinal microbial fermentation or endogenous nitrogen (amino acid) excretion in pigs that thereby reduces the amino acid digestibility. However, there is no positive linear relationship between INSP content and intestinal microbial fermentation. INSP does not affect the endogenous excretion of nitrogen and total nitrogen. When soluble NSPs in growing pig diets are more than 0.8 %, the protein digestibility and growth performance are significantly affected. There is a linear relationship between diet SNSP (X) and ileal excretion of nitrogen (y) as follows: $y = 1.7832X - 8.2074$ ($R^2 = 0.99$). Also, there is a linear relationship between the INSP and ileal excretion of nitrogen as follows: $y = 0.026X + 5.0431$ ($R^2 = 0.43$), which has however no significant correlation. Interestingly, the fecal nitrogen excretion (y) has the similar linear relationship with two types of NSPs (X) as follows: $y = 0.2563X + 0.5773$ ($R^2 = 0.89$, SNSP) and $y = 0.3165X + 1.546$ ($R^2 = 0.35$, INSP). This indicates that the two types of NSPs have similar influence on fecal nitrogen excretion (Yin et al. 2004).

Lastly, the dietary NSPs accelerated the basal metabolism of PDV tissues, thus affecting the utilization of amino acids in feedstuffs.

9.2.3.2 Other Anti-nutritional Factors

Phytic acid in plant feedstuff not only form phytate combined to phosphorus but also form insoluble complexes combined with protein and amino acids, thus reducing the utilization of these nutrients. Studies have showed that some other anti-nutritional factors, including insulin-inhibitory factor, tannin, and so on, can increase the endogenous ileal amino acid and nitrogen content (Huang et al. 2001). Tannin can reduce the true digestibility of amino acids by combination with endogenous nitrogen in digestive tract (Jansman et al. 1995).

9.2.4 Effects of Feed Starch on Amino Acid Metabolism

The synchronized supply of energy and amino acids for tissue protein synthesis is conducive to the optimal utilization of amino acids and thus maximum protein synthesis. Because the different sources of dietary starch release glucose at different rates during digestion in the pig body, the supply of glucose and amino acids have different synchronization levels. The different sources of feed starch can affect in the different degrees the protein digestion and utilization, the uptake capacity and proportion of portal vein amino acids, microbial protein synthesis, and levels of nitrogen excretion in feces and urine.

9.2.4.1 Effects of Starch Sources, Composition, and Structure on Feed Digestion and Absorption of Amino Acids

The type and source of starch have significant impact on the digestibility of protein and amino acid absorption, particularly on ileal digestibility (Li et al. 2007; Laplace et al. 2001). It was reported that the ileal digestibility of the protein and some amino acids (cysteine, serine, threonine, phenylalanine, and tyrosine) were significantly reduced when pig ingested the diet including slow digestion of pea starch (Everts et al. 1996). It was also reported that diets including high level of rapidly digestible amylopectin (glutinous rice) and high level of slowly digestible amylose (resistant starch) not only reduce the apparent and true digestibility of dietary protein but also can reduce the apparent and true ileal digestibility of aspartic acid, glutamic acid, serine, histidine, threonine, arginine, tyrosine, methionine, phenylalanine, leucine, isoleucine, and lysine. Furthermore, when the ratio of amylose to amylopectin was equal to 0.23 in the corn-based diet, the ileal amino acid digestibility was significantly better (Li et al. 2008).

It is believed that the different structures of starch can affect differently the digestion of amino acids. Slowly digestible starch can increase the endogenous nitrogen and amino acid secretion and the microbial nitrogen and amino acid content in digesta because slowly digestible starch has relatively slow digestion property. That is the reason why slowly digestible starch reduces amino acid digestibility. Rapidly digestible starch can accelerate glucose absorption, which competitively inhibits the absorption of amino acids, thereby reducing the digestion and absorption of dietary amino acids (Yin et al. 2001a, b, 2011).

9.2.4.2 Effects of the Starch Sources, Composition, and Structures on the Microbial Nitrogen Content and the Flow of Nitrogen in Fecal and Urine

Starch sources, composition, and structures can affect microbial biomass nitrogen and the flow of nitrogen in feces and urine. It was reported that urinary nitrogen excretion has a downward trend with the increased content of resistant starch diet and increased excretion of fecal nitrogen (Li et al. 2008). There is a quantitative relationship between fecal nitrogen excretion (y) and resistant starch content of the diet (X) as follows: $y = 0.785X + 9.1739$ ($R^2 = 0.997$). It was reported that pigs fed resistant starch diet have higher fecal nitrogen content. It is supposed that there are two reasons for such a result: (1) resistant starch may reduce the intestinal absorption of nitrogen and (2) the microbial fermentation in the large intestine increased microbial protein synthesis (Heijnen and Beynen 1997).

9.2.4.3 Effects of Starch Sources, Composition, and Structures on the Net Absorption and Composition Model of the Portal Vein Amino Acids

Studies have shown that starch sources, composition, and structures can affect the net absorption and amino acid composition in the portal vein. The content of essential amino acids (valine, isoleucine, phenylalanine, tryptophan, arginine, serine, cystine, tyrosine, lysine, histidine) in portal vein of pigs fed pea starch diet was higher than that in pigs fed the corn starch diet (van der Meulen et al. 1997).

The diet, including high percentage of rapidly digestible amylopectin (glutinous rice) and slowly digestible amylose (resistant starch), not only reduced the total absorption of amino acids in the portal venous blood after feed intake but also changed the composition pattern of absorbed portal amino acids, as well as reduced the proportion of EAA in the total pool of absorbed amino acids. However, the total absorption of amino acids in the portal venous blood and the composition pattern of amino acids absorbed in the portal blood were significantly improved when the ratio of amylose to amylopectin was 0.23 in the cornstarch diet (Deng et al. 2010). Since the different starch in feed have different digestibility, the different starch composition will not only affect digestion and absorption of dietary amino acids but also affect amino acid metabolism in intestinal mucosa, thus affecting the net portal absorption and composition pattern of amino acids (Huang et al. 2006).

9.2.4.4 Effects of Starch Sources, Composition, and Structure on Amino Acid Metabolism in Visceral Tissues

Starch source, composition, and structure can affect the amino acid metabolism in visceral tissues. It has been reported that pigs fed corn-based diet (the ratio of amylose to amylopectin is 0.23) have the highest protein synthesis rate (FSR) in PDV tissues and liver. The order of FSR in other diets is as follows: brown rice-based diet > starch-based diet (including high percentage of slowly digestible amylose) > glutinous rice-based diet (including high percentage of rapidly digestible amylopectin). The FSR (%/day) of spleen, pancreas, duodenum, jejunum, ileum, and colon in pigs fed glutinous rice-based diet were significantly lower than those in the corn-based group ($P < 0.05$) and brown rice-based group ($P < 0.05$). The FSR of spleen, pancreas, duodenum, jejunum, ileum, and colon in pigs fed glutinous rice-based diet were lower than corn-based group by 84.76 %, 30.34 %, 46.20 %, 32.19 %, 27.16 %, and 36.02 %, respectively. The FSR were lower than brown rice-based group by 81.97 %, 21.72 %, 46.15 %, 30.48 %, 25.14 %, and 32.78 %, respectively. The FSR also were lower than resistant starch group by 60.49 %, 17.79 %, 34.10 %, 23.23 %, 13 %, and 28.9 %, respectively. Additionally, the liver FSR in pigs fed glutinous rice-based diet were lower than corn, brown rice, and resistant starch groups by 14.99 %, 14.38 %, and 9.27 %, respectively (Deng et al. 2010).

9.3 Regulatory Means of Amino Acid Metabolism in Pigs

9.3.1 Diet Formulation

The measurement of true ileal digestible amino acids can eliminate the impacts of measurement conditions. The values of true ileal digestible amino acids can be additive. The data of true ileal digestible amino acids can make diet formulations more accurate, which can reduce the safety margin and dietary crude protein level without reducing animal performance. Some synthetic amino acids can be supplemented in the diets in order to meet the requirements of some limiting amino acids, thereby improving the overall efficiency of amino acids.

The feasibility of reducing nitrogen excretion by amino acid-balanced low-protein diet was investigated in growing pigs, which was formulated based on the true digestible amino acids and synthetic amino acids (Deng et al. 2007a, b, 2009). Experiment designed 5 crude protein levels, namely, 18.2, 16.5, 15.5, 14.5, and 13.6 %. The results showed that fecal nitrogen, urinary nitrogen, and total nitrogen excretion (g/day) decreased with the reduced dietary crude protein levels. Compared with those of crude protein group of 18.2 % (control group), fecal nitrogen were reduced by 7.45, 13.04, 13.82, and 17.39 % in crude protein groups of 16.5 %, 15.5 %, 14.5 %, and 13.6 %, respectively. Urine nitrogen decreased by 19.25, 26.86, 37.43, and 44.64 %. Total nitrogen decreased by 15.13 %, 22.00 %, 29.18 %, and 35.14 %, respectively. In addition, the ratio of urinary nitrogen to fecal nitrogen levels decreased with reduced dietary crude protein ($P < 0.0001$). The nitrogen retention also decreased with decreased dietary crude protein ($P < 0.001$).

However, the percentage of nitrogen deposition to nitrogen intake increased with the reduced dietary crude protein levels ($P < 0.01$). Dietary crude protein levels did not affect nitrogen digestibility ($P > 0.05$). Nitrogen digestibility in each group fluctuated between 83.86 and 82.19 %. Moreover, the apparent digestibility of nitrogen in groups supplemented with synthetic amino acids was lower than that of control group. But the apparent biological value of nitrogen increased with the decrease of dietary crude protein ($P < 0.001$). From the results of growth performance in different crude protein levels, it was indicated that the production performance of growing pigs was not affected by the decrease of dietary crude protein levels from 18.2 to 16.5 % in the whole trial period. On the contrary, the ADG, average daily feed intake, and the feed conversion rate were enhanced to a certain extent, while the production performance decreased with further reduction of protein levels. Carcass measurement indicated that the back fat thickness of pigs increased with the decrease of dietary protein levels ($P < 0.03$). In summary, the production performance, carcass quality, and nitrogen utilization as well as economic and ecological efficiency can be achieved when dietary crude protein levels reduced from 18.2 to 16.5 % (thus at less than 2 %).

9.3.2 *Regulation of Functional Carbohydrate in Feed on Digestion and Metabolism of Amino Acids*

During several decades, it has been thought that the role of carbohydrates is mainly related to their structure for energy storage material. But in recent years, it has been found that glycosylation in vivo is involved in the synthesis of numerous proteins as well as numerous life processes. In addition, many natural polysaccharides and oligosaccharides can affect various physiological functions and activities of animals and humans. Particularly oligosaccharides can regulate animal growth and metabolism, enhance immune functions, and maintain intestinal health (Huang et al. 2005, 2007; Deng et al. 2007c, d; Kong et al. 2007; Yin et al. 2008). These functions are more and more recognized by scientists. Animal growth, immunity, and gut microflora changes are closely associated with digestion and metabolism of amino acids. Therefore, these functional carbohydrates in feed have an important regulatory role in digestion and metabolism of amino acids.

9.3.2.1 Oligosaccharides Can Improve Digestion and Utilization of Amino Acids and Substitute Antibiotics in Feed

Studies have shown that oligosaccharides can improve the utilization of amino acids in pig diets. For instance, supplementation with chitosan or GMOS in diet decreased plasma urea nitrogen levels, while increased total plasma protein concentration (Tang et al. 2005). Supplementation with 0.1 % GMOS in diet increased the apparent ileal digestibility of most amino acids when compared to the control group (Hou et al. 2005).

Moreover, oligosaccharides can protect the intestinal functions and reduce the gastrointestinal mucosa diastasis, thereby reducing the endogenous ileal amino acid secretion and increasing amino acid digestibility. Supplementation of GMOS and chitosan in the casein-based diets significantly reduced the loss of endogenous amino acids (Tang et al. 2005; Deng et al. 2007c, d).

Long-term supplementation with antibiotics in feed disrupts the diversity in swine intestinal microflora and also the normal immune functions in body. In contrast, oligosaccharides can maintain the normal intestinal microbial community. Compared with antibiotics, oligosaccharides enhanced the cell and humoral immunity in animal body, increased the secretion and expression of growth-related factors, and thus promoted animal growth. It has been reported that oligosaccharides increased the levels of IgA, IgM, IgG, IL-6, IL-1β, and IL-2 in serum

and the gene expression of IL-1β in blood mononuclear cells, intestinal mucosa cells, and intestinal lymph node cells compared with antibiotics group. The serum IGF-I and GH levels and muscle IGF-I gene expression in oligosaccharides group were significantly higher than those in the antibiotic group (Hou et al. 2005; Tang et al. 2005).

Oligosaccharide can be viewed as a new nutritional means for the regulation of amino acid metabolism and livestock production and quality. This may help to reduce nitrogen pollution in pig production and reduce the dosage of antibiotics in feed.

9.3.2.2 NSP and Resistant Starch

Though NSP cannot be digested by pigs, NSP can be fermented and used by pig intestinal microbes. Some NSP (such as inulin) can selectively promote the growth of beneficial bacteria. It is considered that inulin is a bifidus factor which has prebiotic effects. Inulin has the advantages to be produced at a lower cost than oligosaccharides. In addition, NSP can play probiotic-like role by digestion into oligosaccharides in the upper digestive tract.

Like oligosaccharides, resistant starch can regulate the intestinal microbial flora in a way that it may promote intestinal health. Microbial fermentation can produce short-chain fatty acids to reduce the intestinal pH, thus suppressing the activities of pH-sensitive pathogens.

9.3.3 Effects of Enzyme (NSP Enzymes and Phytase) on the Amino Acid Metabolism

Some enzymes are able to destroy anti-nutritional factors in feed and to reduce the viscosity of intestinal contents, therefore improving the feed nutrient utilization. Furthermore, several enzymes play an important regulatory role on the intestinal microflora (Garry et al. 2007). These enzymes have shown great potential in regulating feed amino acid metabolism, eliminating feed anti-nutritional factors, and reducing nitrogen excretion. In recent years, as a metabolic regulator of nutrients, enzymes have been widely applied in feed production.

9.3.3.1 Improvement of NSP Enzymes on Digestion and Utilization of Nitrogen (Amino Acids)

As mentioned above, the soluble NSP in carbohydrates is the main factor for increasing the secretion of intestinal endogenous nitrogen (amino acids), and thereby for reducing digestion and utilization of amino acids. Since there is a lack

of endogenous enzymes to degrade NSP in the digestive tract of pigs, it is necessary to add exogenous enzymes in order to eliminate or reduce anti-nutritional effects of NSP. Several reports have shown that supplementation with NSP enzymes can improve the digestion and utilization of nitrogen (amino acids) in many diets, especially in diets with high NSP content.

Supplementation of xylanase in wheat- or its by-product (wheat bran, sub-powder)-based diets can improve the digestibility of proteins and reduce the loss of endogenous nitrogen (Yin et al. 2000b). Supplementation with β-glucanase or other enzymes (β-glucanase, xylanase, and protease) in shelling barley (Falcon) significantly increased the apparent ileal digestibility of crude protein and amino acid absorption in growing pigs. The supplementation of xylanase also significantly increased the apparent ileal absorption of cystine, isoleucine, lysine, methionine, and tyrosine (Yin et al. 2000a). Supplementation of β-glucanase and xylanase in naked barley diets can improve digestion and utilization of amino acids as measured by an increased plasma urea nitrogen concentration (Yin et al. 2001a, b, c, d). Moreover, supplementation with arabinoxylanase in wheat bran or rice bran diets can reduce the negative nutritional effect of soluble NSP in the small intestine and thereby increase the digestibility of amino acids in proteins (NRC 1998).

Effect of enzymes depends on the types of diet. Different types of feed have inconsistent type and content of NSP. For example, the main NSP of barley is β-glucan although the main NSP of wheat is arabinoxylans. It has been reported that, in wheat bran diet including the highest content of NSP and xylan, xylanase shows the best improvement of the digestibility of nutrients and reduction of the excretion of endogenous nitrogen compared with other diets (Yin et al. 2000b). In five barley diets supplemented with β-glucanase, xylanase together with complex enzyme β-glucanase have the most obvious improvement on apparent ileal amino acid digestibility of several amino acids. In comparison of five barley diets, complex enzyme mixture had better improvement when dietary β-glucan content was higher (Yin et al. 2001a, b, c, d). The improving effect of enzymes in diet containing high content of soluble NSP (0.8 %) in corn and wheat bran diet was obviously better than that of low content of soluble NSP (0.5 %) in corn and rice bran diet (NRC 1998).

NSP-degrading enzyme can cause hydrolysis or dissolution of NSP in the cell wall, thereby reducing or eliminating gel packets, and thus improving the digestion of amino acids in the small intestine (Li et al. 1996). The increase of amino acid ileal digestibility induced by enzyme was mainly due to the decrease in nitrogen excretion, rather than to the increase of protein true digestibility. Indeed, it has been found that the endogenous nitrogen loss is reduced by 19 % in growing pigs fed wheat bran diets supplemented with xylanase, while the ileal true digestibility of crude protein is similar (approximately 92 %) with or without enzyme (Yin et al. 2000a, b). It has been reported that the total protein in pancreatic exocrine secretion was reduced by 17 % in pigs fed with barley-based diet supplemented with enzyme (Jansman et al. 1995). The above studies suggest that the main role of the supplementation with NSP enzymes in the pig diets is to promote the degradation of dietary water-soluble NSP in the intestine. This would allow to increase the

digestion of some nutrients in the small intestine instead of the large intestine, thereby reducing the intestinal fermentation of some substrates, including carbohydrates and amino acids. It would indirectly reduce the excretion of endogenous and microbial amino acids, thus increasing the utilization of amino acids.

9.3.3.2 Improvement of the Utilization of Nitrogen by Phytase

Supplementation of phytase in pig diets can break the union between phytic acid and amino acids, and thus improve utilization of protein and amino acids in feed. It has been reported that ileal apparent digestibility of dry matter, organic matter, and crude protein as well as fecal apparent digestibility of dry material, organic matter, and crude protein are significantly improved in growing pigs supplemented with 500 U/kg phytase. Ileal true digestibility of seven essential and semi-essential amino acids (histidine, arginine, methionine, lysine, isoleucine, leucine, and phenylalanine) and seven nonessential amino acids (aspartic acid, glycine, proline, alanine, threonine, tyrosine, and glutamic acid) were also improved. Nitrogen deposition, nitrogen deposition rate, and net deposition rate of nitrogen were enhanced by 2.8 g/day and 8.81 and 11.02 % compared with the control group without phytase (Fan et al. 2005).

The effect of phytase is dependent on the protein sources and on the dietary amino acids contained in proteins. Since the raw materials used in diet formulations are different, there are different possibilities for the formation of phytic acid and protein complexes with thus different effects of phytase on such substrates.

9.3.4 Regulation of Other Factors on Amino Acid Metabolism

In addition to oligosaccharides, polysaccharides, and enzymes, many other additives, such as probiotics, herbal additives, natural plant extracts, trace elements, amino acid chelators, and active peptides, can exert a regulatory role in amino acid metabolism. They act through different mechanisms that can be divided into the following categories: regulation of the gut microflora, maintenance of intestinal health, improvement of immune functions, regulation of the intestinal metabolism, increase of the activity of digestive enzymes, regulation of expression, and secretion of growth-related factors. These additives, that can directly or indirectly affect the digestion and utilization of amino acids, are considered as effective means to regulate amino acid metabolism.

Some functional amino acids, including arginine, glutamine, and proline, play versatile roles in nutritional metabolism, growth, and immunity. Recently, it has been reported that early weaning stress is associated with marked alterations in amino acid metabolism and some perturbations in population and/or activities of gut microorganisms. These perturbations were manifested in increased levels of organic acids, amino acids, and acetyl-glycoproteins as well as reduced levels of

choline metabolism and lipoproteins. Dietary supplementation with arginine could partially counteract the changes of metabolites induced by weaning stress including lipid and amino acid metabolism (He et al. 2011b).

9.4 Summary

In recent years, the nutritional regulation of amino acid metabolism has been extensively studied and has made great progress in areas such as the cloning of genes related to amino acid transporters, classification of the microbial flora in different intestinal segments, and effects of the different structure of the sugar source on metabolism and utilization of amino acids in the PDV tissues (Krehbiel and Matthews 2003; Wang et al. 2008). These have built the theoretical and technological foundations used for the study of the regulation of amino acid metabolism.

However, digestion of proteins, amino acid absorption, and metabolism of amino acids in tissues are very complex. There are many areas that need further studies, such as intestinal metabolism of amino acids, relationship between amino acids and glucose metabolism, regulation of the supply of dietary amino acid and carbohydrate on the balance, and pattern of nutrients and metabolites in the portal vein. Finally, the establishment of the quality of a given dietary protein quality should take into account as an additional parameter the pattern of the amino acids in portal vein. Better understanding of the regulatory mechanisms involved in amino acid metabolism will lead to more rational and thus better use of amino acids in proteins and supplements for animal production, thus allowing to obtain the optimal economic and social benefits.

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Chapter 10
Amino Acids and Hormone Secretion in Pigs

Bie Tan, Yulong Yin, Yizhen Wang, De Wu, Yingcai Lin, Xuemei Ding, Yuyun Mu, and Jirong Lv

Contents

10.1 Introduction

Amino acids have been implicated in the regulation of hormone synthesis and secretion (Kuhara et al. 1991; Newsholme et al. 2005; Zeng et al. 2012). Amino acids stimulate or inhibit the release of some pituitary, pancreatic, and gastrointestinal hormones by oral or intravenous administration. For example, tyrosine (or

B. Tan • Y. Yin (✉)
Research Center of Healthy Breeding of Livestock and Poultry and Key Laboratory of Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture The Chinese Academy of Sciences, Changsha, Hunan 410125, China
e-mail: yinyulong@isa.ac.cn

Y. Wang
College of Animal Science, Zhejiang University, The Key Laboratory of Molecular Animal Nutrition of Ministry of Education, Hangzhou, P.R. China

D. Wu • X. Ding
Institute of animal Nutrition, Sichuan Agricultural University, Sichuan, China

Y. Lin
Institute of Animal Science, Guangdong Academy of Agricultural Science, Guangdong, China

Y. Mu
Shanghai Xinnong Feed Co. Ltd, Songjiang, China

J. Lv
Chengdu Dadhank Biotech Corp, Chengdu, Sichuan, China

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_10,

phenylalanine) is the precursor for the synthesis of epinephrine, norepinephrine, and thyroid hormones. Arginine stimulates the secretion of insulin, growth hormone (GH), prolactin, glucagon, and placental lactogen (Flynn et al. 2002). Glutamine and leucine also increase insulin release from the pancreatic E-cells (Newsholme et al. 2005). Amino acids also induce the secretion of GI hormones such as glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), and ghrelin (Nakamura et al. 2011). The mechanism of hormones release induced by amino acids may involve membrane depolarization and certain receptors and transporters. It is becoming increasingly recognized that gastrointestinal chemosensing involves additional mechanisms. In this chapter, the regulations and mechanisms of amino acids on GH–IGF-I axis hormones release, pancreatic secretion, and gastrointestinal hormone secretion are discussed.

10.2 Amino Acids and GH/IGF-I Axis

GH and insulin-like growth factor-I (IGF-I) control growth in swine and the GH/IGF-I axis plays an essential and central role in the regulation of body growth and composition, as well as cellular proliferation and differentiation processes (Li et al. 2002; Lucy 2008). Specific amino acids appear to be important in the GH–IGF-I axis. But under normal growing conditions, the GH–IGF-I axis is probably not limited by amino acid availability. However, in conditions of low intake and some pathological states, these signals may become crucial.

The growth-inhibitory effect of amino acid deficiencies might be caused in part by impaired action of GH–IGF-I axis. Bajoria et al. (2002) demonstrated the link between the reduction in certain essential and nonessential amino acids and alterations in fetal circulating levels of IGFBP-I, in growth-restricted twins. Single depletion of arginine, proline, threonine, tryptophan, or Val caused a block of GH-stimulated IGF-I gene expression in cultured pig hepatocytes (Brameld et al. 1999). The GH, IGF-I, and IGFBPs in plasma are sensitive to changes of nutritional status. Many studies have indicated that growth retardation induced by undernutrition is usually followed by an increase of GH and a decrease of IGF-I in plasma of pigs (Vance et al. 1992; Guay and Trottier 2006). Katsumata et al. (2002, 2004) found that a low-lysine or a low-threonine diet significantly reduced plasma IGF-I and IGFBP3 concentrations. The reduction in plasma IGF-I caused by reduced dietary lysine or threonine might have been partly due to suppression of posttranscriptional events of IGF-I expression because low-lysine or low-threonine diet did not affect IGF-I mRNA expression in the liver. An approximately 0.30–0.40 proportional shortage in a single amino acid in a pig diet compared with its requirement does not seems to be severe enough to suppress IGF-I mRNA expression in the liver. Increased clearance rate of circulating IGF-I, indicated by lower IGFBP3 level, may be one reason for the lower plasma IGF-I caused by restriction of a single amino acid in a pig diet. In the study by Wiltafskya et al. (2010), the growth retardation resulting form excess dietary leucine was observed. This might be partially caused by

decreased activity of the GH–IGF-I axis as a result of leucine-induced valine deficiency.

Aspartate and arginine are most effective in stimulating GH secretion (Bequette 2003). It has been proposed that excitatory amino acids (EAA) play an important role in regulating secretion of pituitary hormones including GH (Estienne and Barb 2002). Intravenous administration of 100 and 150 mg/kg BW of aspartate or glutamate to gilts increased GH secretion, and aspartate was found to be a more potent secretagogue of GH secretion than was glutamate (Barb et al. 1996). Elevated plasma levels of arginine have been found to correlate with increased secretion of GH and IGF-I. Maximum GH level and the area under the GH curve (AUC) were increased in a dose-dependent manner in response to arginine (0.5–1 g/kg BW) treatment via the duodenum in pigs. However, GH response to the combined arginine and aspartate treatment was delayed compared to arginine alone and was not dose dependent (Cochard et al. 1998). Saleri et al. (2003) demonstrated that arginine also significantly stimulated GH secretion in pig pituitary cells. But in growing-finishing pigs, dietary 1 % arginine supplementation did not affect serum concentrations of growth hormone and insulin-like growth factor-I (Tan et al. 2009).

GH release has a complex regulation with two antagonistic hypothalamic hormones, growth hormone releasing hormone (GHRH) and somatostatin, as well as the liver-derived hormone IGF-I. It has been reported that the mechanism of regulation of GH secretion by amino acid administration is provided by promotion of secretion of a GHRH and by suppression of somatostatin secretion (Casanueva and Diequez 2004). Arginine stimulates GH secretion probably via a direct cholinergic effect, membrane depolarization, and subsequent calcium influx, and by inhibiting hypothalamic somatostatin release. NO production also appears to be involved in the control of GH release by arginine in pig pituitary cells (Saleri et al. 2003). GH secretion is under the negative feedback control of IGF-I, which was observed in GH response to arginine by Nass et al. (2002). This effect was present before the start of the arginine infusion, and this likely accounts for the lower peak GH response to arginine (Nass et al. 2002). The actions of EAAs on GH secretion are mediated through interaction with different postsynaptic receptors, and the *N*-methyl-D,L-aspartic acid (NMA) has attracted attention (Aguilar et al. 2005; Tena-Sempere et al. 2000). NMA treatment increased circulating GH concentrations in prepubertal gilts (Estienne et al. 1995) and boars (Estienne et al. 2000). Xi et al. (2002) reported that feeding

NMA at a dose of 50 mg/kg to finishing pig significantly also increased the IGF-I level in the serum, liver, and muscle and postulated that this increase is primarily caused by the stimulation of GH release in pituitary.

10.3 Amino Acids and Release of Glucagon and Insulin

In normal physiology, protein meals, with their attendant amino acid loads, stimulate glucagon and insulin secretion (Charlton et al. 1996). Secretion of insulin and glucagon by specific cells in the pancreas is regulated usually in the opposite direction; but a striking exception is that insulin and glucagon secretion are stimulated by amino

acids or a protein meal (Muller et al. 1971). The balance between insulin and glucagon regulates the metabolism of glucose, fat, and protein, thereby promoting a stable inner metabolic milieu; and this action is done via pathways involving cAMP signaling (McKnight et al. 2010). Glucagon activates adenylyl cyclase to generate cAMP, which stimulates protein kinase A (PKA) (Mersmann and Smith 2005). Insulin has opposing effects to glucagon, causing uptake of glucose and amino acid from the blood to various tissues and stimulation of glycogen, protein, and lipid synthesis.

Unlike glucose, which inhibits glucagon secretion and stimulates insulin release, amino acids stimulate release of both hormones. Specific amino acids are known to acutely and chronically regulate insulin secretion from pancreatic β-cells (Newsholme et al. 2005), and leucine, arginine, and lysine are considered as the most potent stimulators of insulin secretion. The accumulation of positively charged amino acids including L-arginine, L-homoarginine, and L-ornithine in islet cells represents an essential determinant of their secretory action (Blachier et al. 1989b). In the presence of D-glucose, L-lysine stimulates insulin secretion to the same extent as L-arginine or L-ornithine, but the hormonal release is not further enhanced by combinations of these cationic amino acids (Blachier et al. 1989a; Sener et al. 1989a). Arginine, alanine, and glutamine are potent stimulators of glucagon secretion from pancreatic α-cells (Kuhara et al. 1991; Dumonteil et al. 2000; Quesada et al. 2008). Östenson and Grebing (1985) showed that glutamine significantly enhanced glucagon release from 297 to 528 pg/μg DNA/h in normal islets and from 553 to 806 pg/μg DNA/h in α-cell rich islets in guinea pigs. Arginine is the amino acid known to stimulate the highest production of both insulin and glucagon. A great deal of studies have showed that arginine stimulates insulin secretion in pigs (Kim and Wu 2004; Yao et al. 2008) and humans (Eaton and Schade 1974), diabetic subjects (Stingl et al. 2002), and obese subjects (Copinschi et al. 1967). In growing-finishing pigs, dietary 1 % arginine supplementation increased serum concentrations of glucagon by 36 %, compared with the control group (Tan et al. 2009). However, intracellular glutamate or glutamate-derived metabolites contribute to fuel-induced hormone secretion in β- but not α-cells (Feldmann et al. 2011). A few amino acids such as isoleucine can inhibit α-cell secretion while leucine has a dual effect depending on its concentrations (Quesada et al. 2008).

Glucose plays opposite roles in the mechanisms leading to amino acid-induced hormone release from the α- and β-cells, functioning as an inhibitor in the first case and as a permissive agent in the second (Pagliara et al. 1974). There are suggestions that arginine stimulates insulin release by modulating the insulinogenic signal evoked by glucose (Efendic et al. 1971). The regulation of glucagon secretion by glutamine appears to be reciprocally related to factors affecting glucose metabolism and ATP-levels in the α-cell (Östenson and Grebing 1985). Certain amino acids also can directly stimulate insulin secretion primarily by increasing $[Ca^{2+}]$ (Flatt et al. 1991; Maechler et al. 2002). The release of insulin evoked by cationic amino acids could be due to depolarization of the plasma membrane with subsequent gating of voltage-sensitive Ca^{2+} channels and/or to some other biophysical effect (Blachier et al. 1989b; Smith et al. 1997). L-Lysine stimulated insulin release

involving the similar biophysical mechanism in pancreatic β-cells and also act as a fuel in islet cells (Sener et al. 1989a). In addition, endogenously formed polyamines and NO may play a role in the secretory response of islet cells to amino acids (Henningsson and Lundquist 1998; Sener et al. 1989b). NO is a negative modulator of insulin release and a positive modulator of glucagon release induced by L-arginine or L-homoarginine. These NO effects are also mainly exerted independently of membrane depolarization events (Henningsson and Lundquist 1998). Recent studies have showed that G-protein-coupled receptors (GPCRs) are involved in the regulation of amino acids-induced insulin and glucagon secretion, which provided potential new treatments for diabetes (Layden et al. 2010; Winzell and Ahrén 2007).

10.4 Amino Acids and Gastrointestinal Hormone Secretion

It has been reported that free amino acids, liberated from protein during digestion, induce the secretion of GI hormones such as GLP-1, CCK, and ghrelin (Nakamura et al. 2011). Ingestion of an amino acid mixture stimulates GLP-1 release in humans (Herrmann et al. 1995). GLUTag cells secrete GLP-1 in response to a range of individual amino acids, including alanine, serine, glutamine, asparagine, and glycine. And glutamine was a more effective stimulus of GLP-1 release than the other amino acids tested (Gameiro et al. 2005; Reimann et al. 2004). Glutamine has also been found to trigger GLP-1 secretion in normal weight and obese human subjects (Greenfield et al. 2009). But in dogs, basal concentrations of CCK and gastrin were not affected by intravenous infusion of amino acids (Fried et al. 1982). Of the amino acids that stimulate CCK secretion, the aromatic amino acids, L-phenylalanine and L-tryptophan, are the most effective (Liddle 1994). With the opposite effect on appetite compared with CCK, ghrelin is increased in plasma in response to oral essential amino acids mixture supplementation in humans (Knerr et al. 2003). High plasma levels of amino acids stimulated ghrelin secretion in horse (Sugino et al. 2010). In weanling pigs, oral tryptophan ingestion also increased plasma ghrelin level and ghrelin expression in gastric fundus (Zhang et al. 2007).

The mechanism for amino acids induced increase of the secretion of GI hormones may involve the membrane depolarization and Ca^{2+}-sensing receptor (CaSR) (Reimann et al. 2006; Wang et al. 2011). The stimuli of certain amino acids result in membrane depolarization and Ca^{2+} entry through voltage-gated Ca^{2+} channels. Alanine and glycine showed great capacity for generating membrane depolarization and increasing intracellular Ca^{2+} in GLUTag cells (Gameiro et al. 2005). Several lines of evidence indicate that intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) is important in stimulating CCK release and that amino acids regulate this signal through CaSR (Cancela and Petersen 2002; Mangel et al. 1995). In a CCK-secreting cell line, phenylalanine was shown to stimulate CCK release and to increase $[Ca^{2+}]_i$ through a mechanism that was dependent on an L-type Ca^{2+}

channel (Mangel et al. 1995). In isolated CCK-secreting cells loaded with a Ca^{2+}-sensitive dye, phenylalanine and tryptophan, but not nonaromatic amino acids, caused an increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ was blocked by the CaSR inhibitor Calhex 231 (Wang et al. 2011). Glutamine triggers depolarization by Na^+-coupled uptake, together with voltage-gated Ca^{2+} entry, while a second pathway involves elevation of intracellular cAMP levels (Diakogiannaki et al. 2011; Tolhurst et al. 2011). Glycine and γ-amino butyric acid (GABA) receptors open Cl^- channels on the surface membrane resulting in Cl^- efflux and subsequent membrane depolarization (Reimann et al. 2006; Tolhurst et al. 2011).

The GI hormones secretion increased by amino acids can be viewed as the effects of chemical "messengers," which signal the gastrointestinal tract and accessory organs to perform different actions (Jahan-Mihan et al. 2011). Chemoreceptors located in the small intestine detect luminal amino acids and trigger the release of gut hormones from mucosal enteroendocrine cells (EECs). These hormones act on gut vagal receptors or are released into the blood and reach the central nervous system. Absorbed amino acids can also induce metabolic signal produced in the periphery or directly in some specific brain area (Tomé et al. 2009). In addition to CaSR described above, metabotropic glutamate receptor subtypes (mGluRs), taste receptor type 1 (T1R1), and G-protein-coupled receptor family C group 6 subtype A (GPCR6A) also partially mediated the chemical sensing between dietary amino acids and EECs (Raybould 2010). The amino acid selectivity profiles for CaSR, GPRC6A, and the T1R1/T1R3 heterodimer indicate that aromatic amino acids such as phenylalanine, tryptophan, and histidine are CaSR specific. Cysteine is both acting on CaSR- and GPRC6A. In contrast, lysine seems to act on GPRC6A (Nakamura et al. 2011). GPR93 has been proposed to act like a taste receptor, on the basis that its expression and activation promote CCK secretion, at least in a CCK-secreting EEC line (Choi et al. 2007). Amino acid transporters also may function as an electrogenic and metabolic transceptors. For example, glutamine triggered GLP-1 release in a murine EEC line upon binding to the Na^+-dependent amino acid transporter SLC38A2 (Reimann et al. 2004). However, the involvement of amino acid metabolic transceptors in mammals in intestinal amino acid sensing has not been fully investigated (Miguel-Aliaga 2012).

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Chapter 11
Amino Acids, Gene Expression, and Cell Signaling in the Pig Intestine

Qiwen Fan, Xianghua Yan, Yulong Yin, and Zhen Liu

Contents

Abbreviations

4E-BP1	The eIF4E-binding proteins
5-HT	5-Hydroxytryptamine
AADR	Amino acid deprivation responses
ASN	L-Asparagine

Q. Fan • X. Yan
Animal Science College, Huazhong Agricultural University, Wuhan 430033, China

Y. Yin (✉)
Research Center for Healthy Breeding of Livestock and Poultry, Hunan Engineering and Research Center of Animal and Poultry Science and Key Laboratory for Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, The Chinese Academy of Sciences, Changsha, Hunan 410125, People's Republic of China
e-mail: yinyulong@isa.ac.cn

Z. Liu
Department of Biotechnology and Environmental Science, Changsha University, Changsha, China

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_11,

ASNase	Asparaginase
ASNS	Asparagine synthase
ASRGL1	Asparaginase-like protein 1
CCBL1	Computational formalization of cognitive binary logic
CHOP	CCAAT/enhancer-binding protein-homologous protein
DEPTOR	DEP domain containing mTOR-interacting protein
EAA	Essential amino acids
EGF	Epidermal growth factor
eIF2	Eukaryotic initiation factor 2
GCN2	General control non-depressible 2
GLN	L-Glutamine
GPx	Glutathione peroxidase
GR	Glutathione reductase
GST	Glutathione-s-transferase
HA	Histamine
HisDC	L-Histidine decarboxylase
HRG	Histidine-rich glycoprotein
IL-8	Interleukin-8
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCTs	Monocarboxylate transporters
mLST8	Mammalian lethal with sec-13 protein 8
MOCS3	Multi-objective clustering selection operator section 3
mSin1	Mammalian stress-activated map kinase-interacting protein 1
mTOR	The mammalian target of rapamycin
mTORC1/2	mTOR complexes 1/2
NAC	*N*-acetylcysteine
NEAA	Nonessential amino acids
NMDAR	*N*-methyl-D-aspartate receptor
NO	Nitric oxide
ODC	Ornithine decarboxylase
PHD	Phenylalanine deficiency
PheH	Phenylalanine hydroxylase
PKU	Phenylketonuria
PRAS40	Proline-rich Akt substrate 40 kDa
protor1/2	Protein observed with rictor 1 and 2
raptor	Regulatory-associated protein of mammalian target of rapamycin
rictor	Rapamycin-insensitive companion of Mtor
S6K	p70 S6 ribosomal kinase
SAA	Sulfur-containing amino acid
SCFAs	Short-chain fatty acids
SOD	Superoxide dismutase
SREBP-2	Sterol regulatory element-binding protein-2

TH	Tyrosine hydroxylase
TLR4	Toll-like receptor-4
TNF-α	Tumor necrosis factor-alpha

11.1 Introduction

As the component of proteins amino acids serve as precursors for nucleic acids, hormones, vitamins, and other important molecules. Amino acids are incorporated into mammalian protein mostly as α-amino acids (Yin 2008). The only exception is proline, which is an α-imino acid. So the general structure of amino acids is made of a carboxyl group, an amino nitrogen group, and a side chain attached to a central α-carbon. Functional differences among the amino acids lie in the structure of their side chains. In addition to differences in size, these side groups carry different charges at physiological pH (e.g., nonpolar, uncharged but polar, negatively charged, positively charged). Some amino acids are hydrophobic (e.g., branched-chain and aromatic amino acids) and some are hydrophilic (most others).

Older views of the nutritional classification of amino acids categorized them into two groups: essential and nonessential. The nine essential amino acids (Table 11.1) are those that have carbon skeletons that cannot be synthesized to meet body needs from simpler molecules in animals, and therefore must be provided in the diet. The small intestine is the primary organ responsible for terminal digestion and absorption of dietary nutrients including protein and amino acids (Yin 2008; Wu 1998). There is a growing awareness that the small intestine may be an important site for the metabolism of drugs and other foreign compounds, particularly orally administered ones because these must traverse mucosal epithelial cells of the intestine before entering the bloodstream and body tissues (Windmueller and Spaeth 1981).

For a long time, it has been assumed that all amino acids can enter the blood when absorbed by intestine epithelial cells. But more recently, it has been found that the small intestine can metabolize to a large extent some amino acids like dietary glutamate and most glutamine in addition to circulating glutamine derived from other tissues (Windmueller and Spaeth 1975). Glutamine (66 %), glutamate (98 %), and asparagine (99 %) are metabolized by the jejunal mucosa after uptake (Windmueller and Spaeth 1975, 1976). Recent in vivo studies have demonstrated extensive catabolism of many amino acids in the pig small intestine in the first pass (Yin et al. 2010). The biochemical bases for the metabolism of nonessential amino acids (NEAA) in pig enterocytes are now well established (Yin 2008). And metabolism of essential amino acids (EAA) is believed to play an important role in regulating the efficiency of utilization of dietary protein and amino acids (Le Floc'h et al. 1997).

Table 11.1 Indispensable, dispensable, and conditionally indispensable amino acids in the human diet (Laidlaw and Kopple 1987)

Indispensable	Dispensable	Conditionally indispensable[a]	Precursors of conditionally indispensable
Histidine[b]	Alanine	Arginine	Glutamine/glutamate, aspartate
Isoleucine	Aspartic acid	Cysteine	Methionine, serine
Leucine	Asparagine	Glutamine	Glutamic acid/ammonia
Lysine	Glutamic acid	Glycine	Serine, choline
Methionine	Serine	Proline	Glutamate
Phenylalanine		Tyrosine	Phenylalanine
Threonine			
Tryptophan			
Valine			

[a]Conditionally indispensable is defined as requiring a dietary source when endogenous synthesis cannot meet metabolic need

[b]Although histidine is considered indispensable, unlike the other eight indispensable amino acids, it does not fulfill the criteria used in this report of reducing protein deposition and inducing negative nitrogen balance promptly upon removal from the diet

Although the classification of the EAA and their assignment into a single category have been maintained in this report, the definition of NEAA has become blurred as more information on the intermediary metabolism and nutritional characteristics of these compounds has accumulated (Nutrition Board and Institute of Medicine 2005). Laidlaw and Kopple (1987) divided dispensable amino acids into two classes: truly dispensable and conditionally indispensable. Five of the amino acids in Table 11.1 are termed dispensable as they can be synthesized in the body from other amino acids.

11.2 The Function of Amino Acids

11.2.1 Metabolism of L-Amino Acid

Although the free amino acids present in the body fluids represent only a very small proportion of the body's total mass of amino acids, they are very important for the nutritional and metabolic control of the body's proteins.

The complex metabolism of amino acids can adapt to the physiological functions of intestinal tract and play important roles in the maintenance of intestinal structure and regulation of intestinal function.

11.2.1.1 Asparagine

L-Asparagine (ASN) is one of the NEAA, but it plays an important role in the metabolism. It can inhibit the function of monocarboxylate transporters (MCTs),

which are sodium-independent transporters that mediate the update of aromatic acid and function as a net efflux pathway for aromatic amino acids in the basolateral epithelial cells (Kim et al. 2001) and regulate carbohydrate transport and metabolism in the body. Through the action of the asparagine synthetase, asparaginase-like protein 1 (ASRGL1) protein is involved in amino acid transport and metabolism (Fresquet et al. 2004; Al Sarraj et al. 2005; Overington et al. 2006). In addition, ASN is involved in the adjustment of energy production and conversion (Oppedisano et al. 2004; Dun et al. 2007).

In the pig intestine, ASN is a more stable amino acid in solution than glutamine but with similar structure. This amino acid can stimulate enterocyte cell proliferation (Rhoads et al. 1995). Asparagine synthetase is increased in cultured mouse spleen lymphocytes after stimulation by phytohemagglutinin, lignins, and lipopolysaccharide (LPS) (Hongo et al. 1989; Suzuki et al. 2002). Those studies suggest the possible link between the stimulated asparagine production and macrophage, lymphocyte activation.

11.2.1.2 Cysteine

Sulfur-containing amino acid (SAA) metabolism has been receiving increased attention because of the link to chronic diseases such as cardiovascular disease, Alzheimer's disease, and diabetes. SAA have been regarded as involved in cardiovascular risk factors (O'Callaghan et al. 2005; Ozkan et al. 2002). In order to get a sulfur atom for subsequent sulfuration reactions, cysteine is first desulfurated by Nfs1 which transfers it onto a cysteine of multi-objective clustering selection operator section 3 (MOCS3), yielding a protein persulfide (Marelja et al. 2008).

L-Cysteine use by gut epithelial cells may play an important role for maintenance of glutathione synthesis and cellular redox function (Bauchart-Thevret et al. 2011). Glutathione synthesis is limited by L-cysteine availability, and there are many studies which have shown the potential for dietary L-cysteine intake to affect overall antioxidant status (Griffith 1999; Boebel and Baker 1983; Burgunder et al. 1989). Research on cysteine in the pig intestine has been often focused in relationship with *N*-acetylcysteine (NAC). This latter compound is the precursor for cysteine and glutathione production, therefore playing an important role in the protection of cells against oxidative stress (Wu et al. 2004). NAC reduces inflammation in the small intestine by regulating redox, epidermal growth factor (EGF), and Toll-like receptor-4 (TLR4) signaling (Hou et al. 2012a), and alleviates the mucosal damage and improves the absorptive function of the small intestine in LPS-challenged piglets (Hou et al. 2012b). When injury occurred in intestinal mucosa, the activities of antioxidant NAC, superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) were enhanced. These enhancements might be related to the elimination of the superoxide radical and of H_2O_2, and are accompanied by a fall in glutathione-s-transferase (GST) and glutathione reductase (GR) activities (Cuzzocrea et al. 2000).

11.2.1.3 Histidine

L-Histidine is one of the EAA for many animals. L-histidine decarboxylase (HisDC) is the enzyme catalyzing the formation of histamine from L-histidine (Mamune-Sato et al. 1992). Recent report shows that histidine-rich glycoprotein (HRG) is an abundant plasma glycoprotein that has been shown to regulate a number of important biological processes, such as angiogenesis, cell adhesion and migration, fibrinolysis and coagulation, complement activation, immune complex clearance, and phagocytosis of apoptotic cells (Jones et al. 2005).

L-Histidine's function can be related to histamine (HA) synthesis. Several reports suggest that HA is involved in the regulation of arousal state (Lin et al. 1990), locomotor activity (Clapham and Kilpatrick 1994), cardiovascular control (Imamura et al. 1996), water intake (Lecklin et al. 1998), and food intake (Leurs et al. 1998). Histamine is present in the mucosa of the gastrointestinal tract (Lorenz et al. 1973) and the intestine (Lee and Silverberg 1976; Linaker et al. 1981; Cooke et al. 1984; McCabe and Smith 1984). Histamine is the main mediator for inflammatory response; it can regulate immune function by stimulating receptors on target cells, these latter including hematopoietic progenitors, macrophages, platelets, dendritic cells, and T cells (Dy and Schneider 2004).

11.2.1.4 Tryptophan

L-Tryptophan is catabolized in seven steps to yield aminomuconate. Intermediates in this process are used for the synthesis of serotonin and kynurenine (Peters 1991). The serotonin 5-hydroxytryptamine (5-HT) neurotransmitter system contributes to various physiological and pathological conditions. 5-HT is the first neurotransmitter for which a developmental role was suspected (Nakamura and Hasegawa 2007).

L-Tryptophan is readily absorbed from the gastrointestinal tract (Yao et al. 2011). This amino acid is extensively bound to serum albumin. It is metabolized to serotonin and other metabolites and excreted in the urine. Pyridoxine and ascorbic acid appear to be related to the metabolism of tryptophan.

11.2.1.5 Tyrosine

L-Tyrosine is an NEAA, but it plays a key role in signaling transduction. Many signaling pathways do so by altering the phosphorylation state of tyrosine, serine, or threonine residues in target proteins (Pawson and Scott 1997). Tyrosine hydroxylase (TH) is the first enzyme in catecholamine biosynthesis, as well as being the rate-limiting enzyme in that process. TH requires tetrahydrobiopterin and uses iron as a cofactor in the 3,4-hydroxylation of tyrosine to produce dopa (Lewis et al. 1993; Flatmark 2000). Four isoforms of TH are expressed in the human brain and all of them possess the enzymatic activity (Lewis et al. 1993; Flatmark 2000).

L-Tyrosine can regulate absorption in small intestine (Musch et al. 1987). This amino acid is the substrate for the biosynthesis of the thyroid hormone thyroxine, the neurotransmitter dopamine, the adrenal hormones, and the pigment molecule melanin (Fitzpatrick 2003). A recent study has shown that acetaldehyde induces tyrosine phosphorylation and disrupts tight junction and adherent junction in human colonic mucosa. These latter effects can be prevented by EGF and glutamine (Basuroy et al. 2005).

11.2.1.6 Lysine

The function of L-lysine has been related to intestinal protection in pigs. L-Lysine may be a partial 5-HT 4 receptor antagonist and suppresses 5-HT 4 receptor-mediated intestinal pathologies (Smriga and Torii 2003). An increase in L-lysine intake might be a useful tool in treating stress-induced anxiety and 5-HT-related diarrhea-type intestinal dysfunctions (Smriga and Torii 2003). Recently the research on L-lysine has focused on L-lysine methylation and L-lysine-specific demethylase (Eliazer et al. 2011; DiTacchio et al. 2011). L-Lysine effect on the proteins of the ubiquitin–proteasome pathway has been described (Kensche et al. 2012; Van der Veen et al. 2011).

11.2.1.7 Methionine

L-Methionine is an EAA necessary for normal growth and development in mammals (Finkelstein 1990). L-Methionine is the precursor for cellular methylation reactions and for the synthesis of cysteine and can thus replace part of the cysteine requirement (Finkelstein et al. 1988; Finkelstein and Martin 1984; Mackay et al. 2012). Methionine salvage is important in humans for the recycling of sulfur that has to be assimilated in energy-consuming reactions (Pirkov et al. 2008; Albers 2009).

It is concluded that the transmethylation–transsulfuration sequence is the major pathway for L-methionine metabolism in the mammalian liver (Finkelstein 1990). L-Methionine is one of the EAA and it has very important functions in the intestine. SAA deficiency up-regulates intestinal L-methionine cycle activity and suppresses epithelial growth in neonatal pigs (Bauchart-Thevret et al. 2009). In proteins, one of the amino acids most easily oxidized is L-methionine, which is converted to L-methionine sulfoxide (Weissbach et al. 2002).

11.2.1.8 Phenylalanine

L-Phenylalanine and tyrosine constitute the two initial steps in the biosynthesis of dopamine, which, in turn, is the metabolic precursor of noradrenaline and

adrenaline (Lou 1994). The extracellular L-phenylalanine concentration influences brain function in phenylalanine deficiency (PHD) by decreasing dopamine synthesis (Lou 1994). Phenylalanine hydroxylase (PheH) is an iron(II)-dependent enzyme that catalyzes the hydroxylation of aromatic amino acid L-phenylalanine to L-tyrosine (Zhang et al. 2011). Adrenaline, dopamine, and norepinephrine can be used as neurotransmitters and involved in the regulation of brain function.

L-Phenylalanine transport is depressed by L-tryptophan and L-methionine (Spencer and Samiy 1961). In the intestine, phenylalanine represents a competitive inhibitor involved in the regulation of the absorption of amino acids. There are also interactions which have been described between phenylalanine and the anionic amino acids, aspartate and glutamate (Philip et al. 2011). Phenylketonuria (PKU) is characterized by phenylalanine accumulation and progressive mental retardation. Phenylalanine assembly can form amyloid-like deposits, suggesting a new amyloidosis-like etiology for PKU (Adler-Abramovich et al. 2012).

11.2.1.9 Threonine

L-Threonine is also an EAA. This amino acid is the second or third limiting amino acid in swine or poultry diets behind the total SAA and lysine. So this amino acid can influence the balance of amino acids in vivo. This nutrient plays a critical role in the maintenance of intestinal mucosal integrity and barrier function (Mao et al. 2011).

Intestinal mucin synthesis is sensitive to dietary threonine supply, which suggests that the gut's requirement for threonine may comprise a significant proportion of the whole body requirement (Nichols and Bertolo 2008). L-Threonine is of great importance for the maintenance of intestinal health. It is an EAA that is abundantly present in glycoproteins produced by the intestine (Sophie RD et al. 2007). The high requirement of the gut for L-threonine has often been ascribed to the synthesis of mucins, which are secreted glycoproteins protecting the intestinal epithelium from injury (Mao et al. 2011; Remond et al. 2009). This requirement could be even greater during intestinal inflammation, when mucin synthesis is enhanced and there is no oxidation of L-threonine by enterocytes (Remond et al. 2009). Because mucin proteins are little digested and reused, intestinal mucin secretion represents a net loss of L-threonine for the body (McCabe and Smith 1984).

11.2.1.10 Alanine

Among the 20 amino acids, L-alanine is the principal amino acid released by muscle, in accordance with the fact that alanine is the principal amino acid extracted by liver for gluconeogenesis (Felig et al. 1970). L-Alanine plays a major role in the transfer of nitrogen from peripheral tissue to the liver. This amino acid is involved in the metabolism of amino acids and derivatives (Yang et al. 2002), vitamins and cofactors (Marelja et al. 2008), nucleotides (Lee et al. 1995; Tamaki

et al. 1990), L-phenylalanine and L-tyrosine catabolism (Wu 1998), (Han et al. 2009; Rossi et al. 2004), L-tryptophan catabolism (Perry et al. 1995), and glyoxylate metabolism (Rodionov et al. 2010). L-alanine is protective against the buildup of toxic substances that are released into muscle cells when muscle proteins are broken down quickly to meet energy needs, as it happens with aerobic exercise (Chang and Goldberg 1978). L-Alanine strengthens the immune system, boosts muscular anaerobic endurance and muscular strength, and increases aerobic endurance (Chang and Goldberg 1978).

11.2.1.11 Arginine

L-Arginine functions in the body are related to its utilization as a free amino acid, as a component of most proteins, and as the substrate for several nonprotein/nitrogen-containing compounds (Nieves and Langkamp-Henken 2002). L-Arginine participates in multiple pathways with important nutritional and physiological functions, including the synthesis of protein, nitric oxide (NO), creatine, proline, glutamate, and polyamines as well as the secretion of hormones (Tan et al. 2009a, b, 2011; He et al. 2009; Li et al. 2007; Yao et al. 2008). L-Arginine which enhances the immune system stimulates cell division, increases wound healing and hormone secretion, and regulates the vascular tone and many other various physiological processes (Nieves and Langkamp-Henken 2002). L-Arginine is the main substance of the urea cycle, which yields urea as the major form in which excess nitrogen is excreted from the human body (Brusilow and Horwich 2001). L-Arginine also is the main substance involved in the metabolism of polyamines (Moinard et al. 2005).

NO, a multifunctional second messenger, is implicated in numerous physiological functions in mammals that range from immune response and potentiation of synaptic transmission to dilation of blood vessels and muscle relaxation (Pacher et al. 2007). NO is also a regulator of intestinal electrolyte transport (MacNaughton 1993), and it has a dual excitatory and inhibitory effect on intestinal motility (Holzer et al. 1997; Tan et al. 2012a, b; Kong et al. 2012; Liu et al. 2012; Wu et al. 2012). L-Arginine is the only substrate for the synthesis of NO. As the precursor of NO, arginine possesses many functions related to effects on the immune system.

11.2.1.12 Aspartate

Aspartate is an NEAA in mammals, being produced from oxaloacetate by transamination. Oxaloacetate is one of several 2-oxoacids that function efficiently in the computational formalization of cognitive binary logic (CCBL1)-mediated transamination (Han and Li 2004) and oxaloacetate is an important substance involved in gluconeogenesis. The active form of the enzyme asparagine synthase (ASNS) is a dimer which can catalyze the reaction of aspartate, glutamine, and ATP to form

asparagine, glutamate, AMP, and pyrophosphate (Van Heeke and Schuster 1989). Moreover, aspartate donates one nitrogen atom in the biosynthesis of inosine, the precursor to the purine bases (Chen et al. 2005). The glutamate *N*-methyl-D-aspartate receptor (NMDAR) plays a critical role in learning and memory (Burgdorf et al. 2011). Aspartate also stimulates NMDAR, though not as strongly as the amino acid neurotransmitter glutamate does (Chen et al. 2005). In the intestine, aspartate can affect the absorption of nutrients and, as the dietary supplement, can modify the intestinal ecosystem.

11.2.1.13 Citrulline

Citrulline is a key intermediate in the urea cycle, the pathway by which mammals excrete ammonia. Citrulline is made from ornithine and carbamoyl phosphate in one of the central reactions in the urea cycle (Rabier and Kamoun 1995). In cells, NO was synthesized from citrulline (via arginine) as well as from arginine, indicating the operation of the citrulline–NO cycle involved in arginine recycling (Mori 2007). Although citrulline is not coded for by DNA directly, several proteins are known to contain citrulline as a result of a posttranslational modification. Circulating citrulline is emerging as an innovating biomarker candidate for assessment of intestinal function (Crenn et al. 2011). Citrulline is a precursor for the production of arginine by the kidney (Crenn et al. 2011).

Glutamine, as arginine is a precursor of ornithine, which can be converted to citrulline by the intestine (van de Poll et al. 2007). Hepatic citrulline uptake limits the amount of gut-derived citrulline reaching the kidney and this may have implications for interventions aimed at increasing systemic arginine concentrations (van de Poll et al. 2007).

11.2.1.14 Glutamate

Monosodium glutamate is the crystalline salt of glutamate often used as a flavor enhancer. In neuroscience, glutamate is the most abundant excitatory neurotransmitter that plays a key role in long-term potentiation as well as in learning and memory (Burgdorf et al. 2011; McEntee and Crook 1993). Glutamate is a key molecule in cellular metabolism and can be involved in the synthesis of some amino acids.

Glutamate is also a major intestinal oxidative fuel and may represent a useful dietary supplement for the infant gut metabolism (Janeczko et al. 2007). Extensive gut metabolism limits the absorption of supplemental dietary glutamate even at excessive intakes (Janeczko et al. 2007).

11.2.1.15 Glutamine

The amino acid glutamine plays a central role in nitrogen transport within the body and is a fuel for rapidly dividing cells, such as in the gut and the immune system (Novak et al. 2002). It has been reported that glutamine has the ability to regulate gene expression in a number of physiological processes (Curi et al. 2007). Glutamine is also involved in amino acid synthesis and interconversion (transamination). These reactions mediate the synthesis of aspartate, asparagine, glutamate, and glutamine from ammonia or the intermediate of glycolysis, and allow the utilization of the carbon atoms from these four amino acids for glucose synthesis under fasting conditions.

Ko et al. (1993) show that L-glutamine, when added in amounts greater than required to prevent apoptosis, is essential for the EGF stimulation of cell proliferation as well as DNA, RNA, and protein synthesis in IEC-6 cells. This in vitro study further extends our knowledge on the important role of GLN in intestinal cell growth and function, and is consistent with a large number of in vivo studies demonstrating gut-trophic effects of this amino acid (Burke et al. 1989; Souba et al. 1992).

11.2.1.16 Ornithine

Ornithine has several common characteristics with citrulline. Both of them play a key role in the urea cycle. Ornithine is also not coded by DNA, and, in that sense, is not involved in protein synthesis (Weber and Miller 1981). Ornithine can realize the reversible transfer of an acetyl-group to an acidic (glutamate) amino acid by employing a common mechanism involving an acetyl-enzyme intermediate, but using different side chain binding modes, which is catalyzed by ornithine acetyltransferases (Iqbal et al. 2011).

Ornithine decarboxylase (ODC) is the first enzyme of the polyamine biosynthetic pathway. ODC is induced by androgens in the mouse kidney. ODC in the small intestine is stimulated three- to tenfold by refeeding or administration of insulin (Maudsley et al. 1976). The upregulation of proglucagon and ODC gene expression may be the mechanism by which short-chain fatty acids (SCFAs) facilitate intestinal adaptation (Tappenden et al. 1996). Systemic administration of SCFAs has been shown to facilitate adaptation to small bowel resection (Tappenden et al. 1996). It has been shown that ODC activity in macroscopically normal tissue of patients with tumors of stomach and large intestine increased as the disease progressed. In addition, there is an inverse relationship between ODC activity in adenocarcinomas and differentiation (Berdinskikh et al. 1991). Thus in the intestine, ODC is indeed an important regulator and plays a role in cell signaling.

11.3 Amino Acids, Gene Expression, and Cell Signaling

Many studies have shown that glutamine modulates expression of genes that are crucial for intestinal metabolism and function. Dietary glutamine supplementation increased intestinal expression (120–124 %) of genes that are necessary for cell growth and removal of oxidants while reducing (34–75 %) expression of genes that promote oxidative stress and immune activation (Wang et al. 2008). Previous studies have shown that glutamine regulates the mitogen-activated protein kinase (MAPK) activation and c-Jun signaling in enterocytes (Rhoads 1999). Son et al. (2005) found that histidine significantly inhibited both hydrogen peroxide and tumor necrosis factor-alpha (TNF-α) induced interleukin-8 (IL-8) secretion as well as mRNA expression in intestinal epithelial-like Caco-2 cells and HT-29 cells. Moreover, other amino acids also contribute to regulate the intestinal metabolism since treatment of human intestinal cell line with branched-chain amino acids (leucine, isoleucine, or valine) down regulated the expression of the sterol regulatory element-binding protein-2 (SREBP-2), a transcription factor involved in cholesterol metabolism (Chen and Reimer 2009). Finally, amino acids such as branched-chain amino acids, glycine, lysine, threonine, and cysteine may also play important regulatory roles in the intestine, particularly in improving the immune function (Li et al. 2007; Calder 2006; Burrin and Stoll 2007; Newsholme et al. 2011; Wang et al. 2009).

It seems that the functions, in which amino acids are implicated as gene expression regulators, involve the activation of different signaling pathways and transcription factors. In fact, the regulation to gene expression is editing the structure of RNA, including deadenylation-dependent mRNA decay, microRNA-mediated RNA cleavage, regulation of mRNA stability, and tRNA aminoacylation. The two main processes by which amino acids regulate gene expression are control of tRNA aminoacylation and regulation of mRNA through signaling pathway.

11.3.1 Amino Acids Regulate Aminoacylation of tRNA

tRNAs are directly involved in the translation process of gene expression. In that way, they can influence the gene expression. tRNA synthetases catalyze the ligation of tRNAs to their cognate amino acids in an ATP-dependent manner. The reaction is shown in Fig. 11.1. The rapid hydrolysis of pyrophosphate makes these reactions essentially irreversible under physiological conditions (Fersht and Kaethner 1976a). Specificity of the tRNA charging reactions is achieved both by specific recognition of amino acid and tRNA substrates, by the synthetase, and by an editing process in which incorrect aminoacyladenylate molecules (e.g., valyladenylate associated with isoleucyl tRNA synthetase) are hydrolyzed rather than conjugated to tRNAs in the second step of the reaction (Baldwin and Berg 1966a, b; Fersht and Kaethner 1976b).

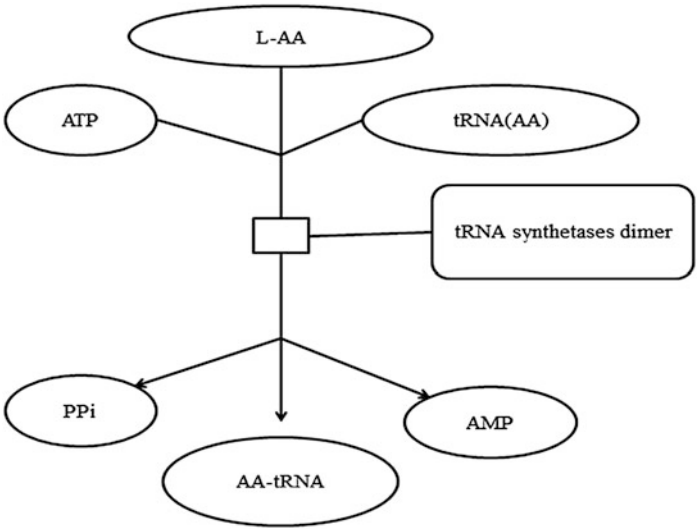

Fig. 11.1 L-AA tRNA aminoacylation. The reaction proceeds in two steps. First, amino acid and ATP form an aminoacyladenylate molecule, releasing pyrophosphate. The aminoacyladenylate remains associated with the synthetase enzyme where in the second step it reacts with tRNA to form aminoacyl tRNA and AMP

The tRNA synthetases can be divided into two structural classes based on conserved amino acid sequence features (Burbaum and Schimmel 1991).

A number of tRNA synthetases are known to have functions distinct from tRNA charging (Park et al. 2005). Additionally, mutations in several of the tRNA synthetases, often affecting protein domains that are dispensable in vitro for aminoacyl tRNA synthesis, are associated with a diverse array of neurological and other diseases (Park et al. 2005, 2008; Antonellis and Green 2008). These findings raise interest into the role of these enzymes in human development and disease.

11.3.2 Amino Acids Regulate Gene Expression Through Cell Signaling Pathway

The best-characterized example of amino acid-induced regulation of a signal transduction pathway is one involving a protein kinase referred to as the mammalian target of rapamycin (mTOR), which acts to modulate the function of proteins engaged in both global mRNA translation and the selection of specific mRNAs for translation (Kimball and Jefferson 2004).

mTOR in most organisms has evolved to be able to make a transition between anabolic and catabolic states. This would allow these organisms to survive and

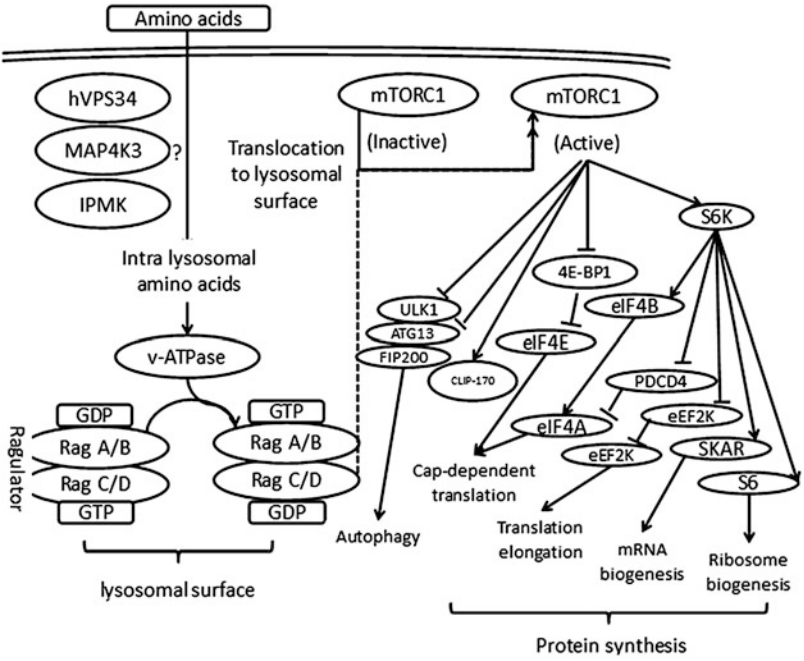

Fig. 11.2 The molecular mechanisms involved in the amino acid regulation of gene expression through the mTORC1 signaling pathway

grow in environments in which nutrient availability is variable (Laplante and Sabatini 2012). There are two mTOR complexes and both mTOR complexes are large. mTORC1 is known to have six and mTORC2 seven known protein components. They share the catalytic mTOR subunit, and also mLST8 (also known as GbL) (Jacinto et al. 2006; Kim et al. 2003), DEP domain containing mTOR-interacting protein (DEPTOR) (Peterson et al. 2009), and the Tti1/Tel2 complex (Kaizuka et al. 2010). In contrast, regulatory-associated protein of mammalian target of rapamycin (raptor) (Hara et al. 2002; Kim et al. 2002) and proline-rich Akt substrate 40 kDa (PRAS40) (Sancak et al. 2007; Thedieck et al. 2007; Wang et al. 2007) are specific to mTORC1, whereas rapamycin-insensitive companion of Mtor (rictor) (Jacinto et al. 2004; Sarbassov et al. 2004), mammalian stress-activated map kinase-interacting protein 1 (mSin1) (Jacinto et al. 2006; Frias et al. 2006), and protein observed with rictor 1 and 2 (protor1/2) (Thedieck et al. 2007; Pearce et al. 2007, 2011) are only part of mTORC2. Figure 11.2 describes the known molecular functions of amino acid-induced regulation of the mTOR complex.

Amino acids are the nutrition signal which can induce activated mTORC1. When activated, mTORC1 promotes protein synthesis mainly by phosphorylating the kinase p70 S6 ribosomal kinase (S6K) and the translation regulator of the eIF4E-binding proteins (4E-BP1) (Ma and Blenis 2009). This complex also induces

lipid biogenesis by activating SREBP1 and PPARg transcription factors (Laplante and Sabatini 2009) and inhibits catabolism by blocking autophagy through the phosphorylation of the ULK1–Atg13–FIP200 complex (Ganley et al. 2009; Hosokawa et al. 2009).

Besides the mTOR pathway, many amino acids show their own way to reflect the cell signaling. In that sense, asparagine and glutamine are good examples. Asparagine starvation by asparaginase (ASNase) enhances phosphorylation of the eukaryotic initiation factor 2 (eIF2) by general control non-depressible 2 (GCN2) kinase, leading to reduced global mRNA translation rates. This conserves energy and allows cells time to reprogram stress-related gene expression to alleviate cell injury (Bunpo et al. 2010). Asparaginase depletes circulating asparagine and glutamine, activating amino acid deprivation responses (AADR) such as phosphorylation of eukaryotic initiation factor 2 (p-eIF2) leading to increased mRNA levels of asparagine synthetase and CCAAT/enhancer-binding protein-homologous protein (CHOP) and decreased mTORC1 signaling (Bunpo et al. 2009). NAC inhibits activation of c-Jun N-terminal kinase, p38 MAP kinase, and redox-sensitive activating protein-1 and nuclear factor kappa B transcription factor activities regulating expression of numerous genes (Zafarullah et al. 2003).

Particular attention has been paid to the effects of glutamine at appropriate concentrations for its capacity to enhance a great number of cell functions via the activation of various transcription factors (Brasse-Lagnel et al. 2009). Glutamine is known to play a regulatory role at the gene and protein level in several cell-specific processes including metabolism (e.g., oxidative fuel, gluconeogenesis precursor, and lipogenic precursor), cell integrity (survival, cell proliferation), protein synthesis and degradation, redox potential, respiratory burst, insulin resistance, insulin secretion, and extracellular matrix synthesis (Curi et al. 2007). Glutamine has been shown to regulate the expression of many genes related to metabolism, signal transduction, and cell defense and repair and to activate intracellular signaling pathways (Curi et al. 2007).

Figure 11.3 is a schematic view of the regulatory functions of glutamate. Both the diversity of the studies into the effect of glutamine and the variety of transcription factors involved in its action can be seen in this figure. Although glutamine deprivation was also able to stimulate the expression of ASNS (Hutson and Kilberg 1994) and CHOP (Huang et al. 1999) genes in different kinds of mammalian cells, the involvement of the NSRE and AARE sequences in these effects was not studied. Moreover, none of these responsive elements were identified in the other target genes studied.

11.4 Summary and Perspectives

EAA cannot be synthesized in vivo, so their functions are often placed at the center of nutrition and animal science. NEAA can be synthesized in the body and used for conversion into other amino acids. In addition, the so-called NEAA can become

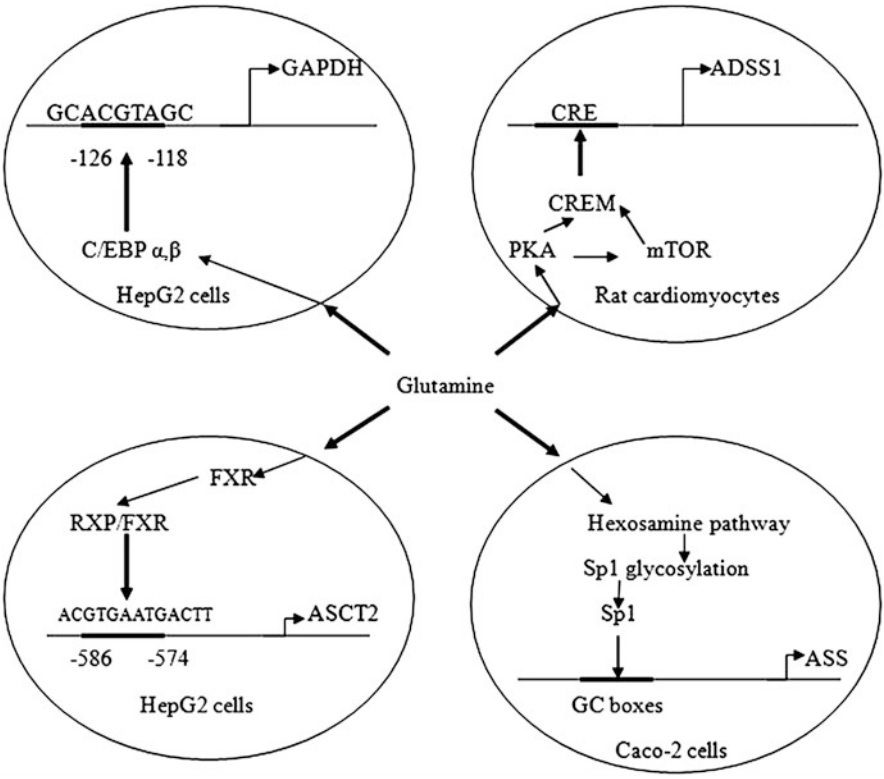

Fig. 11.3 Schematic representation of the influence of glutamine on the transcription of genes involved in intermediary metabolism (Brasse-Lagnel et al. 2009)

essential at some special physiological conditions. The functional amino acids (Wu et al. 2007, 2009), it can be considered that one given functional amino acid can be either a "nonessential" or an "essential" amino acid.

The precise pathways from the entrance of the amino acid into the cell to the activation of gene transcription remain elusive and the molecular mechanisms involved represent an important perspective for future research.

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Chapter 12
Amino Acids and Immune Functions

Bie Tan, Mingyong Xie, and Yulong Yin

Contents

12.1 Introduction

In modern, high-density production systems, swine are challenged by pathogenic microorganisms—bacteria, viruses, and parasites that can cause infectious disease or pathology, especially for neonatal and weaned piglets (Zhang et al. 2012; Ren et al. 2012). In these latter, immune system is not well developed in the first 4 weeks of life (Yang and Schultz 1986). Amino acids have been demonstrated to play important roles in immune responses by regulating (1) the activation of T lymphocytes, B lymphocytes, natural killer cells, and macrophages; (2) cellular redox state, gene expression, and lymphocyte proliferation; and (3) the production of antibodies, cytokines, and other cytotoxic substances (Li et al. 2007b; Kim et al. 2007). A number of studies have showed that dietary specific amino acids

B. Tan • Y. Yin (✉)
Research Center for Healthy Breeding of Livestock and Poultry, Hunan Engineering and Research Center of Animal and Poultry Science and Key Laboratory of Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, The Chinese Academy of Sciences, Changsha, Hunan 410125, People's Republic of China
e-mail: yinyulong@isa.ac.cn

M. Xie
The Key Laboratory of Food Science of Ministry of Education and Department of Food Science and Engineering, Nanchang University, Nanchang 330047, P.R. China

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_12,

supplementation to pigs with malnutrition and infectious diseases enhance the immune status, thereby reducing morbidity and mortality (Ewaschuk et al. 2011; Johnsona et al. 2006; Liu et al. 2008; Han et al. 2008; Tan et al. 2009; Ren et al. 2012a, b). In this chapter, functions of amino acids in regulating the immune system and the amino acids requirements of immune system are described, in the hope of providing great promise in improving health and preventing infectious diseases in animals and humans.

12.2 Porcine Immune System

The two primary immune responses to microorganisms and their antigens are generated by the innate and acquired or adaptive immune systems. The two immune systems interact intimately and fulfill the different needs of the host to control microorganisms. Innate immunity of pigs consists of the similar components described for other mammals. The effector functions are realized through two major mechanisms: (a) the recruitment and activation of cellular components, including macrophages, neutrophils, natural killer (NK) cells, and dendritic cells (DCs) and (b) the release of a broad spectrum of extracellular mediators such as cytokines, chemokines, complement, and antimicrobial peptides (AMPs). The cells of the immune system include the myeloid cells, monocytes, macrophages, dendritic cells, neutrophils, eosinophils, basophils and lymphocytes, of which there are two major types, the B and T lymphocytes. B cells and T cells are responsible for humoral and cell-mediated immunity, respectively, which sum to form adaptive immunity. The porcine immune system differs in many aspects from that of humans and mice, including morphological differences in the lymphatic system, and phenotypic differences in immune cells as well as functional differences in immune cell populations (Rothkötter et al. 2002). Unlike other most species, the lymphocytes enter into the lymphoid organs through the lymphatic vessels and exit directly into the blood in the pig. These differences might contribute to the predisposition to and outcomes of bacterial infections such as Salmonella serovars (Scharek and Tedin 2007).

The gastrointestinal tracts (GITs), one of the largest immunological organs of the body, contain greater than 10^{12} lymphocytes and has a greater concentration of antibodies than any other site in the body (Mayer 2000). With respect to immune function within the GITs, it may be equally important to achieve a homeostatic balance between immune tolerance and immune responsiveness (Artis 2008). The mucosal immune system is adequately equipped to generate a protective immune response directed at harmful pathogens, but it also has the capability to be tolerant of the ubiquitous dietary antigens and normal microbial flora while maintaining the ability to permit the absorption of nutrients. Therefore, the development of the gastrointestinal immune system is important for establishing an effective immunological response to a diverse milieu of dietary and microbial antigenic components (Brandtzaeg and Pabst 2004; Burkey et al. 2009).

The neonatal piglets have little active mucosal immune system with the low level of peripheral lymphocytes, underdeveloped lymphoid nodes, rudimentary jejunal Peyers patches, and the low numbers of effector/memory immune cells (Blecha 2001). Changes in activation of immune system by antigenic stimuli by commensal microbial flora, pathogens, and environmental influences result in the appearance of conventional, activated T and B cells and the influx and expansion of mucosal immune cells and those in the peripheral lymphoid pool (Sinkora et al. 1998, 2005). Piglets receive the passive immunity via colostral immunoglobulins intake. As time progresses up to weaning, the active mucosal system gradually gathers the ability to generate its own antibody molecules in the gut wall. In the first 2 weeks of life, the intestine rapidly becomes colonized with lymphoid cells. Between 14 and 28 days of age, the intestinal mucosa becomes colonized with $CD4^+$ cells, and $CD8^+$ cells begin to appear at 35 days age. The immunological architecture of piglet cannot be considered fully mature until 7 weeks of age (Sinkora and Butler 2009). In commercial practice, piglets are weaned at 3–4 weeks of age, and this results in an increased susceptibility to bacterial infection and acute diarrhea and high mortality rates. The obvious increase of the numbers of $CD2^+$ leucocytes was observed in the intestine (Dréau et al. 1995), and piglets showed reduced ability to react to the lymphocyte mitogen phytohemagglutinin after weaning. With the reduction of IL-2 secreted from systemic T cells, production of specific antibody was also reduced (Butler et al. 2002; Sinkora et al. 2000).

12.3 Amino Acids Metabolism by Cells of the Immune System

The utilization of amino acids by immune cells plays an important role in the function of the immune system (Curi et al. 1997). Glutamine utilization has been linked to functional activities of immune cell function such as cytokine production, nitric oxide production, superoxide production, and phagocytosis. Many of cells of the immune system including lymphocytes, macrophages, and neutrophils utilize glutamine at high rates, which is related to the specific function of these cells in the inflammatory response (Curi et al. 1997, 1999; Wu et al. 2007; Wang et al. 2008; Newsholme et al. 1999; Ren et al. 2013). Therefore, glutamine had to be present at 10- to 100-fold in excess of any other amino acid in culture and cannot be replaced by glutamic acid or glucose. Although the activity of the first enzyme responsible for the metabolism of glutamine, glutaminase, is high in these cells, the rate of oxidation is low. Much of the glutamine is converted to glutamate, aspartate, lactate, and in appropriate conditions to CO_2 (Newsholme 2001). Koch et al. (1990) demonstrated that glutamine provides N- and C-atoms required for the synthesis of macromolecules and energy while leucine provides more precursors for incorporation into protein in peripheral lymphocytes.

Cell culture studies have showed that BCAA are absolutely essential for lymphocytes to synthesize protein, RNA, and DNA and to divide in response to stimulation (Calder 2006). Immune cells are able to incorporate BCAA into proteins

and oxidize BCAA (Calder 2006; Burns 1975). Walrand et al. (2004) reported that leucine incorporation into immune cell proteins was linear over time with a comparable slope. Incorporation of isoleucine into proteins by lymphocytes is greatest, followed by eosinophils, and then by neutrophils (Burns 1975). Immune cells express branched chain alpha keto acid dehydrogenase and decarboxylase activities and so can oxidize BCAA. Lymphocytes take up and oxidize leucine and isoleucine in vitro (Calder 2006). Mitogen stimulation of lymphocytes increases leucine transport by 270 %, leucine transamination by 195 %, and leucine oxidation by 122 % (Koch et al. 1990). Isoleucine is oxidized by neutrophils and lymphocytes through the Krebs cycle after decarboxylation and lymphocytes are able to oxidize isoleucine eight times more rapidly than neutrophils (Burns 1975). In a B cell line, the pattern of uptake of all three BCAAs through the cell cycle is the same, and the order of the rate of uptake is leucine, isoleucine, and valine (Glassy and Fur long 1981).

Another important immuno-enhancing amino acid, arginine, is metabolized either by inducible nitric oxide synthases (iNOS) or by arginase 1 in immune cells. These enzymes are stimulated by T helper 1 or 2 cytokines, respectively. In the absence of immune stimulation, little arginine is used by immune cells due to a lack of expression of major arginine metabolizing enzymes, iNOS and arginase 1 (Bernard et al. 2001). Myeloid cells expressing arginase 1 are described in a growing number of disease processes, prominently in cancer, autoimmune diseases, and in graft vs. host disease (Bronte and Zanovello 2005; Rodriguez et al. 2005; Serafini et al. 2006; Popovic et al. 2007). The accumulation of arginase 1-expressing myeloid cells in spleens in mice after surgical trauma has also been observed (Makarenkova et al. 2006). Myeloid suppressor cells (MSC) efficiently deplete arginine and generate ornithine. Arginase 1 expression is also detected in mononuclear cells after trauma or surgery. Unlike arginase 1, iNOS is regulated by opposing stimuli. It has been shown that inflammatory stimuli induce the expression of iNOS in myeloid cells and other cell types (Hibbs 1991; Popovic et al. 2007; Johansson et al. 2002, 2010). iNOS can be induced in response to various cytokines such as IL-1, TNFα, and IFNγ, or bacterial products such as LPS. In vivo, IFNγ is the most potent and prevailing inducer of iNOS (Johansson et al. 2002). iNOS exerts a regulatory effect on arginase activity through the production of hydroxy-L-arginine, an intermediate product in the generation of NO. Arginase 1 in turn regulates NO through depletion of arginine availability (Morris 2004). Neither iNOS nor arginase 1 is induced in T lymphocytes, which represents a marked difference between these cells and myeloid cells. T lymphocytes depend on arginine for proliferation, ζ-chain peptide, and T cell receptor complex expression.

12.4 Impact of Amino Acids on Immune Function: Pig Studies

Amino acids affect immune system function usually through actions at several levels in the gastrointestinal tract, thymus, spleen, regional lymph nodes, and immune cells of the circulating blood (Cunningham-Rundles 2002). The regulation

of amino acids on the immune function can be considered from two perspectives, namely, the enhancement of the immune response that protects individuals from infections and malignant neoplasms and the reduction of over-responses such as inflammation and autoimmunity (Yoneda et al. 2009). The roles of glutamine, arginine, threonine, methionine, cysteine, and tryptophan in enhancing the immune function in pigs have been well established (Johnsona et al. 2006; Ewaschuk et al. 2011; Tan et al. 2008; Li et al. 2007b; Wang et al. 2006; Grimble 2006; Le Floc'h and Sève 2007).

Glutamine is required to support optimal lymphocyte proliferation and production of cytokines by lymphocytes and macrophages (Wu 1996; Yoo et al. 1997; Yu et al. 2002; Calder and Yaqoob 1999). Adding glutamine to the weaning diet of pigs significantly modified immune cells in the mesenteric lymph nodes, in a potentially beneficial manner by preventing an increase in antigen naive CD4+ cells, increasing the proliferative response to pokeweed mitogen, and supporting a Th-1 type cytokine response after T cell stimulation (Johnsona et al. 2006). Glutamine is preferentially metabolized by the intestinal mucosa and by lymphocytes. As a precursor for glutathione (GSH) it helps maintain the antioxidant status of cells, improving the gut barrier function against bacterial infection. So glutamine supplementation is useful in reducing early steps in weaning-related gastrointestinal infections (Ewaschuk et al. 2011; Liu et al. 2002). Intestinal tissue from control, but not from Gln-supplemented, pigs responded to *Escherichia coli* with a significant increase in mucosal cytokine mRNA (IL-1β, IL-6, transforming growth factor-β, and IL-10) and a decrease in tight-junction protein expression (claudin-1 and occludin) (Ewaschuk et al. 2011).

As a precursor for nitric oxide and polyamine synthesis, arginine profoundly influences immune function (Kelly et al. 1995; Wu and Meininger 2002). Arginine has been demonstrated to exert beneficial effects on pregnant sows (Kim et al. 2006) and weaned pigs (Tan et al. 2009), on LPS-immunostimulated pigs (Liu et al. 2008), and on cyclophosphamide-immunosuppressed pigs (Han et al. 2009). This amino acid reduces morbidity and mortality in response to infectious pathogens. Administration of arginine increased thymus size, weight and cellularity, enhanced lymphocyte proliferation in response to mitogen and alloantigen, augmented macrophage and natural killer (NK)-cell-induced lysis of tumor cells, and increased IL-2 production by lymphocytes and receptor activity (Han et al. 2009; Kim et al. 2006; Tan et al. 2009). Arginine supplementation improves the development of digestive tract, prevents intestinal villous atrophy, and decreases the expression of intestinal pro-inflammatory cytokines, thereby enhancing the mucosal immune status in early-weaned piglets and alleviating mucosal injury of LPS-challenged pigs (Liu et al. 2008; Tan et al. 2008).

Threonine plays an important role in the production of antibodies and in providing overall immune system support. A significant part of the threonine intake is utilized by the gut itself and is used for the synthesis of endogenous secretions, particularly mucus, which is important to maintain the gut barrier. Threonine supplementation can regulate the innate immune function of IPEC-J2 cells infected with Pseudorabies Virus at molecular level, and can inhibit expression of genes corresponding to IL-1β,

TNF-α, and TGF-β1, but enhance expression of gene corresponding to IL-6 and IL-15, while the effects were found to vary with time (Han et al. 2012). Dietary supplementation with threonine also has been demonstrated to promote serum levels of IgG in sows (Cuaron et al. 1984) and increase antibody production, serum IgG levels, and jejunal mucosal concentrations of IgG and IgA, while decreasing jejunal mucosal concentrations of IL-6 in young pigs challenged with *E. coli* (Li et al. 1999; Wang et al. 2006).

Sulfur amino acids, methionine and cysteine, have indeed been shown to be beneficial for the immune system, aside from their role in protein synthesis (Grimble 2006). Additional dietary intake of methionine plus cysteine can reduce the adverse effects of immune system stimulation on whole body protein deposition in growing pigs, and probably accelerates the recovery (Litvak et al. 2011). Dietary supplementation with *N*-acetylcysteine (a stable precursor of cysteine) is highly effective in enhancing immune functions under various disease states (Geudens et al. 2008; Grimble 2001). However, improvement of immune function in pigs challenged with aflatoxin was not observed with supplementation of 0, 0.15, 0.30, or 0.45 % methionine to a basal diet containing 0.33 % methionine (Van Heugten et al. 1994). Methionine and cysteine are precursors of important molecules and important for intestinal mucosal function. We recently demonstrated that a sulfur amino acid-free diet administered enterally to piglets for 7 days led to a reduced intestinal mucosal growth associated with villus atrophy, reduced epithelial cell proliferation, lower goblet cell number, and diminished small intestinal redox capacity (Bauchart-Thevret et al. 2009).

Tryptophan plays an important role in the defense of the body and immune response modulation (Moffet and Namboodiri 2003; Le Floc'h and Sève 2007), in relation with the kynurenine pathway. In pigs, Melchior et al. (2004, 2005) showed that this metabolic pathway is involved in tryptophan metabolism disturbances associated with an inflammatory response. Pigs suffering from lung inflammation had lower plasma tryptophan concentrations and higher IDO activity in lungs and associated lymph nodes, than pair-fed healthy control piglets (Le Floc'h et al. 2004; Melchior et al. 2004, 2005).

12.5 Amino Acids Requirements to Optimize Immunity

A deficiency of dietary protein or amino acids has long been demonstrated to impair immune function and increase the sensitivity of animals to infectious challenges or stressful conditions (Le Floc'h et al. 2004). For example, deficiency of branched chain amino acids and of arginine + lysine increased splenocyte proliferation, but sulfur amino acid deficiency decreased splenocyte and lymphocyte proliferation (Konashi et al. 2000). The study with piglets has shown that threonine deficiency caused higher nitrogen excretion, blood urea, and lower number of acidic mucin-producing goblet cells in the small intestine (Wang et al. 2007). Dietary supplementation with amino acids beyond their requirements for growth deposition might

thus be useful depending on environmental conditions particularly during periods of stress and when the immune system is challenged (Reeds and Jahoor 2001).

It is now clear that immune system stimulation (ISS) can cause morphological and physiological changes in the gastrointestinal tract and impact nutrient utilization in pigs (Le Floc'h et al. 2004; Mani et al. 2012). During immunological stress, amino acids are redistributed away from protein production towards tissues involved in inflammation and immune response (Bruins et al. 2000, 2002; Webster et al. 2002). They are used for the synthesis of inflammatory and immune proteins, to support immune cell proliferation, and for the synthesis of other compounds important for body defense functions (Le Floc'h et al. 2004; Webster et al. 2002). Immune activation appears to alter glutamine and arginine metabolism. Bruins et al. (2000) and Deutz et al. (1992) have shown that surgery and endotoxemia induced by LPS injection results in an increase in glutamine efflux from the hindquarter and intestine and in glutamine uptake by the liver and spleen. During the peak of an immune response, the requirement for those nonessential amino acids (glutamine, arginine, cysteine, and so on) increases 2- to 3-fold (Wilmore and Shabert 1998; Pond and Newsholme 1999), becoming, at least potentially, limiting (Reeds and Jahoor 2001). ISS does not change the apparent ileal digestibility (AID) of amino acids but alters the partitioning of sulfur amino acids in favor of nonprotein body stores in growing pigs (Rakhshandeh et al. 2010). These findings reflect an increased need for dietary sulfur amino acids to support the immune response during immune system stimulation in growing pigs (Rakhshandeh et al. 2010). In pigs injected with turpentine, fibrinogen plasma concentrations increase by 30 % and fibrinogen synthesis increases by 140 % (Jahoor et al. 1999). And in pigs with a lung inflammation induced by intravenous injection of complete Freund's adjuvant, plasma tryptophan concentrations declined for 10 days. Therefore, the increase in protein synthesis may require a great quantity of tyrosine, phenylalanine, and tryptophan (Le Floc'h et al. 2004; Melchior et al. 2004, 2005). Li et al. (1999) reported that although maximum growth rate of 17–31 kg pigs occurred at a dietary threonine level of 6.8 g kg^{-1}, higher threonine levels were needed to maximize humoral antibody production and IgG levels. To optimize immunity of 10–25 kg pigs, 6.6 g per day of true ileal digestible threonine should be fed (Wang et al. 2006). Li et al. (2007a) showed that the ideal amino acid pattern of lysine/methionine/threonine/tryptophan on the digestible basis was 100:27:29:59 for 10 kg pigs under immune stress and 100:30:21:61 for piglets under normal conditions.

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Chapter 13
The Pig Model for Studying Amino Acid-Related Human Diseases: Amino Acids and Intestinal Diseases in Preterm Infants

Caihong Hu, Yulong Yin, Anshan Shan, Xingguo He, Weifen Li, and Jun Fang

Contents

C. Hu • W. Li
The Key Laboratory of Molecular Animal Nutrition of Ministry of Education, College of Animal Science, Zhejiang University, Hangzhou, P. R. China

Y. Yin (✉)
Research Center for Healthy Breeding of Livestock and Poultry, Hunan Engineering and Research Center of Animal and Poultry Science and Key Laboratory for Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, The Chinese Academy of Sciences, Changsha, Hunan 410125, P. R. China
e-mail: yinyulong@isa.ac.cn

A. Shan
Institute of Animal Nutrition, Northeast Agricultural University, Harbin 150030, P. R. China

X. He
Changsha Luye Biotechnology Co., Ltd, Changsha, Hunan 410125, P. R. China

J. Fang
College of Bioscience and Biotechnology, Hunan Agricultural University, Changsha, Hunan 410125, P. R. China

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_13,

13.1 Preterm Delivery

Preterm delivery in humans, defined as birth before 90 % gestation, is a leading cause of infant morbidity and mortality worldwide, occurring in approximately 10 % of all pregnancies (McIntire et al. 1999). Infants born prematurely account for the majority of all neonatal deaths. Not surprisingly, preterm infants show various signs of organ immaturity and this may make preterm neonates more sensitive to serious feeding-induced gastrointestinal complications (Siggers et al. 2011). The immature gastrointestinal tract is less able to deal with microbiology, immunology, and nutrition-related challenges of postnatal life as a result of deficiencies in intestinal structural integrity, digestive capacity, and intestinal immunity (Neu 2007). Such deficiencies are associated with increased enteric disease susceptibility in preterm versus term neonates. Thus, investigating means of improving these deficiencies will aid in improving the maturation of the preterm gastrointestinal tract, in reducing gut inflammation, and in optimizing nutrition and health in this compromised population.

13.2 Animal Models

However, less detailed information is available from human infants partly due to the difficulties in performing well-controlled studies on this vulnerable population of infants. In addition, it is neither ethical nor practical to conduct these experiments with the human fetus or infant.

Often, the more suitable approach is the use of animal models. While some earlier studies have focused on information derived mainly from rodent models (e.g., rats and mice) (Sodhi et al. 2008), it is difficult to conduct experiments with laboratory rodents because of their small body size and immature organs at birth, and this makes the large farm animals (e.g., pigs, cattle, sheep) more attractive models in this field.

Although no animal model will ever perfectly mimic the human condition, the pig has emerged as a superior non-primate experimental animal model because of similarities in anatomy, development, nutrition, and physiology between the pig and the human (Ball et al. 1996; Clouard et al. 2012). Pigs are also the only widely utilized animal model that is truly omnivorous, and they have strikingly similar nutritional requirements to that of humans (Patterson et al. 2008). The gut in the newborn pig is more mature than in newborn rodents, although less mature than in infants (Sangild 2006). Thus, in pigs, preterm delivery at 90 % gestation is comparable to preterm infants born at approximately 75 % gestation (30 weeks) (Siggers et al. 2011). In contrast to rodent models, the size of the newborn pig easily allows for clinically tissue collections and experimental manipulation of physiologic conditions. Besides, the ontogeny, the physiology of digestion, and associated metabolic processes are very similar between humans and pigs (Patterson et al. 2008; Patrycja and

Barbara 2008), which makes the pig an attractive animal model for further studies on intestinal complications related to preterm birth.

13.3 Determinants in Intestinal Inflammatory Diseases

Preterm infants suffer from numerous devastating intestinal diseases, including necrotizing enterocolitis (NEC). Nutritional, microbial, and immunological dysfunctions may all play a role in disease progression. The lowered digestive and nutrient absorptive function, impaired intestinal epithelial barrier, inappropriate bacterial colonization, and a dysregulated mucosal immune system may add to this increased susceptibility to enteric disease.

13.3.1 Immature Digestion and Nutrient Absorption in Preterm Neonates

Preterm birth is associated with immature motility, digestive capacity, and nutrient absorption, thus leading to nutrient fermentation, bacterial overgrowth, and mucosal inflammation.

Gastrointestinal motility is limited largely to infants less than 34 weeks gestation (Riezzo et al. 2009). The motility is found considerably less organized in premature infants (Neu 2007), probably, because of the intrinsic immaturity of the enteric neurons. Also in preterm piglets, bowel movements are not well developed during the first days of enteral feeding (Sangild et al. 2002a, b, c). The incomplete innervation and poor motility of the immature gut, thereby, may lead to stasis, nutrient fermentation, inappropriate colonization, and further contribute to the development of enteric disease in preterm infants (Neu 2007; Oste et al. 2005).

Studies in both animals and infants indicate that immature brush-border enzyme activities following preterm delivery may result in maldigestion, excessive nutrient fermentation, intestinal distension, and mucosal ischemia in preterm infants. In preterm infants, the intestine is relatively short (Weaver et al. 1991) and may have a reduced absorptive area, consistent with studies in pigs (Sangild et al. 2000, 2002a, b, c). Besides, preterm-delivered pigs also differ from term neonates in their intestinal cell proliferative and apoptotic responses (Burrin et al. 2000; Bittrich et al. 2004). The impaired ability of the immature intestine to increase cell proliferation, decrease apoptosis, and regulate the mesenteric blood flow (Crissinger et al. 1994; Clark et al. 2005; Dyess et al. 1993) may lead to mucosal atrophy, dysfunction, and necrosis in preterm neonates. In addition to ontogenetic immaturity of enterocyte function, the possible hypoxia, hypothermia, altered endocrine and metabolic status may make preterm neonates more sensitive to serious feeding-induced complications (Sangild 2006).

These deficiencies in intestinal structure and function appear to be one of the most critical problems resulting in feeding intolerance, a commonly encountered problem

in neonatal care. Formula-fed preterm neonates are thus at increased risk of developing diseases due to their compromised digestive system. Similar results are observed in preterm newborn pigs. Preterm birth affects the intestinal response to enteral nutrition in newborn piglets. Formula feeding in preterm newborn pigs leads to a diminished intestinal trophic responses relative to colostrum (Bjornvad et al. 2005; Oste et al. 2005; Sangild et al. 2006), marked atrophy of the mucosal surface, and increased permeability (Rouwet et al. 2002). Preterm neonates, thus, may require a period of total parenteral nutrition (TPN) before enteral nutrition is administered (Heird and Gomez 1996; Sangild et al. 2002a, b, c). Nevertheless, parenteral nutrition has significant detrimental side effects, including intestinal atrophy, malfunction, and sepsis leading to increased susceptibility to inflammatory stimuli and the development of intestinal inflammation (Siggers et al. 2011). This is consistent with the observation that TPN increases the risk of NEC in prematurely born piglets.

13.3.2 Deficient Host-Associated Defense Mechanisms in Preterm Neonates

Relative to term neonates, preterm neonates show immature intestinal barrier that lacks several key protective mechanisms that normally prevent invasion by luminal bacteria. The premature gastrointestinal tract also has increased intestinal permeability (Neu 1996) commonly observed in preterm neonates (Neu 2007), making the immature intestine more permeable to macromolecules, while little is known about the maturation of tight junction proteins such as occludin and claudins, which constitute the major paracellular barrier of the epithelium (Nusrat et al. 2000). The mucus layer forms a physical barrier between the underlying epithelium and the lumen of the gastrointestinal tract (Atumal et al. 2001), protecting the epithelium against noxious agents and pathogenic bacteria. However, the production of mucous was reported to be immature in preterm infants (Claud and Walker 2001; Omari and Davidson 2003; Sangild 2006), leading to a diminished intestinal barrier function, impaired mucosal repair, and lowered degradation of bacterial toxins.

Additionally, impaired functioning of immune defenses in preterms (Sangild et al. 2002a, b, c; Baxter 2010), as well as lower levels of immunoglobulins (e.g., IgA, IgM, IgG) (Lin 2004), gut B and T lymphocytes, makes premature neonates particularly susceptible to enteric inflammation and injury during the early postnatal period (Claud and Walker 2001; Kuitunen and Savilahti 1995). Coupled with the increased intestinal mucosal permeability in preterms (Rouwet et al. 2002; van Elburg et al. 2003), this impairment leads to transmural translocation of microbes or their toxic products into the immature intestinal mucosal barrier in neonates. This may, in turn, further compromise intestinal defense mechanisms and eventually culminate in an inflammatory cascade, leading to NEC (Berman and Moss 2011). Furthermore, the premature gastrointestinal tract also has decreased regenerative capabilities, and the imbalance between epithelial cell injury and repair usually leads to a vicious cycle of maldigestion, impaired mucosal protection, immune activation and results in a greater potential for tissue damage (Siggers et al. 2011).

13.3.3 Gut Microflora and Inflammatory Responses in Preterm Neonates

Several studies in both human infants and piglets show that assemblages of gut bacteria differ markedly between preterm and term neonates (Schmidt et al. 2008; Cilieborg et al. 2010). While the full-term infants are rapidly colonized with a more diverse microbiota, preterm neonates have a slow bacterial colonization and decreased bacterial diversity present, which may predispose the premature gut to bacterial overgrowth by pathogenic bacteria (Arboleya et al. 2012; Fanaro et al. 2003; Schwiertz et al. 2003), and this difference may be directly related to the degree of intestinal prematurity, the deficient mechanical defense barriers, as well as the environmental factors (Hallstrom et al. 2004). Wang et al (2011) showed that intestinal microbiota had important functions in host energy metabolism, amino acid nutrition, immunity, and health.

Moreover, the nature of the enteral foods may affect initial bacterial colonization patterns in preterm infants (Caicedo et al. 2005; Claud and Walker 2001) and pigs (Shulman 2002; Wang et al. 2011), and the use of parenteral nutrition further delay colonization (Fanaro et al. 2003; Caicedo et al. 2005) in neonates following preterm delivery. Maldigestion and disturbances in intestinal barrier function may lead to bacterial overgrowth and excessive nutrient fermentation, thereby rendering the mucosa more susceptible to bacterial infections and further initiating uncontrolled inflammatory reactions (Siggers et al. 2011).

A disordered enterocyte signaling to bacterial toxins, via the production of various pro-inflammatory cytokines (e.g., IL-1, IL-6, IL-8, TNF-alpha), is thought to be crucial in the development of NEC in the susceptible preterm infants (Hunter et al. 2008). Those pro-inflammatory mediators may further initiate the inflammatory cascade, thus favoring mucosal barrier disruption and adversely affecting mucosal repair. Toll-like receptors are identified among the immunological components of the early mucosal dysfunction. It has been shown that TLRs (2 and 4) are upregulated in intestinal tissue from preterm pigs with NEC (Sangild 2006). In addition, p38 kinase, cyclooxygenase-2, and NF-kB signaling pathways may all be involved in mucosal inflammation (Grishin et al. 2006; Wang et al. 2010). It remains difficult, however, to further understand why the premature newborn is susceptible to NEC as well as other inflammatory bowel diseases, which may need further study in preterm animal models.

13.4 Nutritional Modulation of Mucosal Defense and Immunology in Preterm Neonates

As previously stated, the immature neonates are prone to bacterial infection and exaggerated immune responses, potentially resulting in irreversible tissue damage. Mother's milk and colostrum contains numerous bioactive factors, including growth factors, immunoglobulins, anti-inflammatory components, as well as amino acids

(Møller et al. 2011; Claud et al. 2003), which help to protect against the development of intestinal diseases. Immunonutrition with specific nutrients may also be effective to modulate the activity of the immune system. Some dietary components, such as amino acids, have already been shown to enhance mucosal barrier function and immunologic responses in animal models and in humans (Stechmiller et al. 2004). Kim et al (2007) reported that provision of amino acids and fatty acids with specific functions may enhance the performance of pregnant and lactating sows by modulating key metabolic pathways by which can enhance conception rates, embryogenesis, blood flow, antioxidant activity, appetite, translation initiation for protein synthesis, immune cell proliferation, and intestinal development. Increasing evidence shows that dietary supplementation of specific amino acids to animals and humans with malnutrition and infectious disease enhances the immune status, thereby reducing morbidity and mortality (Li et al. 2007; Yin et al. 2010).

13.4.1 Arginine

The arginine, which is nutritionally essential for neonates, is involved in a number of biological and physiological processes. Arginine is crucial for the synthesis of protein and molecules (e.g., nitric oxide (NO), creatine, and polyamines) with enormous physiological importance (Flynn et al. 2002; Rhoads et al. 2004; He et al. 2009; Kim et al. 2007; Li et al. 2007; Wu et al. 2009). Nitric oxide is a vasodilator involved in intestinal permeability, mucosal integrity, and barrier function (Upperman et al. 2005; Wu et al. 2007). Moderate levels of NO are important for regulation of mesenteric blood flow and protect the mucosa from injury. Polyamines are involved in the regulation of gene expression, DNA and protein synthesis, apoptosis, as well as cellular division (Flynn et al. 2002). Moreover, arginine stimulates the secretion of growth hormone and insulin in preterm infants (Vlaardingerbroek et al. 2011), thereby playing an important role in regulating nutrients metabolism (Liu et al. 2008; Yao et al. 2008, 2011; Yin and Tan 2010; Tan et al. 2009, 2011).

Intestinal amino acid metabolism differs between preterm and term birth. A significant nutritional problem in preterm infants is a severe deficiency of arginine (hypoargininemia), which occurs in more than 50 % of the preterm infant population (Wu et al. 2004). Arginine deficiency may result in hyperammonemia as well as intestinal, immunological, and neurological dysfunction (Flynn et al. 2002) and it is often associated with an increased incidence of NEC in preterm infants (Becker et al. 2000). Thus, the knowledge of arginine metabolism and physiological effects is beneficial for optimizing neonatal survival and health in this compromised population.

Wu et al. (1999) reported that the amino acid composition of the fetal pig was similar to that of human fetus. It has been reported that endogenous synthesis of arginine is important for maintaining arginine homeostasis in the neonatal pigs (Flynn and Wu 1996), and the underdevelopment of intestinal arginine synthesis

may be primarily responsible for hypoargininemia in preterm neonates (Dekaney et al. 2003). Glutamine and proline are major substrates for intestinal synthesis of citrulline in pigs (Wu et al. 2000a, b, 2009; Wang et al. 2008). However, synthesis of citrulline is low and there is little conversion of citrulline into arginine in enterocytes of preterm neonates owing to the limited expression of the genes for key enzymes (e.g., pyrroline-5-carboxylate synthase, argininosuccinate synthase, and lyase), thereby contributing to hypoargininemia (Wu et al. 2004). Furthermore, the possible increase in whole-body arginine catabolism, as well as absence of perinatal cortisol surge due to premature delivery, may also be responsible for the limited endogenous synthesis of arginine. In preterm piglets, low rates of intestinal arginine synthesis are associated with low plasma arginine concentrations (Urschel et al. 2007). Plasma levels of citrulline, arginine, and glutamine are lower in premature neonates with NEC compared with healthy infants (Wu et al. 2001; Becker et al. 2000); and provision of exogenous arginine prevents hyperammonemia and reduces NEC (Amin et al. 2002). Thus, an enhancement of endogenous arginine synthesis in preterm neonates may be obtained by the promotion of the maturation of intestinal arginine-synthetic enzymes.

Glucocorticoids play a crucial role in advancing the maturation of intestinal arginine synthesis and possibly decrease the incidence of NEC in preterm infants (Bauer et al. 1984). Administration of cortisol is effective to advance the maturation of intestinal arginine synthesis in preterm neonates (Wu et al. 2004). Another promising candidate is glucagon-like peptide-2 (GLP-2), a nutrient-responsive gut hormone, which may exert multiple effects on intestinal mucosa growth in preterm neonates (Estall and Drucker 2005). In neonates, proline is a dietary precursor for arginine and is dependent on intact gut metabolism (Vlaardingerbroek et al. 2011). Preterm infants receiving PN are unable to synthesize sufficient proline de novo (Miller et al. 1995). Future research is needed to define mechanisms for arginine metabolism and develop strategies for arginine deficiency in preterm infants.

Arginine plays an important role in improving intestinal function and regulating nutrient metabolism, but the underlying mechanisms are largely unknown (Liu et al. 2008). He et al. (2009) conducted metabolomic analysis of the response of growing pigs to dietary L-arginine and found that arginine alters the catabolism of fat and amino acids in the whole body, enhances protein synthesis in skeletal muscle, and modulates intestinal microbial metabolism. Tan et al (2011) indicated that Arg differentially regulates expression of fat-metabolic genes and increases mTOR signaling activity in skeletal muscle (Yao et al. 2008) and white adipose tissue, therefore favoring lipogenesis in muscle but lipolysis in adipose tissue.

13.4.2 Glutamine

Glutamine is the preferred fuel for rapidly proliferating cells including enterocytes (Chauhan et al. 2008). It is essential for many metabolic processes, and supplementation with this amino acid has been demonstrated to improve mucosal integrity and intestinal barrier function in critically ill patients. In vitro, glutamine is required for

barrier function (Ewaschuk et al. 2011) and helps recovery from loss of transepithelial resistance and increase of permeability induced by stress in Caco-2 cells (Li et al. 2003). Wang et al (2008) found that early weaning resulted in increased expression of genes related to oxidative stress and immune activation but decreased expression of genes related to macronutrient metabolism and cell proliferation in the gut. Dietary glutamine supplementation increased intestinal expression (120–124 %) of genes that are necessary for cell growth and removal of oxidants, while reducing (34–75 %) expression of genes that promote oxidative stress and immune activation. Functionally, the glutamine treatment enhanced intestinal oxidative-defense capacity, prevented jejunal atrophy, and promoted small intestine growth and body weight gain in weaned piglets. These findings reveal coordinate alterations of gene expression in response to weaning and aid in providing molecular mechanisms for the beneficial effect of dietary glutamine supplementation to improve nutrition status in young mammals.

Glutamine is abundant in mother's milk but present in much lower levels in formula milk (Agostoni et al. 2000). Thus, neonates, prematurely born infants, would benefit from glutamine addition. However, clinical studies of glutamine supplementation remain inconclusive. Parenteral glutamine appears to be well tolerated and safe in preterm neonates and this amino acid reduces the time to achieve full enteral nutrition (Thompson et al. 2003). Provision of glutamine showed some beneficial effects such as inhibition of whole body protein breakdown (Kadrofske et al. 2006) and activation of immune system in preterm infants (Parimi and Kalhan 2007). However, Tubman and Thompson (2001) reported that no additional benefit of the addition of glutamine to preterm infants was observed in their study. More studies are needed to evaluate the efficacy of this amino acid in neonatal nutrition and to understand the mechanism of glutamine dysfunction-related pathology.

13.4.3 Methionine and Cysteine

Methionine, an essential amino acid, is also a source through cysteine production for the synthesis of glutathione (GSH). This latter compound plays a crucial role in reducing intestinal oxidative damage and inflammation (Thomas et al. 2008). The rates of transsulfuration of methionine are high in prematurely born low birth weight infants (Maaike et al. 2007a, b). This may reflect high demands for glutathione (GSH) and methionine in parenteral amino acid mixtures for premature babies. It is reported that general cysteine requirement is less than 18 mg/kg per day and that cysteine is probably not a conditionally essential amino acid in the prematurely born infant (Maaike et al. 2007a, b). Regardless of the adequate GSH and protein synthesis, methionine has been implicated in increased homocysteine concentration (Courtney-Martin et al. 2008; Shoveller et al. 2004) in the neonate. Therefore, a balance between methionine and cysteine should be taken in consideration to provide the adequate total sulfur amino acid (SAA) in neonatal nutrition (Courtney-Martin et al. 2010).

13.4.4 Branched-Chain Amino Acids

The essential branched-chain amino acids (BCAAs), leucine, isoleucine, and valine, are used for incorporation into body protein (Maingay-de Groof et al. 2010; Li et al. 2011), and utilization by intestine is also high. The uptakes of total leucine and valine carbon are relatively large and the oxidation rates of these essential branched-chain amino acids are high in fetuses and neonates (van den Akker et al. 2011). Among the BCAAs, leucine can act as a nutrient signal and stimulates protein synthesis via the activation of translation initiation factors (Vlaardingerbroek et al. 2011). Recent work with young pigs shows that reducing dietary protein intake can improve gut function after weaning but result in inadequate provision of essential amino acids for muscle growth. Yin et al. (2010) reported that supplementing L-leucine to a low-protein diet may maintain the activation of translation initiation factors and adequate protein synthesis in multiple organs of post-weaning pigs. This novel finding provides a molecular basis for designing effective nutritional means to increase the efficiency of nutrient utilization for protein accretion in neonates. Leucine is not only a substrate for protein synthesis of skeletal muscle but also plays as signaling molecules to affect feeding behavior, energy balance, and fuel efficiency (Li et al. 2011). Leucine activates signaling factor of mammalian target of rapamycin (mTOR) to promote protein synthesis in skeletal muscle and in adipose tissue. It is also a major regulator of the mTOR sensitive response of food intake to high protein diet. Meanwhile, leucine regulates blood glucose level by promoting gluconeogenesis and aids in the retention of lean mass in a hypocaloric state. It is beneficial to animal nutrition and clinical application and extrapolation to humans (Li et al. 2011). Studies in newborn pigs suggest that enteral leucine supplementation may have a beneficial effect on neonatal growth as it may enhance protein synthesis in an mTORC1-dependent pathway (Suryawan et al. 2012; Li et al. 2011). The gut has a high demand for leucine and protein synthesis has been found to be limited by deficient leucine intake (Elango et al. 2002). BCAA-enriched parenteral nutrition in preterm neonates might influence functional outcome in the direct postnatal phase. The requirements of the individual BCAAs are almost twice the current recommendations (Maingay-de Groof et al. 2010). To optimize current parenteral and enteral feeding, the optimal BCAA ratio should be determined for both. Further studies are required to better understand the role of BCAAs in the regulation of neonatal growth.

13.4.5 Threonine

Threonine is an indispensable amino acid that must come from dietary sources. It is critical in the production of mucins in the gut (Schaart et al. 2009). Therefore, this amino acid is of presumably vital nutritional importance to maintain the protective mucus layer and thus the intestinal barrier function. Lack of threonine can result in

diarrhea and reduced mucin production, indicating the important role of threonine in the structure and function of the gut (Vlaardingerbroek et al. 2011). However, prolonged dietary excess of threonine fed to neonates, may have negative behavioral consequences and may induce serious metabolic disturbances (Chapman et al. 2009). Dietary threonine imbalance is known to reduce the growth of the small intestine, liver, and skeletal muscle in young animals. Using the pig model, Wang et al. (2007) found that either a deficiency or an excess of dietary threonine impairs protein synthesis in these tissues. This finding provides a mechanism for the low growth performance of animals fed a threonine-imbalanced diet. Currently, neonatal amino acid solutions provide intakes of threonine (111–165 mg/kg per day) are greater than an infant's enteral intake from breast milk (76 mg/kg per day) (WHO 2007). Chapman et al. (2009) concluded that current parenteral solutions should be revised to incorporate the population-safe requirements of threonine to promote optimum metabolic and neurologic growth in neonates.

13.4.6 Tryptophan

Tryptophan is an essential amino acid while the concentration is low in plasma and content low in proteins compared with the other essential amino acids (Vlaardingerbroek et al. 2011). Intestinal inflammation, malnutrition, and pro-inflammatory situation may result in tryptophan depletion (Christmas et al. 2011), thus affecting the weight gain and nitrogen balance in neonates. In young pigs, experimental inflammation was associated to a decrease in plasma tryptophan concentrations compared with healthy piglets (Le Floc'h et al. 2008). Furthermore, studies showed that in neonatal piglets, no difference is found in tryptophan requirements when enteral and parenteral feeding are compared (Alegria et al. 1999). Therefore, most parenteral neonatal amino acid solutions contain similar concentrations of tryptophan compared with human breast milk (Cvitkovic et al. 2004). Lastly, the commercially available preterm formula content ranges from 18 to 36 mg/kg per day when infants receive 160 ml/kg per day of milk (Vlaardingerbroek et al. 2011).

13.4.7 Other Amino Acids

Taurine is considered conditionally essential because needs are not met when intake is low (Verner et al. 2007). It has important roles in intestinal absorption, membrane stability, and visual development in preterm infants. Currently, taurine concentrations in modern parenteral amino acid solutions are more than sufficient to meet recommendations (Verner et al. 2007). Tyrosine is also a conditionally essential amino acid resulting from the insufficient enzymatic activity in preterm infants. Due to poor tyrosine solubility in parenteral nutrition (Roberts et al. 2001), hydroxylation of phenylalanine to tyrosine may be a good way to provide tyrosine when the diet is tyrosine-deficient.

13.5 Conclusion

Deficiencies in intestinal integrity, barrier function, digestive capacities, and intestinal immunity, make preterm neonates more susceptible to inflammatory diseases. Using preterm pigs, recent evidence suggests that nutritional modulation has great potential to improve neonatal intestinal development, manipulate the gut microbiota, in parallel with direct effects on the mucosal immune system, to prevent the onset of NEC. Some amino acids have been shown to enhance mucosal barrier function, immunologic responses, and NEC resistance in piglets and in neonates. However, it always remains a challenge to translate data generated from animal models to corresponding conditions in humans. Understanding the unique function of amino acids could eventually play a pivotal role in improving future nutritional strategies for premature infants. Clearly, further studies involving amino acids as compounds for prevention and clinical therapies against devastating intestinal diseases are needed, both in parenterally and enterally fed infants. Such work will greatly advance our knowledge with regard to the "optimal" amino acid pattern and it will also be beneficial for designing the next generation of amino acid supplemental solutions to optimize survival and health in preterm neonates.

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Chapter 14
Amino Acids and Obesity, Diabetes, and Dyslipidemia in Pigs and Other Mammals

Wu Xin, Li Fengna, and Yulong Yin

Contents

14.1 Introduction

The incidence of diabetes, obesity, and dyslipidemia dramatically increased in all parts of the world, a fact largely attributed to dietary and lifestyle changes that come with economic development (Nguyen et al. 2008). Obesity is closely linked with diabetes and can lead to endocrine, cerebrovascular diseases. Various complications induced by diabetes and obesity are endangering public health and have become one of the most challenging problems facing the world today (Nguyen et al. 2008).

Regional body fat distribution has an important influence on metabolic and cardiovascular risk factors. Increased abdominal (visceral) fat accumulation is a risk factor for type 2 diabetes, dyslipidemia, hypertension, stroke, and coronary artery disease (CAD) (Franssen et al. 2011; Repas 2011).

The primary dyslipidemia related to obesity is characterized by increased triglycerides, decreased HDL levels, and abnormal LDL composition. The recent emphasis on treatment of the dyslipidemia of the metabolic syndrome

W. Xin • L. Fengna • Y. Yin (✉)
Research Center for Healthy Breeding of Livestock and Poultry, Hunan Engineering and Research Center of Animal and Poultry Science and Key Laboratory for Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, The Chinese Academy of Sciences, Changsha, Hunan 410125, People's Republic of China
e-mail: yinyulong@isa.ac.cn

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_14,

(hypertriglyceridemia, reduced high-density lipoprotein, and increased small, dense low-density lipoprotein particle number) has compelled practitioners to consider lipid-lowering therapy in a greater number of their patients, as one in two individuals over age 50 has the metabolic syndrome (He et al. 2012a, b). Individuals with the metabolic syndrome typically have normal low-density lipoprotein cholesterol levels, and current lipid-lowering guidelines may underestimate their cardiovascular risk. Two subgroups of patients with the metabolic syndrome are at particularly high risk for premature CAD. One, individuals with type 2 diabetes, accounts for 20–30 % of early cardiovascular disease. The second, familial combined hyperlipidemia, accounts for an additional 10–20 % of premature CAD. Familial-combined hyperlipidemia is characterized by the metabolic syndrome in addition to a disproportionate elevation of apolipoprotein B levels. The measurement of fasting glucose and apolipoprotein B, in addition to the fasting lipid profile, can help to estimate CAD risk in patients with the metabolic syndrome.

In recent years, amino acids, especially functional amino acids have attracted increased attention among the public and the medical community because of accumulated evidence from a large body of literature suggesting that functional amino acids may benefit human health (Newsholme et al. 2011; Kim et al. 2007a, b, c; Tan et al. 2009, 2011; Wu et al. 2007; Yao et al. 2008; Yin and Tan 2010; Li et al. 2011; Yin et al. 2010). Amino acid metabolism can be split into those 20 amino acids used for protein biosynthesis and play important role related with a wide variety of cells and tissues and relate these changes to alterations in endocrine, physiologic, and immune function (Newsholme et al. 2011). Substantial data from epidemiologic surveys and nutritional intervention studies in humans and animals suggest that dietary amino acids have protective effects against a variety of disorders, including cardiovascular disease, hyperlipidemia, cancer, osteoporosis, and various forms of chronic renal disease (Eizirik et al. 1988; Sulochana et al. 1998).

Amino acid metabolism is complex because of the large number of metabolites involved. Evidence is also emerging that consumption or supplementation of amino acids may have a beneficial effect on diabetes mellitus and obesity in animals and humans. Results showed that plasma spectrum of 20 kinds of amino acid metabolism and blood glucose values have a strong correlation between high and low, of which 7 amino acids (Arg, Cit, Asp, Asn, Thr, Leu, Trp) on the blood glucose level of grouping the greatest contribution. Several studies in humans and animals have shown that amino acids reduce plasma total cholesterol and LDL cholesterol (Hagemeister et al. 1990).

This chapter examines the evidence for a possible role of dietary amino acids in diabetes mellitus and obesity and discusses various mechanisms by which this class of amino acids may affect glucose and lipid metabolism and improve the control of body weight and glucose homeostasis as well as dyslipidemia.

14.2 Amino Acids and Obesity

The last 25 years have seen a great increase of the incidence of obesity in the world. Obesity is increasing, probably as a consequence of easily available, fat-rich food and an increasingly sedentary lifestyle. Obesity has reached epidemic proportions

and is strongly linked to a number of chronic diseases, including diabetes, dyslipidemia, cardiovascular disease, and so on (Hedley et al. 2004).

Branched-chain amino acids (BCAA) cannot be made by the body so they are labeled "essential amino acids" and include leucine, isoleucine, and valine. It has been found that increasing intake of branched-chain amino acids may help in maintaining a healthy weight (Qin et al. 2011). Possible proposed mechanisms of branched-chain amino acids on obesity include the effect of leucine supplementation on the anti-hunger hormone leptin (Lynch et al. 2006), as well as the ability of branched-chain amino acids to participate in the control of blood sugar and in the maintenance of a healthy weight (Mobbs et al. 2005).

Leucine supplementation markedly reduced diet-induced obesity when fed with high-fat feed. Supplementation with leucine leads to a 32 % reduction of weight gain and a 25 % decrease in adiposity in mice fed high-fat diet. Leucine also decreases food intake and BW in normal rats and *ob*/*ob* mice. It is similar to that achieved by high protein diet, also indicating that leucine is a major component of the effects of a high protein diet. Moreover, leucine and high protein diet reduce AMPK but promote mTOR activity in the hypothalamus, resulting in inhibition of neuropeptide Y and stimulation of pro-opiomelanocortin expression. It suggests that a cross-regulation between AMPK and mTOR exists to regulate feeding in a leucine-dependent manner.

Furthermore, the relationship between obesity and amino acid metabolism displays important physiological and clinical implications. Patterson et al. (2002) studied the effect of obesity on regional skeletal muscle and adipose amino acid metabolism using a combination of stable isotope tracer and arterio-venous balance methods (Patterson et al. 2002). The rate of leucine release from forearm and adipose tissues in obese women is lower than in lean subjects, and obesity is associated with a lower fractional contribution from skeletal muscle to systemic leucine rate of appearance. It helps to illustrate the reasons why obese persons are more effective than lean ones in preserving body protein during fasting.

For amino acids, changes in blood concentrations of select essential amino acids and their derivatives, in particular BCAA, sulfur amino acids, tyrosine, and phenylalanine, are apparent with obesity (Adams 2011). Metabolomic profiling of obese versus lean humans reveals a BCAA-related metabolite signature that is suggestive of increased catabolism of BCAA and correlated with insulin resistance. This comprehensive metabolic profiling was performed on74 obese (median BMI of 36.6 kg/m^2) and 67 lean (median BMI of 23.2 kg/m^2) subjects (Table 14.1) (Newgard et al. 2009). The data showed that levels of the BCAAs valine and leucine/isoleucine were 20 % and 14 % higher, respectively, in obese compared to lean subjects ($P < 0.0001$). It is hypothesized that these changes may reflect an overload of BCAA catabolism in obese subjects. In growing pigs, by comparing genetically obese and lean strains, it was found that threonine, tyrosine, and creatine are higher in obese than in lean pigs, while urea is lower in obese pigs suggesting that obese pigs have different nitrogenous metabolism when compared with lean animals (He et al. 2012a, b).

Table 14.1 Amino acids concentration in the serum detected by tandem mass spectrometry

Amino acids (mM)	Obese ($n = 74$)	Lean ($n = 67$)	P value
Valine	281.4 (249.2, 332.9)	235.3 (204.1, 257.0)	<0.0001
Leucine/Isoleucine	170.0 (150.2, 200.8)	149.0 (132.5, 176.6)	<0.0001
Glutamate/Glutamine	118.4 (91.4, 143.7)	81.2 (66.7, 95.2)	<0.0001
Glycine	282.6 (245.6, 319.6)	328.4 (265.6, 403.0)	0.0007
Alanine	433.4 (394.5, 492.3)	367.3 (297.1, 420.0)	<0.0001
Phenylalanine	72.6 (66.3, 78.9)	61.6 (55.1, 68.8)	<0.0001
Tyrosine	79.5 (68.5, 90.0)	67.1 (56.7, 73.5)	<0.0001
Aspartate/Asparagine	20.1 (17.3, 23.8)	16.5 (13.5, 19.7)	<0.0001
Arginine	135.2 (116.5, 148.5)	115.3 (101.6, 137.0)	0.0007
Citrulline	32.0 (27.9, 40.3)	36.3 (30.5, 40.7)	0.04
Histidine	81.6 (73.5,88.9)	81.9 (71.9, 91.6)	0.57
Methionine	27.5 (25.1, 30.7)	27.6 (24.2, 30.9)	0.68
Omithine	69.6 (55.3, 80.8)	64.8 (53.3, 71.8)	0.18
Proline	176.4 (155.4, 231.5)	158.1 (138.4, 201.7)	0.02
Serine	115.6 (100.7, 131.9)	116.7 (102.2, 138.6)	0.69

Note: The obese subjects were comprised of 70 % women and 41 % African Americans, whereas the lean subjects were 57 % women and 45 % African Americans (Newgard et al. 2009)

Tryptophan and phenylalanine may help in the fight against obesity by burning fat more efficiently and suppressing appetite. Many researchers suggest that certain supplements, amino acids in particular, may be effective in weight loss programs. Tryptophan supplements tend to diminish the desire for carbohydrates. And since most overweight people seem to favor sweet, processed carbohydrate foods, it may be helpful in weight loss. Phenylalanine suppresses the appetite by increasing the production of the neurotransmitter norepinephrine.

In another hand, the prevalence of pre-pregnancy obesity has increased and is estimated to be almost 30 % among reproductive aged women (Artal et al. 2010; Kim et al. 2007a, b, c; Ogden et al. 2006).

Maternal obesity is associated with increased placental amino acid transport and hyperleptinemia. The study of placental amino acid transport and the effect of leptin on placental amino acid transport in vitro in the setting of maternal obesity was conducted. The obese group had decreased placental sodium-dependent neutral amino acid transporter (SNAT) activity (Fig. 14.1), maternal hyperleptinemia, and decreased syncytiotrophoblast expression of leptin receptor and SNAT-4. Placental amino acid uptake was significantly stimulated by leptin in the lean group as compared to the obese group. Maternal weight gain and offspring birth weights were not different between groups. Maternal obesity was accompanied by decreased placental SNAT activity associated with maternal hyperleptinemia and placental leptin resistance in spite of appropriate maternal weight gain and normally grown neonates (Farley et al. 2010). These findings suggest altered placental function that may have clinical implications in obese pregnant women.

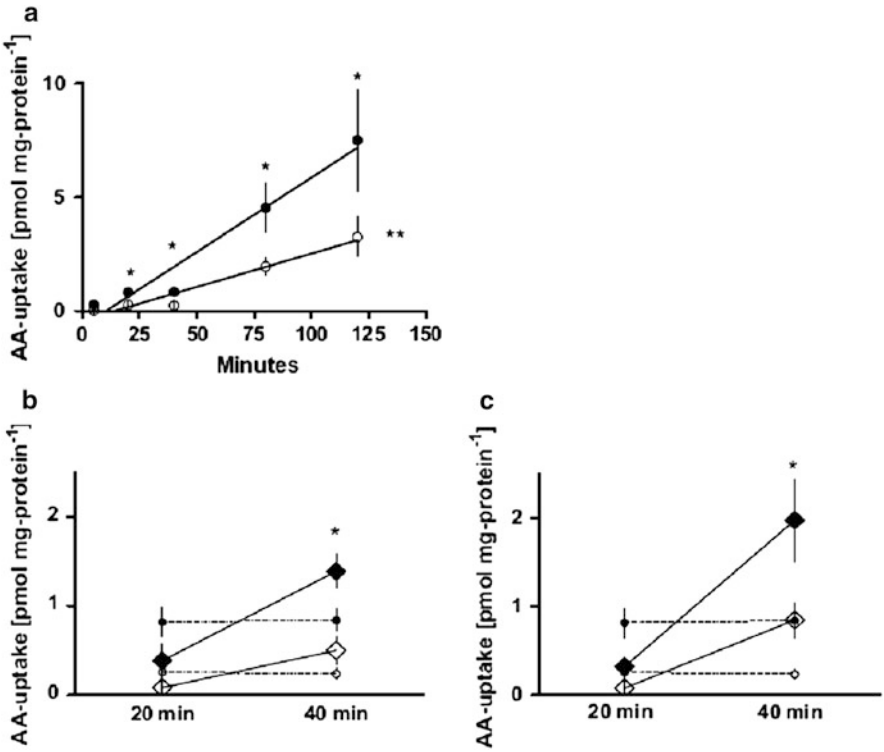

Fig. 14.1 (**a**) Placental SNAT activity (pmol mg/protein/min), term placental villous fragments. Lean (**c**, $n = 1{,}017$), obese (**b**, $n = 7$). *$P < 0.05$ (20, 40, 80, 120 min time points e lean vs. obese), **$P < 0.005$ (SNAT activity lean vs. obese), amino acid (AA). (**b**, **c**) Effect of leptin stimulation on placental SNAT activity (pmol mg/protein/min), at concentrations of 100 ng/mL (**b**, lean vs. obese $P < 0.07$), 500 ng/mL (**c**, lean vs. obese $P < 0.10$) in term placental villous fragments (Farley et al. 2010)

14.3 Amino Acids and Diabetes

Diabetes is a typical metabolic disease, with a variety of complications, an important factor in the quality of life of patients, early diabetes. Thus ultra-early detection and prevention are particularly important.

Amino acids are the building blocks of proteins and are also used in the synthesis of DNA, glucose, and ATP. Amino acids as important metabolic network metabolites are associated with diabetes mellitus (Chen et al. 2011; Newsholme et al. 2011). Amino acids may play a direct or indirect (via generation of putative messengers of mitochondrial origin) role in insulin secretion (Newsholme et al. 2007). Amino acids have antidiabetic effects (Sulochana et al. 1998). Specific amino acids are known to acutely and chronically regulate insulin secretion from pancreatic cells in vivo and in vitro (Smith et al. 1997). Mitochondrial metabolism is crucial for the coupling of amino acid and glucose recognition to exocytosis of insulin

granules. Mitochondria generate ATP, which is the main coupling messenger in insulin secretion, and other coupling factors, which serve as sensors for the control of the exocytotic process.

Numerous studies have sought to identify the factors that mediate the key amplifying pathway over the Ca^{2+} signal in nutrient-stimulated insulin secretion. Predominantly, these factors are nucleotides (ATP, GTP, cAMP, and NADPH), although metabolites have also been proposed, such as long-chain acyl-CoA derivatives and glutamate (Newsholme et al. 2005). This scenario further highlights the importance of the key enzymes or transporters, e.g., glutamate dehydrogenase, the aspartate and alanine aminotransferases, and the malate–aspartate shuttle in the control of insulin secretion. In addition, after chronic exposure, amino acids may influence gene expression in the pancreatic beta cells, which subsequently alters levels of insulin secretion. Only a relatively small number of amino acids promote or synergistically enhance insulin release from pancreatic beta cells (Fajans et al. 1967). Four amino acids were found to be particularly important for stimulating beta cell electrical activity, essential for insulin secretion (leucine, isoleucine, alanine, and arginine) (Bolea et al. 1997).

The mechanisms by which amino acids enhance insulin secretion are varied. The cationically charged amino acid, L-arginine, does so by accumulating in beta cells provoking direct depolarization of the plasma membrane at neutral pH but only in the presence of glucose (Blachier et al. 1989), whereas other amino acids, which are co-transported with Na^+, can also depolarize the cell membrane as a consequence of Na^+ transport and thus induce insulin secretion by activating voltage-dependent calcium channels. Metabolism, resulting in partial oxidation, e.g., L-alanine (Brennan et al. 2002), may initially increase the cellular content of ATP, leading to closure of the ATP-sensitive K^+ (KATP) channel, depolarization of the plasma membrane, activation of the voltage-activated Ca^{2+} channel, Ca^{2+} influx, and insulin exocytosis. Additional mitochondrial signals may be generated that affect insulin secretion (Maechler 2002). A summary of potential regulatory mechanisms with respect to amino acid-stimulated insulin secretion is illustrated in Fig. 14.2.

In vitro studies have shown that amino acids generate signals transduced by the serine/threonine kinase mammalian target of rapamycin (mTOR) to regulate protein synthesis and cell proliferation as mediated by S6 kinase 1 (S6K1) and eukaryotic initiation factor 4EeC binding protein 1 (4E-BP1) (Reimann et al. 2004). Thus, mTOR appears to sense the availability of nutrients, most notably amino acids. It is interesting that activation of the mTOR pathway by prolonged insulin stimulation or increased amino acid availability induces insulin resistance in muscle cells and adipocytes in vitro (10eC12). Activation of mTOR and/or its downstream target, S6K1, leads to serine/threonine phosphorylation and consequently proteosomal degradation of insulin receptor substrate (IRS)-1 (10eC12). This in turn results in inhibition of phosphoinositide (PI) 3-kinase activity (Fajans et al. 1967), an essential step in insulin-mediated glucose metabolism. We further demonstrated that amino acid-induced mTOR/S6K1 activation speeds up insulin-dependent PI3-kinase deactivation in cultured myocytes even before detectable IRS-1 degradation, suggesting that uncoupling of IRS-1/PI3-kinase signaling is

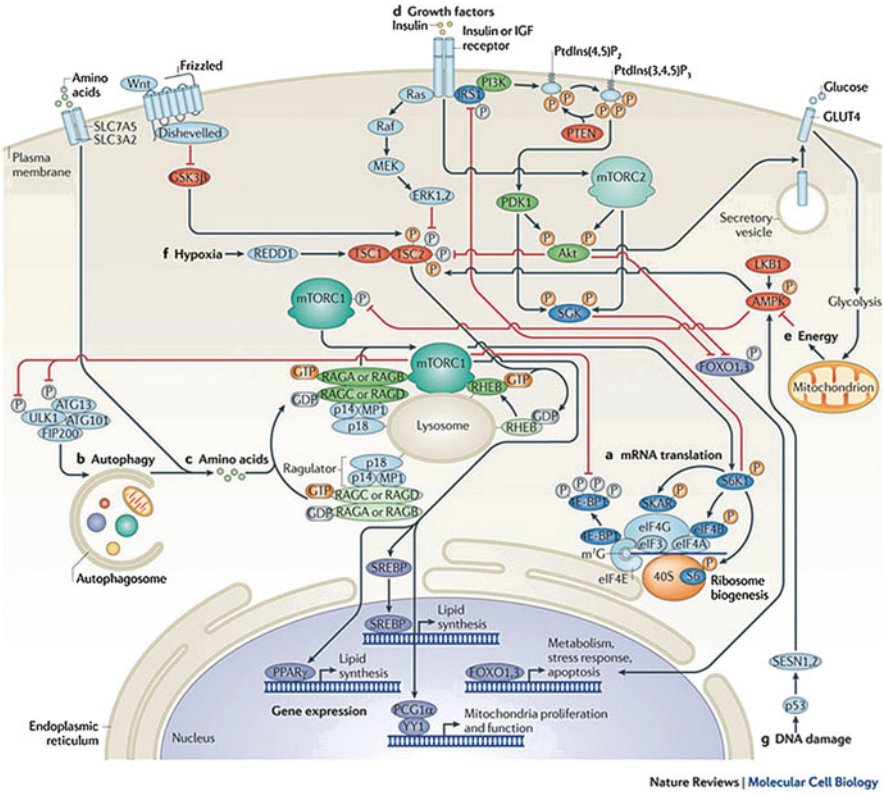

Fig. 14.2 ThemTORsignalling pathway. Mammalian target of rapamycin complex 1 (mTORC1) promotes mRNA translation (**a**) and inhibits autophagy (**b**) by integrating nutrient signals that are generated by amino acids (**c**), growth factors such as insulin and insulin-like growth factors (IGFs) (**d**), energy signals that act through AMP-activated kinase (AMPK) (**e**), and various stressors including hypoxia (**f**) and DNA damage (**g**). Signal integration occurs at the level of the tuberous sclerosis 1 (TSC1; also known as hamartin)-TSC2 (also known as tuberin) complex. Akt and extracellular regulated kinase 1 (ERK1) and ERK2 phosphorylate TSC2, thus inhibiting the GTPase activating protein (GAP) activity of TSC1–TSC2 towards Ras homologue enriched in brain (RHEB). By contrast, phosphorylation of TSC2 by AMPK and glycogen synthase kinase 3β (GSK3β) results in the activation of TSC1–TSC2. The hypoxic factor protein regulated in development and DNA damage response 1 (REDD1; also known as DDIT4) promotes the assembly and activation of TSC1–TSC2. A second level of integration occurs at the lysosome: the Rag GTPases (which are held in place by the Ragulator, which consists of p18, p14, and MAPK scaffold protein 1 (MP1)) recruit mTORC1 to the lysosomal surface in response to amino acids (**c**); in turn, lysosomal recruitment enables mTORC1 to interact with GTP-bound RHEB, the end point of growth factor (**d**), energy (**e**), and stress (**f**, **g**) inputs. Growth factor receptors activate mTORC2 near the plasma membrane (**d**), where mTORC2 may be recruited through binding of mammalian stress-activated map kinase-interacting protein 1 (mSIN1; also known as MAPKAP1) to phospholipids. Because of its role in phosphorylating and activating Akt, mTORC2 forms a core component of the phosphoinositide 3-kinase (PI3K) pathway. Activating and inhibitory phosphates are *orange* and *gray*, respectively. *4E-BP1* eIF4E-binding protein 1, *ATG* autophagy-related, *CBP80* 80 kDa nuclear cap-binding protein, *eEF2K* eukaryotic elongation factor 2 kinase, *eIF* eukaryotic translation initiation factor, *FIP200* 200 kDa FAK family kinase-interacting protein, *FOXO* forkhead

an early event in the interaction between amino acids and insulin action (Fajans et al. 1967).

Supplemental dietary Arg can decrease AGE–RAGE interactions and consequently reduce tissue damage in rats with type 2 diabetes (Pai et al. 2010).

14.4 Amino Acids and Dyslipidemia

Dyslipidemia is characterized by an abnormal amount of lipids (e.g., cholesterol and/or triglycerides) in the blood, which represents risk factors associated with atherosclerosis and cardiovascular disease and remains the most common cause of death.

Studies have shown that severity of hypocholesterolemia in sepsis is quantifiably related to changes in plasma amino acids (Chiarla et al. 2004). It has been reported that cholesterol can regulate AA metabolism and availability in various tissues of piglets. In brain, cholesterol supplementation reduced ($P < 0.05$) concentrations of glutamate, serine, glutamine, threonine, beta-alanine, alanine, methionine, isoleucine, leucine, and gamma-aminobutyrate but increased ($P < 0.05$) concentrations of glycine and lysine (Li et al. 2009).

Also, some amino acids also affect cholesterol metabolism (Blachier et al. 2010). Excessive cystine and histidine increased serum cholesterol and alpha-tocopherol (Katayama et al. 1990). Met (used at a 1 % level) enhanced and Cys reduced the plasma cholesterol level (Sugiyama et al. 1986). Liver cholesterol was also markedly decreased by the addition of Gly to casein, but not methionine nor cystine (Tanaka and Sugano 1989). Adding methionine or cystine to a 10 % soybean protein isolate diet increases cholesterol 7alpha-hydroxylase activity (Taniguchi et al. 2008).

14.5 Summary and Perspectives

As the fundamental building blocks of protein and as precursors of numerous metabolites, amino acids metabolism play important roles related with a wide variety of cell and tissue functions and relate these changes to alterations in endocrine, physiologic, and immune functions. Further research works are needed

Fig. 14.2 (continued) box protein O, *IRS1*, insulin receptor substrate 1, *MEK* MAPK/ERK kinase, *PDK1* 3-phosphoinositide-dependent protein kinase 1, *PGC1α* PPARγcoactivator 1α, *PKC* protein kinase C, *PPARγ* peroxisome proliferator-activated receptor-γ, *PTEN* phosphatase and tensin homologue, *S6K1* S6 kinase 1, *SESN* sestrin, *SGK* serum- and glucocorticoid-regulated kinase, *SREBP* sterol regulatory element-binding protein, *SKAR* S6K1 Aly/REF-like target (also known as POLDIP3)

which would aim at deciphering effects of amino acids in the variety of disorders, including cardiovascular diseases, hyperlipidemia, osteoporosis, and various forms of chronic renal disease.

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Chapter 15
Methods of Amino Acid Analysis

Xia Xiong, Yulong Yin, Yiqiang Huang, Yongfei Wang, Qingqi Wen, Yuyun Mu, Xugang Shu, Zhichun Zhan, Ying Zhou, and Guixiong Qiu

Contents

X. Xiong • Y. Yin (✉)
Research Center for Healthy Breeding of Livestock and Poultry, Hunan Engineering and Research Center of Animal and Poultry Science, Key Laboratory of Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Scientific Observing and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Chinese Academy of Sciences, Changsha, Hunan 410125, People's Republic of China
e-mail: yinyulong@isa.ac.cn

Y. Huang
Xingjia Bio-engineering Co., Ltd, Changsha, Hunan 410011, China

Y. Wang
Research and Development Center, Twins Group Co., Ltd, Nanchang, Jiangxi 330096, China

Q. Wen
Research and Development Center, Twins Group Co., Ltd, Nanchang, Jiangxi 330096, China

Fujian Aonong Biotechnology Corporation, Xiamen, Fujian 361007, China

Y. Mu
Shangshai Xinnong Feed Co. Ltd, Shanghai 201613, China

X. Shu • G. Qiu
Guang Zhou Tanke International Ltd, GuangZhou, Guangdong 510627, China

Z. Zhan • Y. Zhou
Wuhan Sunhy Biology Co., Ltd, Wuhan 430070, Hubei

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_15,

Abbreviations

AA	Amino acid
AAA	Amino acid analysis
HPLC	High-performance liquid chromatography
NMR	Nuclear magnetic resonance
GC-MS	Gas chromatography-mass spectrometry
GE-MS	Capillary electrophoresis mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
CZE	Capillary zone electrophoresis
AQC	6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate
OPA	*o*-Phthalaldehyde reagent
PITC	Phenyl isothiocyanate
FMOC-Cl	9-Fluorenylmethyloxycarbonyl chloride
Dansyl-Cl	Dansyl chloride

15.1 Introduction

There is growing recognition that besides amino acids (AA) as building blocks of proteins and polypeptides, AA act also as cell signaling molecules and regulators of gene expression (Wu 2009; Yin and Tan 2010; Zeng et al. 2012; Kim et al. 2007). Additionally, they are also a main source of energy and serve as precursors for the biosynthesis of numerous compounds including neurotransmitters, porphyrins, polyamines, nitric oxide, etc. (Kaspar et al. 2009a, b; Zhang et al. 2012). AA are important compounds for metabolic profiling, and their quantitative analysis is important for some disease diagnostics as well as for elucidating nutritional influences on physiology. Amino acid analysis (AAA) is used to determine the AA content of amino acid-, peptide-, and protein-containing samples.

Since Moore and Stein (1948) released AA from proteins using 6 M HCl acid at 110 °C for 22 h and separated the AA by ion-exchange chromatography with postcolumn derivatization in the early 1950s, different methods for analysis of amino acids have been developed, including amino acid analyzer, gas chromatography, reversed-phase (RP) high-performance liquid chromatography (HPLC) with precolumn derivatization, capillary electrophoresis, and so on. For several technical and practical advantages, amino acid separations have been carried out mainly by ion-exchange chromatography with postcolumn derivatization and RP-HPLC with precolumn derivatization. Many reviews concerning the analytical methods of AA have been published (Kaspar et al. 2009a, b; Davidson and O'Connor 2008; Rutherfurd and Gilani 2009). The analytical methods of choice for the analysis of AA include gas chromatography-mass spectrometry (GC-MS) , nuclear magnetic resonance (NMR) spectroscopy, capillary electrophoresis mass spectrometry (CE-MS), and liquid chromatography-tandem mass spectrometry(LC-MS/MS). The goal of the present chapter is to briefly compare the established methods and to present new approaches to amino acid analysis.

Quantitative analysis of amino acids is required in several fields, including clinical diagnostics of inborn errors of AA metabolism, biomedical research, bioengineering, and food science (Kaspar et al. 2009a, b). The amino acid analysis in animal nutrition is mainly used not only to determine the composition of AA in feed ingredients for animal diets but also for AA determination in physiological fluids. The AA analysis includes sample preparation and analysis.

15.2 Sample Preparation

15.2.1 Hydrolysis of Proteins and Peptides

Digestion of protein to amino acids requires hydrolysis. Accurate results from AAA require purified protein and peptide samples. Buffer components (e.g., detergents, urea, salts) can interfere with the amino acid analysis and must be removed from the sample before analysis. There are many useful methods including acidic hydrolysis, basic hydrolysis, enzymatic hydrolysis, microwave radiation-induced hydrolysis, and so on. Comparative studies have shown that errors in the performance of the hydrolysis are primarily responsible for variations in the determined compositions (Yuksel et al. 1995; Yin et al. 1991).

15.2.1.1 Acid Hydrolysis

Acid hydrolysis is the most common method for hydrolyzing protein in a biological sample before amino acid analysis. Protein in samples can be hydrolyzed in 6 M HCl at 110 °C for 24 h under N_2 (Wu 1993, 1997; Yin et al. 1994; Fan et al. 1996; Fan and Sauer 2002; Li et al. 2011). However only aspartic acid, glutamic acid, proline, glycine, alanine, leucine, phenylalanine, histidine, arginine, and hydroxyproline can be determined quantitatively during acid hydrolysis (Rutherfurd and Gilani 2009). Under the condition of acidic hydrolysis, asparagine and glutamine are hydrolyzed quantitatively to aspartic acid and glutamic acid. Darragh et al applied a series of hydrolysis times to study the amino acid losses during acid hydrolysis. Most of the residues underwent some degree of loss and the greatest loss was observed with cysteic acid and serine (Darragh et al. 1996). Tryptophan is also largely destroyed, and cysteine cannot be directly determined from the acid-hydrolyzed samples. Alkaline hydrolysis is most commonly used to quantitatively hydrolyze tryptophan (Wu et al. 1999; Li et al. 2011). Methionine and cysteine contents can be determined as methionine, sulfone, and cysteic acid after oxidation with performic acid. The oxidized samples can be then hydrolyzed and analyzed in the same manner as the samples which were not oxidized (Fan et al. 1996; Fan and Sauer 2002). Losses of threonine and serine also occur, and represents usually around 5 and 10 %, respectively (Ozols 1990). To reduce AA losses, various reagents, such as phenol, mercaptoethanol, thioglycolic acid, and indole (Ravindran and Bryden 2005) can be added to the HCl to stabilize the side chains of specific amino acids.

15.2.1.2 Alkaline Hydrolysis

For alkaline hydrolysis, the samples are hydrolyzed at 110 °C for 20 h in 4.2 M NaOH plus 25 % thiodiglycol. The disadvantage of basic hydrolysis is that the serine, threonine, arginine, and cysteine are destroyed. Alkaline hydrolysis is applied if the protein sample contains a large percentage of carbohydrates and is also used to analysis the phospho-amino acid (Ekman and Jager 1993; Fountoulakis and Lahm 1998). In the recent years, only few articles on alkaline hydrolysis of proteins for amino acid analysis were published.

15.2.1.3 Enzymatic Hydrolysis

Enzymatic hydrolysis has the advantage that it allows the quantification of asparagine and glutamine and of other sensitive residues, which are otherwise destroyed during chemical digestion of residues carrying side-chain modifications and this kind of hydrolysis does not induce racemization during digestion. Enzymatic hydrolysis of proteins for amino acid analysis is seldom applied.

15.2.1.4 Microwave Hydrolysis

Acid hydrolysis of protein samples has traditionally been the rate limiting step in amino acid analysis. Several reports over the past 20 years have advocated the use of microwave irradiation as a tool to speed up the hydrolysis of proteins and peptides under acidic conditions (Weiss et al. 1998; Zhong et al. 2005; Damm et al. 2011).

Some samples contain complex mixture of compounds, including protein, carbohydrate, fat, vitamins, and minerals. If the feeds contain >10 % fat, it may be necessary to defat the sample prior to hydrolysis, particularly for tryptophan analysis (Rutherfurd and Gilani 2009). For example, the milk needs to be defatted by centrifugation in studies of free and protein-bound amino acids in sow's colostrum and milk (Wu and Knabe 1994). Then analysis of free amino acids in biological fluids and tissue often requires deproteinization prior to analysis by compounds such as sulfosalicylic acid, trichloroacetic acid, or $HCLO_4$, followed by ultrafiltration.

15.2.2 Derivatization of Free Amino Acids

Since amino acids are nonvolatile compounds and most of them have little UV absorbance, amino acids have been commonly analyzed by ion-exchange chromatography with postcolumn derivatization or high-performance liquid chromatography (HPLC) methods with precolumn derivatization using UV chromophore or fluorophore reagents (Yin et al. 1993a, b).

15.3 Chromatographic Separation and Detection

Many techniques for amino acid analysis exist, and the choice of one given technique often depends on the sensitivity required for the assay.

15.3.1 Ion-Exchange Chromatography with Postcolumn Derivatization

For over 30 years, most AA analysis has been performed by ion-exchange chromatography with postcolumn derivatization with ninhydrin or *o*-phthalaldehyde. Ion-exchange chromatography with postcolumn ninhydrin detection is one of the most common methods employed for quantitative amino acid analysis (Yin et al. 2010, 2011). As a rule, a Li-base cation-exchange system is employed for the analysis of the more complex samples, and the faster Na-based cation-exchange system is used for the more simple amino acid mixtures obtained with protein hydrolysates (typically containing 17 amino acid components). Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength.

The reaction of ninhydrin with primary amino groups to form the purple dye called Ruhemann's purple (RP) was discovered by Siegfried Ruhemann in 1910. Ninhydrin reacts with primary amines at elevated temperatures (100–120 °C) and approximately pH 5. The absorption peak is found at 570 nm, and the absorption intensity is proportional to the concentration of amino acids. In addition, ninhydrin also reacts with secondary amines in compounds such as proline, arginine, asparagine, tryptophan, and cysteine. It is worth noting that ninhydrin reagent is unstable, limiting the use of the ninhydrin/acetate buffer mixture to ~2 weeks. The classic method has been considerably improved with the availability of modern dedicated AA analyzers (Osowska et al. 2004; Miyaji et al. 2010; Boutry et al. 2011, 2012). Despite considerable improvements (Cynober et al. 1985), the run time is still lengthy and when ninhydrin-based detection is used, the method is poorly sensitive for AA present at low concentrations, such as aspartic acid, citrulline, methionine, and tryptophan (Cynober et al. 1987). Detection limit is considered to be 10 pmol for most of the amino acid derivatives, but 50 pmol for proline.

In addition, OPA is also often used as precolumn derivatization reagent. OPA-amino acid derivative is a fluorogenic compound. OPA is 5–10 times more sensitive than either fluorescamine or ninhydrin (Bensen and Hare 1975). However, OPA and its analogs fail to react with secondary amino acids, and the derivatives are often unstable. Postcolumn derivatization reaction occurs after the separation of the amino acids, avoiding the interference with other substances. This technique is suitable for the analysis of amino acids in complex samples and can be used with sample that contain small amounts of buffer components, such as salts and urea, and generally require between 5 and 10 μg of protein sample

per analysis. This method is often cited for its accuracy and reproducibility, but RP-HPLC methods with precolumn derivatization are often used because they are faster, more sensitive, and less costly for the analysis of amino acids.

15.3.2 Reversed-Phase Liquid Chromatography with Precolumn Derivatization

In comparison with ion-exchange chromatographic RP-HPLC with precolumn derivatization has the advantages of reducing analysis times, enhancing sensitivity and flexibility, and lowering cost of instrumentation and maintenance (Ziegler et al. 1992; Jacques et al. 1997). However the precolumn derivatization methods have some disadvantages. This includes derivative instability, derivatization by-product interference with chromatographic separation, and lack of reaction with secondary amino acids. Also, precolumn derivatization techniques may be influenced by buffer salts in the samples. RP chromatography methods were also applied to the separation of amino acid enantiomers (Waldhier et al. 2009). Factors such as mobile phase composition, flow rate, temperature, pH, gradient program, and column type and length affect the performance of RP-HPLC with different degrees of sensitivities. To optimize precolumn derivatization with phenylisothiocyanate, the methodological design has been used to optimize amino acid separation by RP-HPLC (Gheshlaghi et al. 2008).

Different reagents have been used for precolumn derivatization reagents such as 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) (Bosch et al. 2006; Cohen 1994), *o*-phthalaldehyde reagent (OPA) (Wu and Knabe 1994; Molnár-Perl 2001, 2003), phenyl isothiocyanate (PITC) (Battaqlia et al. 1999; Komarova et al. 2004), 9-fluorenylmethyloxycarbonyl chloride (FMOC) (Jambor and Molnar-Perl 2009) and Dansyl chloride (Dansyl-Cl) (Marquez et al. 1986; Furst et al. 1990). The standard to select derivatization reagents is to consider that derived products have different degrees of stability and do not produce interferences. Moreover, the reagent itself should not be fluorescent and consequently should not produce interfering peaks (Liu 2000). Depending on the number of compounds to be measured, chromatographic run time ranges for instance from 13 min for 23 compounds to 95 min for 38 compounds (Kaspar et al. 2009a, b). Some derivatization reagents are summarized in Table 15.1. OPA and FMOC-Cl are the most frequently applied derivatization reagents in the analysis of AA because of their selectivity and sensitivity (Molnar-Perl 2011). OPA method for routine analyses of primary free amino acids (except cystine) in biological fluids. Combination of the OPA and FMOC-Cl techniques are used to determined the secondary amino acids. The use of the dansyl-Cl technique is suggested when the determination of free cystine or cystine-containing short-chain peptides is required (Furst et al. 1990).

Table 15.1 Comparison of precolumn derivatization reagents for analysis of amino acids

Derivative reagent	Detection	Sensitivity	Reproducibility	Stable derivative	Removal of redundant reagent by drying	Removal of redundant reagent by solvent extraction	Interfering side-products	Secondary amino acids detected
OPA	Fluor	fmol	Good	No	No	No	No	No
PITC	UV	pmol	Good	Yes	Yes	No	No	Yes
FMOC-Cl	Fluor	fmol	Good	Yes	No	Yes	Yes	Yes
Dansyl-CL	UV/Fluor	pmol	Good	Yes	No	No	Yes	Yes
FDNB	UV	pmol	Good	Yes	Yes	No	Yes	Yes
FDNPAA	UV	pmol	Good	Yes	Yes	No	Yes	Yes
FDNDEA	UV	pmol	Good	Yes	Yes	No	Yes	Yes
DABS-Cl	UV/Fluor	pmol	Good	Yes				Yes
AQC	UV/Fluor	fmol	Good	Yes	No	Yes	No	Yes
PITC	UV	pmol	Good	Yes	Yes	No	No	Yes

15.3.3 Capillary Electrophoresis

Capillary electrophoresis (CE) has been developed in the 1980s as a high-performance liquid technology. It is based on the use of an electric field to separate charged compounds such as amino acids. It has proven to be a powerful technique for the rapid and highly efficient separation of minute amounts of protein and amino acid sample (Issaq 2001). The analysis of AA by CE has been mainly performed with precolumn, postcolumn, and on-column derivatization techniques with UV chromophore or fluorophore reagents to provide better resolution and sensitivity. Komarova et al. (2004) used pre-capillary derivatization of amino acids with PITC and separation of PTC-derivatives by capillary zone electrophoresis (CZE) and detection at 254 nm. This method allows to widen a list of detectable components up to 19 (without tryptophan) and significantly improve detection limits. Thorough discussion of this method for AAA has been published (Poinsot et al. 2012). Derivatization of amino acids prior to chromatography with chiral reagents has gained in popularity, because the diastereomers formed can be resolved on conventional RP-HPL. Compared with conventional electrophoresis and HPLC, this method has the advantages of high sensitivity and high degree of automation. CE does not require derivatization, but sensitivity of CE-UV analysis can be increased by introduction of a UV-active label. Most of derivatization reagents used for HPLC method can be used for CE method.

The literature of amino acid analysis using CE has been extensively reviewed (Smith 1997, 1999; Poinsot et al. 2006). The new developments of CE, especially those concerning the novelties in detection methods and chirality and the different biological applications have been recently described (Poinsot et al. 2012).

15.3.4 Mass Spectrometry

Ion-exchange chromatography is limited by long run time and by the fact that co-eluting compounds may prevent accurate quantification in some cases. Some amino acids commonly have overlapping retention times by ion-exchange chromatography with postcolumn ninhydrin detection. For example, this is the case for methionine, phenylalanine, and histidine. Mass spectrometry methods, for instance GC-MS (Revelsky et al. 2003; Fiamegos and Stalikas 2006), LC-MS (Molnár-Perl 2003), and GE-MS have the potential to avoid these limitations.

15.3.4.1 GC-MS

Derivatization of amino acids needs to be performed before analysis to produce volatile compounds which are analyzed by gas chromatographic method. The main derivatization methods include silylation, alkylation (Marquez et al. 1994), and acylation. The silylation is the main derivatization method. Silylation is carried out using BSTFA and MTBSTFA under anhydrous conditions and heating or

esterification/acylation in two successive steps (Fiamegos and Stalikas 2006). GC is easily combined with mass spectrometry and a vast number of methods for the preparation of volatile derivatives suitable for GC and GE-MS analysis of amino acids have been reported. Kataoka et al have summarized these approaches (Kataoka et al. 2000). GC/MS methods are known to have good precision and accuracy. Duncan et al have employed isotope dilution gas chromatography/electron capture negative ionization/mass spectrometry (GC/ECNI/MS) to provide accurate and reliable data on <100 fmol of material (Duncan and Poljak 1998; Fan et al. 2006).

15.3.4.2 LC-MS/MS

LC-MS/MS is comparable to traditional LC-ninhydrin detection method. Mass spectral detection shortens analysis times and reduces interference in analysis involved in the detection of inborn metabolic errors (Dietzen et al. 2008). Both GC-MS and iTRAQ-LC-MS/MS are suited for high-throughput amino acid analysis, with the former offering at present higher reproducibility in a completely automated sample pretreatment method, while the latter covers more amino acids and related amines (Kaspar et al. 2009a, b).

15.3.4.3 CE-MS

CE-MS is based on CE as a front-end fractionation coupled to a mass spectrometer. This method provides fast separation and high resolution and is compatible with most buffers and compounds to be analyzed. The method for the determination of underivatized amino acids based on capillary electrophoresis coupled to electrospray ionization mass spectrometry (CE-ESI-MS) has been described (Soga 2000; Soga et al. 2004). GE-MS is a valid alternative to GC-MS for targeted profiling of metabolites, such as amino acids, and possesses some significant advantages over GC-MS (Williams et al. 2007). A review related to the use of CE-MS in the field of metabolomics has been published (Ramautar et al. 2009).

15.3.4.4 NMR-Spectroscopy

Metabonomics provides a useful systems approach to understand global changes in metabolites in animals in response to alterations in genetics, nutrition, environments, and gut microbiota. He et al. have, for instance, compared the metabonome of serum between the genetically obese and lean pigs using a NMR-based metabolomic method (He et al. 2009, 2012) and compared serum and jejunum metabonome in the situation of intrauterine growth restriction of piglets (He et al. 2011a, b, c)

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Chapter 16
Surgical Techniques Used in Amino Acid Nutrition Research

Yonggang Zhang, Yulong Yin, Bo Deng, Ruilin Huang, Tiejun Li, Xugang Shu, Guixiong Qiu, and Martin Nyachoti

Contents

Y. Zhang • Y. Yin (✉) • R. Huang • T. Li
Chinese Academy of Sciences, Institute of Subtropical Agriculture, Research Center for Healthy Breeding of Livestock and Poultry, Hunan Engineering and Research Center of Animal and Poultry Science, Key Laboratory for Agri-ecological Processes in Subtropical Region, Scientific Observing and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Furong Road #644, Changsha 410125, Hunan, People's Republic of China
e-mail: yinyulong@isa.ac.cn

B. Deng
Institute of Animal Husbandry and Veterinary Science, Zhejiang Academy of Agricultural Sciences, Hang Zhou, Zhe Jiang Province 310021, Peoples Republic of China

X. Shu • G. Qiu
Guangzhou Tanke Bio-tech Co., LTD, Room 2801, East Tower of Huihao Building, No. 519 Machang Road, Pearl River New City, Guangzhou, People's Republic of China

M. Nyachoti
Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada R3T 2N2

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_16,

16.1 Introduction

The measurement of the ileal digestibility of amino acids is a convenient method for measuring bioavailability of amino acids in feedstuffs for pigs (Yin 2008). Because of the presence and metabolic activity of microbes, as well as the absence of amino acid absorption by the large intestine (except in the neonatal period), amino acid digestibility determined at the distal ileum is more accurate than fecal digestibility (Zebrowska 1973; Yin and McCracken 1996). That is why many scientists developed numerous surgical techniques to collect ileal digesta, including the slaughter and cannulation techniques. These latter cannulation techniques include:

1. Blood catheter
2. T-cannulation

   Simple T-cannulation (STC)
   Post-cecum valve T-cannulation (PVTC)
   Steered ileo-cecal valve cannulation (SICV)

3. Re-entrant cannulation

   Fore-valve re-entrant cannulation (FVRC)

   Ileo-ileal re-entrant cannulation (IIRC)
   Ileo-cecal re-entrant cannulation (ICRC)
   Ileo-colic re-entrant cannulation (IORC)
   Ileo-rectal re-entrant cannulation (IRRC)

   Post-ileo-colic valve cannulation (PICVC)

4. Ileo-rectal anastomosis

   Simple end-to-side without ileo-cecal valve (ES-IRA)
   End-to-end without ileo-cecal valve (EE-IRA)
   End-to-side including ileo-cecal valve (ESV-IRA)
   End-to-end including ileo-cecal valve (EEV-IRA)

16.2 Blood Catheter Methods

The method of using catheters for blood collection was described firstly by Rerat et al. (1980) and was further developed and improved by Manolas et al. (1983) and Yen and Killefer (1987). Further improvements were then introduced by Huang et al. (2003) and Yin et al. (2010).

The hepatic portal vein carries blood coming from the gastrointestinal tract, pancreas, and spleen; thus it is the major pathway for transporting the nutrients absorbed from the gastrointestinal tract (Huang et al. 2003). Amino acids absorption into the portal vein per unit of time can be measured using the portal blood flow rate (PVBF) and the differences in nutrient concentrations between the portal vein and systemic arterial blood (Rerat et al. 1980; Yen and Killefer 1987).

The whole surgical procedure including preoperative preparation of the animal, implantation of catheters into portal and ileal mesenterium veins and transonic probe, implantation of catheters into the carotid artery, final exteriorization and anchoring of these catheters, postoperative management, and measurement of portal vein blood flow rate, can be done according to the procedure described by Huang et al. (2003).

Furthermore, the portal vein net amino acid or related metabolite net absorption can be calculated according to the following two equations:

$$\mathrm{PVBF} = C_{\mathrm{i}} \times \mathrm{IR} \times \left(\mathrm{PAH_{pv}}(100 - \mathrm{PCVpv}) - \mathrm{PAH_a}(100 - \mathrm{PCV_a})\right)^{-1\times 100} \tag{16.1}$$

$$q = (C_{\mathrm{p}} - C_{\mathrm{a}}) \times \mathrm{PVBF} \times \mathrm{d}t \tag{16.2}$$

where PVBF is portal vein blood flow (ml/min); C_{i} is concentration of PAH infusion solution (mg/ml); IR is portal plasma PAH concentration (ml/min); $\mathrm{PAH_{pv}}$ is portal plasma PAH concentration (mg/ml); $\mathrm{PAH_a}$ is carotid arterial plasma PAH concentration (mg/ml); PCVpv is portal venous blood packed volume, $\mathrm{PCV_a}$ is arterial blood packed volume; q is net absorption; C_{p} is concentration of the nutrient in portal plasma; and C_{a} is concentration of the nutrient in carotid arterial plasma (Huang et al. 2003).

16.2.1 Advantages of the Blood Catheter Method

The blood catheter method allows us to measure the net absorption of amino acids and their metabolites from the gastrointestinal tract.

16.2.2 Disadvantages of the Blood Catheter Method

The whole procedure is much complicated. It needs sophisticated methods and great technical expertise to do the surgery. Also, the expense of the whole surgery is much higher than other methods.

16.3 Slaughter Method

The slaughter method was first developed by Cori (1925). This procedure includes euthanasia of the pig through an approved method followed by removal of the ileum and subsequent collection of digesta (Yin et al. 2001; Tan et al. 2009).

The length of the small intestine removed may affect protein digestibility estimates. So the longer the section of intestine removed, the greater the chances that complete absorption has not occurred. After eating, feed takes about 6 h to reach the distal ileum in pigs (Donkoh et al. 1994). So the time point for digesta collecting is very important.

16.3.1 Advantages of the Slaughter Method

1. Although the slaughter method does have the advantage of causing minimal interference with the animal's digestive tract prior to the time of sampling, it also allows us to take digesta from several parts of the digestive tract (Donkoh et al. 1994).
2. Any kind of diet can be used to determine amino acid digestibilities.
3. Experimental period is shorter and the procedure is relatively simple.

16.3.2 Disadvantage of the Slaughter Method

1. It is difficult to obtain representative digesta samples, because the optimal sampling time is easily influenced by diet type and other factors (Buraczewski et al. 1971; Rerat 1972). In addition, only one sample of ileal digesta can be obtained per animal. So this method needs more animals than other methods to obtain reliable results.
2. Furthermore, there may be shedding of mucosal cells into the gut lumen at death, which interferes with digesta nitrogen (N) content (Badawy et al. 1957, 1958; Fell 1961). Especially electrical current may cause cell sloughing in the gastrointestinal tract leading to contamination of the amino acid content of the digesta (Sauer and de Lange 1993; Albin et al. 1999).

16.4 Cannulation Technique

Due to the disadvantages of slaughter method, cannulation techniques were developed. Cannulation techniques consist of inserting a device (cannula) into the ileum to allow access to digesta in the gastrointestinal tract from the outside of the body directly and it has become a popular method in the determination of nutrient digestibility in pigs (Yin et al. 1993a, 2002).

16.4.1 Simple T-Cannulation

The Simple T-cannulation (STC) method was invented by Cunninghem et al. (1963) and later improved by Dierick et al. (1983). This procedure mainly includes insertion of a T-cannula into the terminal ileum. The T-cannula could be a full- or a half-round

T-cannula. The full round T-cannula forces digesta to travel through the lumen of the cannula at all times, which can lead to increased blockage problems, especially with high fiber diets. In order to reduce the incidences of blockage of full-round T-cannula, the half-round T-cannula was developed (Sauer and de Lange 1993; Albin et al. 1999). The flange of the cannula is inserted into the ileal lumen and the barrel is exteriorized through the body wall, usually on the right side in a region bordered by the last rib, kidney, and the point of the hip (Sauer and de Lange 1993).

16.4.1.1 Advantage of the STC Technique

The STC method is widely used and has many beneficial characteristics, including the following:

1. It is a relatively quick and simple surgical procedure.
2. It little influences on the process of digestion when sampling.
3. It maintains a normal physiological state, which is advantageous for research involving the gastrointestinal tract (Sauer and de Lange 1992).
4. Digesta blockage and leakage risks remain low when compared with the FVRC technique, especially when half-round T-cannula is used.
5. It does not transect the small intestine wall, a practice that leaves the myoelectric complex intact (Sauer and de Lange 1992).

16.4.1.2 Disadvantages of the STC Technique

1. It remains difficult to quantitatively sample ileal digesta because of the unequal distribution of the liquid and solid phase of digesta and the possibility of blocking digesta when a full-round T-cannula is used (Graham and Aman 1986; Zebrowska 1978; Sauer and Ozimek 1986; Schroeder et al. 1989; Leterme et al. 1990). In addition, because of the position of the cannula, the digesta collected has not passed through the entire length of the small intestine.
2. In order to measure digestibility, an indigestible marker has to be used.
3. When high levels of fibrous dietary ingredients are used, blockage and leakage occur frequently so that the accuracy of results may not be optimal.

16.4.1.3 Cannula Design

Some points must be considered in order to design the cannula so as to improve its practical use.

1. In order to make room for more threads making the ring more stable, a thicker inner edge incorporated into the ring has to be designed.
2. Taking into account the animal care and protection, the ring must have a smooth outer edge in order to protect the skin.

3. Two holes can be placed in the ring to facilitate the use of a spanner wrench for ring adjustments, thus eliminating the need for notches on the outer edge of the ring.
4. Generally, a cannula barrel plug is made from nylon which can fit the cannula barrel very well. So the plug prevents digesta from entering and blocking the cannula barrel between collection periods.
5. The length and width of the cannula flange must be designed according to the pig age and body size to make sure it is suitable for placement in the distal ileum (Wubben et al. 2001; Walker et al. 1986).

16.4.2 Postvalvular T-Cecum Cannulation

The Postvalvular T-cecum Cannulation (PVTC) was developed by van Leeuwen et al. on the basis of the simple T-cannula method (van Leeuwen et al. 1988a, b). Later on, de Lange et al. (1989), Jørgensen et al. (1992), and Yin et al. (2000a) used these techniques to measure the digestibility of different nutrients and experienced some problems such as. . .. According to the limitations and problems, Jennifer et al. (2001) added some modifications to the PVTC technique with success to determine nutrient digestibility in pigs.

This technique involves placement of a full-round T-cannula at the ileo-cecal junction (Köhler et al. 1992a, b; Sauer and de Lange 1993; Albin et al. 1999). After removal of the cecum, the T-cannula is placed between the ileum and the large intestine, allowing digesta to flow from the ileum to the large intestine or out of the pig into a collection bag.

16.4.2.1 Advantage of the PVTC Technique

This technique has similar advantages to those given above for the STC technique. However, additional advantages include the following:

1. It can allow almost quantitative samples collection (Yin et al. 2000b).
2. In the PVTC technique, a larger diameter T-cannula can be used (Sauer and de Lange 1993; Albin et al. 1999).
3. This method reduces variation among animals and the number of animals required to complete an experiment (Wubben et al. 2001)

16.4.2.2 Disadvantage of the PVTC Technique

1. It includes a more complex surgical procedure and removal of the cecum (Radcliffe 1999) which represents an unphysiological situation.
2. As well as in the situation of ileo-rectal anastomosis (IRA), it remains unclear if the remaining intestine has similar function when a segment of the gastro-intestinal tract is removed (Radcliffe 1999).

16.4.2.3 Surgical Procedures for the PVTC Technique

Surgery Preparation

Surgery is performed on healthy pigs that have been fasted for 12 h prior to surgery. Pigs are premedicated by intramuscular injection of 4 mg Azaperone (Stresnil®) and 0.05 mg Atropine sulfate per kg bodyweight (Van Leeuwen et al. 1991) or 1.5 ml of a telazol–ketamine–xylazine solution (Wubben et al. 2001). The surgical table is prepared with a heating pad covered by a clean towel to keep the animal warm and dry during surgery (Wubben et al. 2001; Walker et al. 1986). The pigs are placed in the left lateral recumbency and the right side of the animals is washed with a detergent solution to remove any external manure or dirt. The surgical area (a 30 × 30-cm area surrounding the last two ribs) is shaved by using an electric razor (Wubben et al. 2001, Walker et al. 1986).

After anesthesia, the pigs are left with inhalation of O_2/NO in a ratio of 3:2, Halothane (Fluothane) (Van Leeuwen et al. 1991; Wubben et al. 2001). Wubben et al. (2001) has suggested to maintain the initial 5 % (v/v) halothane concentration for 20 min. The halothane concentration can then be reduced to 1.5–2 % to maintain general anesthesia for the remainder of the surgery. Wubben et al. (2001) suggested that to provide a snug fit, the mask should include a flexible rubber portion next to the pig's snout. Of course, it is difficult to be completely sure that there is no anesthesia gases escaping into the surgery room, so it is necessary that safety procedures are put in place to protect surgeons from the anesthesia gases. Such procedures may include an exhaust fan with an attached exhaust tube placed near the pig's head to vent the gas outside (Wubben et al. 2001). Pig's breathing must be monitored during the entire surgery.

Obviously, all the surgical instruments, surgical drapes, and gowns must be sterilized by autoclaving to avoid infection during and after surgery. Cannulas are sterilized by immersion into a 2 % (v/v) solution of glutaraldehyde for at least 60 min.

Before cannulas are inserted into the ileum, they must be rinsed with sterile saline. The skin around the surgical area on the animal must be washed with 70 % ethanol and iodine. Sterile drapes are placed over the surgical area and the front and back legs (Wubben et al. 2001).

Surgical Procedures

Lidocaine with noradrenaline are administered in the area of the incision subcutaneously and intramuscularly for local anesthesia (Van Leeuwen et al. 1991). The detail surgical procedure can be done according to the procedures described by Wubben et al. (2001).

16.4.3 Steered Ileo-Cecal Valve Cannulation

A more recent technique for total collection of ileal digesta is the ileo-colic post-valve procedure which was developed by Darcy et al. (1980). This method

maintains the integrity of the ileum and preserves the functional role of the ileo-cecal sphincter. Later Mroz et al. (1996) implemented a new cannulation technique called steered ileo-cecal valve cannulation (SICV). This method allows for a quantitative collection of ileal digesta.

The SICV cannula comprises of five parts: an inner cannula barrel, an outer cannula barrel, an internal ring attached to a nylon cord, an external ring, and a cylindrical stopper (Radcliffe et al. 2005). The cecum is not cut off when using the SICV cannulation method. The SICV cannula can be moved by using two rings; the inner ring is for the ileum and its distance to the ileo-cecal valve must be about 10 cm, depending on the age of the pigs. To let inner ring stay at the end of ileum, the external ring is made much smaller than inner ring. The external ring is at the end of ileum and attaches to the wall of cecum tightly. The T-cannula is fixed outside of the cut which is about 2–3 cm in cecum. At the time of sampling, the ileo-cecal valve will get into the T-cannula by pulling the nylon cord, meanwhile the external ring will block the space between the colon and the cecum. Mroz et al. (1991) compared the effect of the SICV and PVTC methods on growth performance and digestibility and found that the growth performance of SICV group is better, but the apparent digestibility of DM, organic matter and chloride is lower. The reason is likely that PVTC cannot get total digesta or that flow rate of digesta is different. Mroz et al. (1991) propose to use the SICV method only for 6–8 weeks after surgery because fibrous tissue between two rings provokes hyperplasia 13–14 weeks after surgery. Later Zhang et al. (2004) compared the method of SICV-cannula and T-cannula and found that ileal digesta samples from SICV-cannula are more homogenous than those from the T-cannula.

16.4.3.1 Advantages of the SICV Method

1. Sample collection is easy.
2. This method can maintain the integrity of the ileum and preserves the functional role of the ileo-cecal sphincter.
3. This method allows for a quantitative collection of ileal digesta.

16.4.3.2 Disadvantages of the SICV Method

1. SICV needs complicated surgery procedure and more labor and time cost than other methods.
2. SICV could only be used for 6–7 weeks after surgery, because of the fibrous tissue that develops between two rings due to hyperplasia 13–14 weeks after surgery (Mroz et al. 1991).

16.4.4 The Re-entrant Cannulation Technique

The re-entrant cannulation technique is also one of those that allow for total ileal digesta sample collection. This method consists of fitting two T-cannulas at two

different sites of intestine and connecting the two cannulas using a bridge cannula. When getting samples, the bridge cannula is removed. Then, total digesta can be recovered from the proximal cannula. The digesta after analysis is warmed and put back into intestine through the distal cannula which allows for a situation that is close to the normal physiology and metabolism in pigs (Yin et al. 1991).

Depending on to the position of the cannulas, the re-entrant cannulation technique can be divided into two fore-valve re-entrant cannulation (FVRC) and post-ileo-colic valve cannulation (PICV).

16.4.4.1 Fore-Valve Re-entrant Cannulation Technique

The FVRC is when the proximal cannulation is made before the ileo-cecal valve. The FVRC can be divided into IIRC, ICRC, IORC, and IRRC. The detailed operation procedure can be found in previous studies (Cunninghem et al. 1962; Easter and Tanksley 1973; Laplace et al. 1985). van Leeuwen et al. (1988a, b) who designed the ileo-cecal re-entrant cannula for piglets have described in detail the technique used.

Advantages of the Re-entrant Cannulation Technique

1. Re-entrant cannulation is easy to operate.
2. The accuracy of data obtained by using re-entrant cannulation is very good.
3. When using the re-entrant cannulation technique, there is no need for a digestibility marker, thus avoiding the challenges associated with maker analysis and recovery.

Disadvantages of the FVRC Technique

1. Because the small intestine is completely cut off, the ileo-cecal valve does not work, which affects the normal excitation–contraction coupling which control the normal digesta flow in the small intestine.
2. The bridge cannula can easily drop off, with resultant problems of blockage and leakage, especially with diets containing high levels of non-starch polysaccharides.

16.4.4.2 Post-ileo-colic Valve Cannulation

Darcy et al. (1980) developed the ileo-colic post-valve cannulation technique. PICVC is when the proximal cannula is placed after the ileo-cecal valve. The technique involves the transsection of the intestine and the collection of digesta after the ileo-cecal valve. Although two cannulas are used in this technique, the re-entrant flow of digesta into the colon is not spontaneous. Also the surgery involved in this technique is rather difficult (Darcy et al. 1980). PICVC keeps the integrity of whole ileum, including the ileo-colic valve. However, the cecum is almost cut off totally and the proximal cannula is put into the residential cecum which is behind ileo-colic valve.

Advantages of PICVC

1. PICVC keeps the integrity of the whole ileum.
2. The result accuracy is higher than FVRC.

Disadvantages of PICVC

1. Cecum is cut off totally, so the digestibility of amino acids in proteins is impacted.
2. Blockage can happen.
3. The digesta can easily be contaminated by microbes.
4. Surgery procedure is much complicated, hard to operate, and is time consuming.

16.4.5 Ileo-Rectal Anastomosis

As a novel means of avoiding problems with cannulation, the IRA technique has been reported by Fuller and Livingstone (1982), Picard et al. (1984), Darcy-Vrillon and Laplace (1985), Souffrant et al. (1985), Hennig et al. (1986), and Yin et al. (1993a).

The procedure for IRA involves transecting the small intestine just proximal to the ileo-cecal valve and reattaching it to the distal colon (Sauer and de Lange 1993). Picard et al. (1984) also reported another modified version of this technique in which a T-cannula is placed in the colon to evacuate fermentation gases. Pigs with IRA are relatively easy to maintain and can be fed with diets of any texture (Sauer and de Lange 1992).

The function of the remaining intact intestine is altered over time due to the removal of the large intestine and cecum. The reason of such alteration may be related to the fact that the functional role of the small intestine is changed to compensate for the missing colon. This may alter the amino acid composition of the digesta and observed digestibilities compared with that obtained when using other methods (Fuller and Cadenhead 1991). Köhler et al. (1992a, b) found that N retention of ileo-rectal anastomosed pigs decreased compared to normal pigs and pigs fitted with post-valve T-cecal cannulas, meanwhile Sauer and de Lange (1993) found that nutrients absorption was changed.

According to the position of anastomosis and to the maintenance of the ileo-cecal valve, IRA could be divided into ES-IRA, EE-IRA, ESV-IRA, and EEV-IRA (Laplace et al. 1994). The characteristics of IRA is summarized in Table 16.1.

16.4.5.1 Advantages of the IRA Technique

1. The most important advantage of IRA is that digesta can be collected quantitatively via the anus.

Table 16.1 The kind and features of IRA

Kind of IRA	ES-IRA	ESV-IRA	EE-IRA	EEV-IRA
Style of anastomosis	End-to-side	End-to-side	End-to-end	End-to-end
Position of anastomosis	Ileum end–rectum side	Ileum end–rectum side	Ileum end–rectum end	Ileum end–rectum end
Descending colon is opened or not to rectum	Opened	Opened	No	No
With or without ileo-caecal valves post-surgery	Without	With	Without	With
Degree of backflow of digesta	Significant	medium	Little significant	Not significant
Side effects[a]	Significant	medium	Little significant	Not significant
Data resource	Fuller and Livingstone (1982)	Souffrant et al. (1985)	Picard et al. (1984) and Green et al. (1987)	Green et al. (1988)

[a]Side effects are related to the level of the changes in the microflora activity in digesta and to the digestibility of amino acids with the extension of time post-surgery. Redlich et al. (1997) have compared the morphometry of the small intestine in pigs with four different IRA (i.e., EEV, EE, ESV, and ES) with intact pigs. They concluded that there was no major disturbance after IRA, and the different procedure used are not deleterious to the structural integrity of the small intestine

2. Using this technique, problems such as blockages and leakages which have been described for re-entrant cannulation (Sauer 1976; Just et al. 1980) do not occur.
3. The surgery is simpler compared with other techniques, and pigs recover rapidly.
4. All kinds of diets can be used for tests

16.4.5.2 Disadvantages of the IRA Technique

The normal physiology of swine is altered after surgery, because large intestine is dissociated. As a result, the energy metabolism as well as nitrogen metabolism and water and electrolyte movements may change.

Green et al. (1987) used IRA with the technique of complete isolation of the large intestine to study the digestibility of amino acids in maize, wheat, or barley meals. Endogenous secretion was different when fed different diets. The choice of factor for the correction of apparent digestibility to true digestibility was based upon CF intake of cereal diet.

Redlich et al. (1997) measured villus length, crypt depth, and mucosal thickness and found that despite several minor changes in the morphological characteristics of the small intestine after IRA, there was no major disturbance undermining the validity of the IRA method. Among the four different surgical procedures tested, the data obtained suggested that the end to end procedure (EE) is the most beneficial for the maintenance of the structural integrity of the small intestine.

16.5 Comparison of Different Techniques

16.5.1 Intact Pig and STC in Pigs

Some studies (Laplace and Borgida 1976; Sauer et al. 1979) found some adverse effects of cannulation on nutrient digestibility. Livingstone and Mcwilliam (1985) showed an effect of STC on pig growth which may have been due to decreased nutrient digestibility or utilization. Moughan and Smith (1987) compared the apparent ileal amino acid digestibility between normal pigs and pigs with T-cannulation. They found that there was good agreement between the ileal digestibilities of the amino acids as determined with intact and cannulated pigs with no significant effect of animal type on digestibility.

16.5.2 Slaughter Method and the STC Technique

Donkoh et al. (1994) studied the effect of slaughter method and STC on terminal ileum amino acid digestibility. They found that there was no significant effect of digesta collection procedure on the apparent ileal nitrogen or amino acid digestibility coefficients.

16.5.3 Comparison Among IRA Methods

Green (1988) measured and compared amino acids digestibility in pigs with EE-IRA and in pigs with EEV-IRA. There was no difference in amino acid digestibility when using these two anastomosis. Later Hennig et al. (1992) compared amino acid absorption of swine with ESV-IRA and EEV-IRA, and found about 5 % difference in amino acids absorption and finally proposed to use the EEV-IRA. Laplace et al. (1994), when comparing four IRA (i.e., EEV, ESV, EE, and ES), found that the ES models do not provide correct digestibility values due to backflow and bacterial metabolism. The EE models seem to be the most appropriate to study ileal digestibility of proteins, even in the long term. The EE models can also be used to measure the ileal digestibility of fiber, but only in short term experiments and, in this case, the preservation of the valve appears of interest.

16.5.4 IRA and STC Methods

Leterme et al. (1990) compared the effect of IRA and T-cannulas on the digestibility of amino acids. These authors found that among amino acids, the differences

were only significant for the apparent as well as the true digestibilities of methionine, proline, and tryptophan. Apparently, the observed differences originated from the method used for the collection of the samples from the cannulated pigs. In order to understand why there are differences in amino acid digestibility between IRA and T-cannulas, Leterme et al. (1991) compared the emptying rates with both methods and found as a result that the emptying rate of IRA is significantly faster than T-cannulas. However it is worth to note that Fuller et al. (1994) found that there was no significant difference in ileal amino acid digestibility between IRA and T-cannulas.

16.5.5 PVTC and IRA Methods

Köhler et al. (1991a, b, c, 1992a, b) have compared the effect of PVTC and IRA on the digestibility of nutrients in pigs. They found that there were significant differences in the digestibility of dry matter, nitrogen, and amino acids between PVTC-pigs and IRA-pigs. Meanwhile, they indicated that PVTC is much better for determining the digestibility of nutrients than IRA.

16.5.6 IRA and ICPV Methods

Darcy-Vrillon and Laplace (1985, 1990) have studied the effects of IRA and ICPV on the digestibility of nutrients. They found that the values which were determined by using ICPV were higher than the values which were determined by using ICPV. In addition, the differences were found to change with different diets. Later on, Fuller et al. (1994) reported similar results in their study.

16.5.7 IRA and FVRC Methods

Picard et al. (1984) conducted 53 experiments to compare the difference of IRA and IIRC in the digestibility of amino acids. They found differences only in the digestibility of cystine. Later Hennig et al. (1991) have compared the difference of ES-IRA and ICRC by using ten diets. In all the 20 amino acids of the 10 diets, the differences were higher than 5 %. Especially, the digestibility of methionine which was determined by using ES-IRA was 15 % lower than the value which was determined by using the ICRC technique. Hennig et al. (1993) when studying the effect of ES-IRA and ICRC on the digestibility of amino acids got similar results, but nonetheless proposed to use EE-IRA to replace ES-IRA, because the former may decrease the effect of intestine microbiota on amino acid digestibility.

Yin et al. (1993a, b) also compared ES-IRA and ICRC methods for the measurement of amino acid digestibility and found that there was no significant difference between ES-IRA and ICRC techniques.

16.5.8 STC and FVRC Methods

Zebrowska (1978) compared the difference between STC and FVRC on amino acids digestibility and found some differences, although the reasons explaining these differences remain unclear. Michael et al. (1983) compared the STC and FVRC techniques using different diets and found that the values of amino acid digestibility determined with the STC technique were higher than the values determined with the FVRC technique.

den Hartog et al. (1988) reported that there was no significant difference in the apparent digestibility of dry matter and nitrogen when using PVTC, STC, or FVRC. Later, Köhler et al. (1990) compared the effects of STC, PVTC, and ICRC techniques on nutrients digestibility. Some differences among different techniques were noted, and for some items there were indeed significant differences. The authors suggested that the differences observed originate from the recovery of indicator. In fact, in practice, the recovery of the indicator (i.e., digestibility marker) was lower than 100 %, but when the recovery of indicator was corrected up to 100 %, the difference was found to decrease.

Yin et al. (1991) found that the ICRC technique could not replace STC when abundant cell wall is present in experimental diets. Fuller et al (1994) reported that the values of protein digestibility which were determined by using PVTC are higher than the value which were determined by using T-cannula.

16.6 Conclusion

Above all, it is much likely that the measurement of amino acid digestibility gives different results according to the different techniques used. As shown above, each technique has its advantages and disadvantages. Extensive comparisons between different methods do not allow to define precisely what is the best method to be used when measuring the digestibility of amino acids using different methods.

Only optimal physiological status of animals after surgery allows the measurement of “physiological” digestibility. In other words, it is mandatory to maintain the function and structure of the gastrointestinal tract as normal as possible. In the process of quantitative and representative sample collection, T-cannulation and slaughter technique only get a part of digest. Furthermore, these two methods require the utilization a digestibility marker. In contrast, IRA and re-entrant cannulation allow to collect the whole digesta. In terms of operability, IRA is more simple compared with other techniques, and pigs can recover quickly. In terms of the type of diets tested, only IRA and slaughter technique can be used with any kind of diets. So IRA appears one good choice in determining amino acids digestibility, but the effect of IRA on the animal physiology should be further studied.

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Chapter 17
Determination of Protein Digestibility in the Growing Pig

Paul J. Moughan and Warren Miner-Williams

Contents

17.1 Introduction

The cost of feed is usually the most significant variable cost in modern intensive pork production, and protein is the most expensive component of the diet. There is thus a considerable financial incentive to maximise the efficiency of utilisation of the dietary amino acids, and especially the dietary first-limiting amino acid. The dietary first-limiting amino acid for the weaned and growing pig is often lysine. It is particularly important that the supply of dietary amino acids to the body tissues (i.e. absorbed amino acids) closely matches the amino acid requirements of the tissues for protein synthesis, to avoid unnecessary wastage.

P.J. Moughan (✉) • W. Miner-Williams
Riddet Institute, Massey University, Private Bag 11-222, Palmerston North, New Zealand
e-mail: P.J.Moughan@massey.ac.nz

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_17,

For the first-half of the twentieth century, animal nutritionists focussed on dietary crude protein content and its optimal supply. With the advent of the automated analysis of amino acids, following the groundbreaking work of Moore and Stein in the USA in the 1950s and 1960s, emphasis in the latter half of the twentieth century shifted to dietary amino acids. Initially, faecal amino acid digestibility was determined with animals to allow the prediction of amino acid uptake from the digestive tract, but this was superseded by analysis of amino acid uptake based on amino acids remaining undigested at the end of the small intestine (ileal digestibility). This significant technical advance was based on work from a number of groups undertaken mainly in the 1980s and 1990s.

The aim of this contribution is to review aspects of the ileal measure of amino acid digestibility, and discussion will relate especially to methods that have been developed for the collection of ileal digesta from pigs.

17.2 Ileal Amino Acid Digestibility

From a nutritional standpoint, the digestion of dietary protein is largely complete by the end of the ileum. Protein, peptides and amino acids entering the hindgut (colon and caecum) of the growing pig are subjected to extensive microbial metabolism. Much material is catabolised with end-products such as ammonia and amines. Also, nitrogen (both from the breakdown of undigested dietary and endogenous protein and from urea entering the digestive tract from the blood) is used by the hindgut microbes to synthesise bacterial protein.

Any uptake of amino acids (as such) from the hind gut of the pig is believed to be nutritionally inconsequential. Moreover, faecal protein is predominantly microbial protein, the amino acid composition of which bears no direct relationship to the amino acid composition of the undigested dietary protein that enters the colon. Thus, the determination of amino acid digestibility based on the total collection of faecal output (faecal digestibility) is considered to be flawed (Hodgkinson and Moughan 2000a; Moughan 2003; Fuller and Tomé 2005; Hendriks et al. 2012). Determination of the amounts of dietary amino acids remaining unabsorbed at the terminal ileum, leading to coefficients of ileal digestibility is the preferred approach. There is comprehensive published evidence demonstrating that ileal amino acid digestibility coefficients are more accurate than their faecal counterparts for predicting amino acid uptake into the pig's body (Just et al. 1985; Moughan and Smith 1985; Moughan et al. 1991; Rutherfurd et al. 1997a, b; Columbus and de Lange 2012).

It is now common practice in pig nutrition for protein and amino acid digestibility to be determined based on the collection of ileal digesta rather than faeces (Moughan 2003). Faecal amino acid digestibility values may be quite misleading, either overestimating (which is usually the case) or underestimating (sometimes seen especially for methionine) digestibility. Whenever digestibility is determined based on digesta contents collected from the terminal ileum, however, the digesta

amino acids need to be corrected for amino acids of endogenous origin present in the digesta along with undigested dietary amino acids (Skilton et al. 1988).

If total amino acids are deducted from the dietary amino acids ingested, without any correction for the endogenous amino acids, then "apparent" estimates of digestibility are obtained. Apparent estimates of digestibility are influenced by the protein content of the test diet (Donkoh and Moughan 1994) and can be misleading especially for low dietary protein or amino acid contents. "True" estimates of amino acid digestibility (corrected for the endogenous amino acids), however, are independent of the dietary protein concentration and are a fundamental property of the food itself. True ileal digestibility is the preferred approach for determining amino acid digestibility in the growing pig (Boisen and Moughan 1996; Hodgkinson and Moughan 2000b; Moughan 2003).

Where there is an agreed approach to determining ileal endogenous amino acids, and the ileal endogenous component is corrected for, the term "standardised" digestibility is used instead of "true" (Stein et al. 2007a, b).

Currently in pig production and where the "standardised" amino acid digestibility method is adopted, the traditional protein-free diet method is used to derive estimates of ileal endogenous amino acid flow (Stein et al. 2007a). This is justified based on the work of Jansman et al (2002), where a comparison was made of the overall mean ileal endogenous amino acid losses for pigs given a protein-free diet versus comparable flows for pigs given protein-containing diets, determined over several studies. The flows for the protein-containing diets were higher, but not greatly so compared to when a protein-free diet had been given. There is a need for caution, however, as when one examines data within studies, for protein-free versus protein-containing diets, in most cases dietary protein causes a statistically significantly higher endogenous amino acid flow at the terminal ileum, and furthermore such differences are practically meaningful.

Quite in addition to an increased amount of endogenous protein it may well be that the pattern of amino acids in the ileal endogenous protein also differs when the animal consumes a protein-containing rather than a protein-free diet, thus affecting relative differences among amino acid digestibility coefficients. This aspect is worthy of further investigation. Not only is endogenous ileal amino acid flow higher when protein is present in the diet, but it has also been shown in both the growing rat and pig that the dietary concentration of protein influences the endogenous amino acid loss with greater losses at higher dietary protein or peptide concentrations (Hodgkinson et al. 2000; Hodgkinson and Moughan 2007). The various methods for determining ileal endogenous amino acid flows have been reviewed (Hodgkinson and Moughan 2000a; Moughan et al. 1998).

In the case of processed feedstuffs or feedstuffs that have been stored for long time periods, proteins may have undergone structural changes, which have implications for the determination of amino acid digestibility. This is particularly important for the amino acid lysine. Here, true ileal lysine digestibility or standardised ileal lysine digestibility are still appropriate measures, but the traditional chemical analysis of lysine shouldn't be used. Rather the protein should be reacted with the reagent *o*-methylisourea before acid hydrolysis of the protein, to allow detection of "reactive lysine", and the ileal digestibility of reactive lysine

Table 17.1 Mean true ileal digestible lysine contents[a] compared with true ileal digestible reactive lysine[b] in some processed feedstuffs

	True ileal digestible lysine (g/kg)	
	Traditional analysis	Reactive lysine
Wheat	3.2	2.9***
Maize	2.6	1.9***
Skim milk powder	19.8	16.6***
Cottonseed meal	12.9	10.3***
Evaporated milk	23.4	20.5***
Whole milk powder	26.2	24.0***
Lactose hydrolyzed milk powder	27.2	25.1***
Popped rice cereal product	0.7	0.3***
Grain-based cereal product	1.2	0.5***
Whole grain bread	2.4	2.0**

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

[a]Based on traditional amino acid analysis

[b]Based on reactive lysine determined after reacting the protein with *o*-methylisourea (Moughan and Rutherfurd 1996)

should be determined (Rutherfurd and Moughan 1990, 1998; Moughan and Rutherfurd 1996, 2008; Rutherfurd et al. 1997a, b; Moughan 2005). Damaged proteins are commonly encountered in the feed proteins used in preparing compound pig feeds, and the differences between traditionally determined ileal lysine digestibility and the true ileal digestibility of reactive lysine (available lysine) can be considerable (Table 17.1).

The determination of standardised ileal amino acid digestibility coefficients for pig feeds is one of the major technical advances in pig science over the last few decades. There are economically important relative differences in determined amino acid contents between gross and digestible amino acids. However, to be able to determine ileal amino acid digestibility, digesta from the terminal small intestine (final 10–20 cm of ileum) need to be collected either in total or by sampling along with the use of a marker compound in the feed. The collection of digesta from the pig has spawned much research over the years, and there is a considerable body of knowledge on the subject. Digesta can be obtained either by dissecting the ileum from the body of the pig while under deep anaesthesia (sometimes called the "slaughter method") or by collecting digesta from the ileo-rectal anastomised animal or from a cannulated animal. The various collection methods used in the pig have been the subject of review (Fuller 1991; Yin and McCracken 1996)

17.3 Collection of Digesta: Slaughter Method

The most straightforward way of collecting digesta from the end of the ileum is to dissect the ileum from a deeply anaesthetised animal or immediately upon euthanasia. This method is commonly used in smaller animals such as rats and chickens

where the animal is killed (Payne et al. 1968; Moughan et al. 1984), and in early work with the growing pig using this approach, digesta were removed immediately after death of the animal. It is for this reason that the method is often referred to as the "slaughter method". Because only a sample of digesta is collected with this technique, a marker compound (such as chromic oxide or titanium dioxide) needs to be included in the test diet. With the growing pig an adequate amount of digesta is usually found in the final 10–20 cm of ileum, unless a very highly digestible diet has been fed to the animal, whereupon only small quantities of digesta may be recovered. The technique has successfully been used with milk-fed piglets (Moughan et al. 1990).

There is a need, with this technique, for careful handling of the ileal tissue and attention to how the animal is killed or anaesthetised. Mucosal cells are rapidly shed into the gut lumen following death, thus increasing the protein content of the digesta. The potential problem of cell shedding can be avoided if barbiturates are used to kill the animal and digesta are collected immediately (Badawy et al. 1957; Thorpe and Tomlinson 1967), or if the ileum is removed from the anaesthetised animal.

When using the slaughter method, a frequent (usually hourly) feeding regimen may be employed, or digesta may be collected at a set time after ingestion of a single meal, to coincide with the peak digesta flow. Work has been conducted, often with the rat, studying aspects of digesta sampling (Donkoh et al. 1994a; Van Wijk et al. 1998; James et al. 2002a, b; Butts et al. 2002; Hodgkinson et al. 2002).

The advantage of this approach is its simplicity. There is minimal interference with the digestive tract prior to sampling, there are no limitations on the type of diet fed to the animal (confer cannula blockages), and it is perhaps more acceptable ethically in comparison with cannulation and anastomosis. Digesta can be sampled from several parts of the digestive tract, which is sometimes useful, but dietary markers must be used and only one terminal ileal digesta sample can be obtained per animal, so the animal cannot be used as its own control. However, the major advantage remains, that the anatomical, histological, microbial and physiological changes that can occur over time with cannulation or anastomosis are avoided with this technique.

Controlled studies with the growing pig comparing dietary protein digestibility values obtained either with the "slaughter method" or by using simple T cannulated animals have shown similar mean values and similar variances around the mean (Moughan and Smith 1987; Donkoh et al. 1994b).

17.4 Collection of Digesta: Ileo-Rectal Anastomosis and Cannulation Techniques

17.4.1 Ileo-Rectal Anastomosis

To avoid interference from colonic microflora and to allow repeated sampling of digesta over time, ileo-rectal anastomoses (IRA), have in the past been used to collect pre-caecal digesta from the terminal ileum. Numerous IRA configurations

have been tried since they were first proposed in the early 1980s (Fuller and Livingstone 1982; Laplace et al. 1985; Picard et al. 1984). Digestibility estimates obtained using these procedures vary depending on the configuration used (Darcy-Vrillon and Laplace 1990). In all of the ileo-rectal configurations the normal functions associated with the large intestine are interrupted, and will therefore affect the absorption of water and minerals (Köhler et al. 1991). Since the first use by Fuller and Livingstone (1982) little research has been conducted to determine the effects of IRA on intestinal morphology. Salgado et al. (2002) found that anastomosed pigs had higher spleen and small intestine weights and lower large intestine weights than intact pigs. They also discovered that IRA influenced intestinal villus and crypt architecture although it had no significant effect on the activities of intestinal enzymes. Marinho et al. (2007) also found that IRA modified the intestinal villus and crypt architecture in the duodenum and ileum.

Although there are advantages of IRA there are also many disadvantages. The method has been deemed unethical by some researchers and is not permitted in some jurisdictions. Despite this the method still finds application (Branner et al. 2004; Bohmer et al. 2005; Fontes et al. 2007; Hennig et al. 2006, 2008; Hackl et al. 2010).

17.4.2 T-Cannulae

Classically, methods to obtain ileal digesta samples have involved the use of cannulae, where the lumen of the distal ileum has been exteriorised.

Cannulation techniques can be broadly divided into two categories, those where the experimental animal is fitted with a single cannula and those fitted with two, the so called re-entrant variants. Compared with single cannulation techniques, re-entrant cannulation has been thought to give more reliable nutrient digestibility values since this technique allows the collection of total ileal digesta samples within the experimental time period and this obviates the need for an indigestible marker (Yin and McCracken 1996). However, both approaches have been criticised on the grounds that such procedures have a modifying effect on the processes of digestion and absorption (Sauer and Ozimek 1986) and that differences in the determined digestibility may occur depending on the technique used (Darcy-Vrillon and Laplace 1990). Double re-entrant cannulae are rarely used today because of the complex and expensive surgical procedures.

Studies comparing the different techniques have been undertaken by a number of researchers who have all reported some disparity in the calculated digestibility coefficients (Donkoh et al. 1994b; Köhler et al. 1990; Yin and McCracken 1996; Yin et al. 2000a, b), although some of these differences may be attributable to other secondary factors including the composition of the diet, the amount of dietary fibre or the nature of the indigestible marker (Laplace et al. 1994; Sauer and Ozimek 1986; Yin et al. 2000a, b). Further discussion on the merits of these techniques is therefore required.

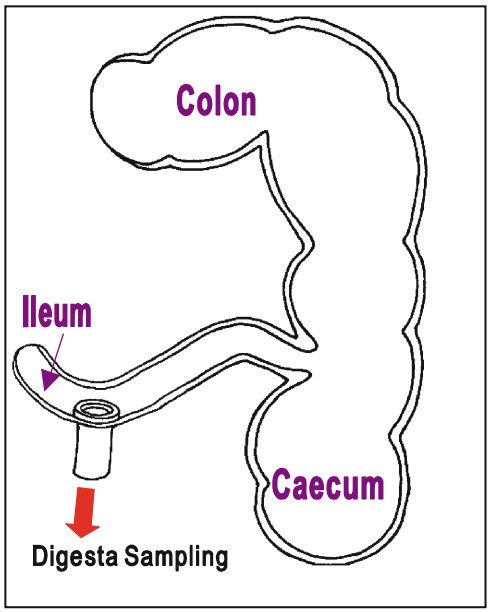

Fig. 17.1 Simple "T" cannula

17.4.3 Simple "T" Cannula

First proposed by Livingstone et al. (1977a) this technique utilised a "T"-shaped cannula made of annealed Pyrex glass that had an internal diameter of just 16–20 mm (Fig. 17.1). The cannula was secured from becoming internalised by one or more Perspex washers and a self-locking nylon strap. The shank of the cannula was threaded to take an impact-resistant plastic cap. The cannula was inserted 15 cm proximal to the ileo-caecal valve. Livingstone reported that the cannulae remained intact and functional when fitted to pigs, housed in smooth-walled pens, growing to a live weight of 175 kg. Trauma from the surgery of fitting the cannulae was reported to be minimal and the intestinal activity, appetite, and growth rate of the animals was unaffected by the cannulation. They concluded that pigs fitted with such a cannula were physiologically similar to intact pigs (Livingstone et al. 1977a, b). As with all single cannula techniques, not all of the digesta flows through the cannula and the incorporation of an indigestible dietary marker to determine the total flow of digesta is essential. It is assumed that the composition of the digesta collected via the T-cannula is representative of the total quantity of digesta flowing through the distal part of the ileum, and that no fractionation of its contents, particularly between the solid and liquid phases occurs. However, this has been questioned by a number of researchers (Fuller et al. 1994; Sauer et al. 1989; Yin and McCracken 1996). Differences in the recovery of indigestible markers have indicated that phase separation may indeed occur and that this may be one reason why a greater variability in results was observed by

Köhler et al. (1990) when they compared a simple T-cannula with a post-valve T-caecum cannula and a re-entrant cannula. They suggested that a reduction in the homogeneity of the samples recovered through the T-cannula may be attributable to changes in pressure at the base of the open cannula, forcing a separation of coarse and fine insoluble particles (Schröder 1988), and this might explain differences in the digestibility coefficients observed in diets rich in fibre. Although no incidence of the cannula blocking was reported by Livingstone et al. (1977a, b) this has been noted by Potkins et al. (1991), who had to change their experimental design owing to the frequency of blockages at the site of the cannula and the consequential inability to collect digesta when their experimental animals were fed a diet rich in bran fibre. Fernández et al. (2001) also reported problems with the simple T-cannulation technique, finding unreasonably large deviations in results when studying fibre rich materials. However, and despite its few disadvantages the use of simple T cannulae, for the determination of nutrient digestibilities, is one of the most commonly used cannulation techniques (Goerke et al. 2012). Today, flexible plastic and rubber T cannulae have replaced the early glass models.

17.4.4 Post-valve T-Caecum Cannula

Developed by van Leeuwen et al. (1988) in the late 1980s at the Institute for Animal Nutrition and Physiology in the Netherlands, the Post-valve T-caecum cannula (PVTC) is made of medical grade silicon tubing, with an internal diameter of 25 mm and is secured by an exterior ring and self-locking nylon straps (Fig. 17.2). The technique involves the removal of part of the caecum and installation of the cannula opposite the ileo-caecal valve. One hour before the collection of digesta starts, the cannula is opened to allow the ileo-caecal valve to protrude into the lumen of the cannula. Although this ensures that more of the digesta flowing from the ileum is collected, this technique cannot guarantee a complete quantitative collection of ileal digesta and so the inclusion of an indigestible dietary marker is advised (van Leeuwen et al. 1991). Having studied more than 100 animals fitted with PVTC cannulae, with live weights ranging from 8 to 100 kg, van Leeuwen's group found that post-operative recovery was swift and that the animals displayed no signs of discomfort or a reduction in appetite, even over long periods of time. Following the partial caecectomy and insertion of the cannula into what remains of the caecum, the physiology of both the ileum and the colon appear to be normal. As the gut is not transected, there appears to be no interference with the myoelectric innervation of the intestines that may reduce intestinal motility, points that have been corroborated by a number of other researchers (Köhler et al. 1990, 1992a, 1992b; Yin and McCracken 1996; Yin et al. 2000b). Although few digestibility studies have compared PVTC cannulae with other methods of collection, those that have concluded that results, using the PVTC technique, are comparable with those obtained using simple T-cannula (Köhler et al. 1990; Yin and McCracken 1996; Yin et al. 2000b).

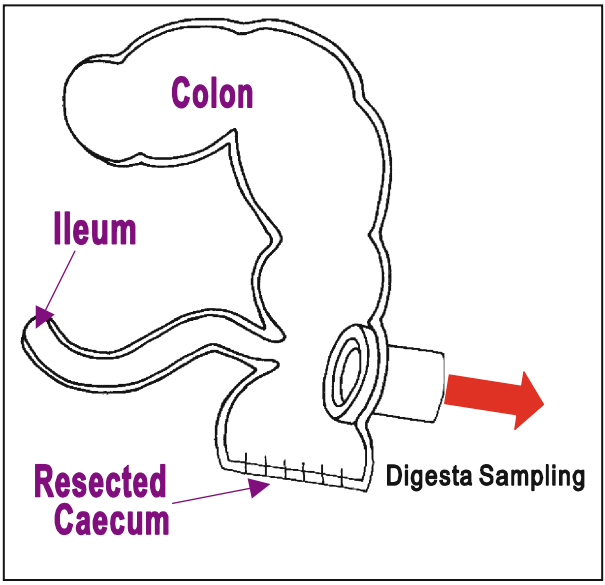

Fig. 17.2 Post-valve T-caecum cannula

In one long-term study by Kohler et al. (1992a, b) using 23 pigs (9 with IRAs, 7 with PVTCs and 6 intact pigs), no differences were found in growth performance, body nitrogen retention, blood variables and mineral balances, between intact animals and those fitted with PVTCs.

Greater precision can be achieved using the PVTC method for most diets and this is thought to be attributable to greater consistency in the recovery of the indigestible marker, specifically titanium dioxide. However, with high fibre diets some variability in the amount of digesta recovered has been recorded, which confirms the necessity of using an indigestible marker (Köhler et al. 1990, 1991; van Leeuwen et al. 1991; Yin et al. 2000a) Although the PVTC cannulation technique has been found to have a minor effect on total tract nutrient digestibility (Lindberg 1997), it is at present considered to give the most satisfactory results for ileal digestibility determination (Hodgkinson and Moughan 2000a; Presto et al. 2011).

17.4.5 Steered Ileo-Caecal Valve

Mroz et al. (1994, 1996) developed a variant of the PVTC technique which they termed the steered ileo-caecal valve (SICV), cannulation (Fig. 17.3). This technique permits the collection of digesta, with both low and high fibre diets, and maintains the normal physiological function of the gut as it does not require the removal or transection of any part of the GIT. Although this technique was thought to allow the

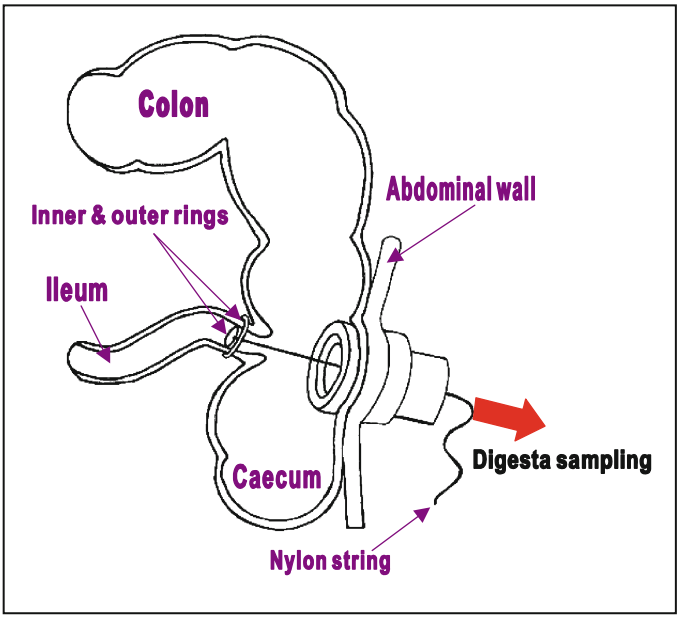

Fig. 17.3 Steered ileo-caecal valve

quantitative collection of digesta, the recovery of a chromium marker from the faeces of cannulated pigs was approximately 20 % less than in intact pigs. Therefore under the conditions of their study, the use of an inert marker was deemed advisable for determination of the total tract digestibility and ileal digestibility of nutrients. Changes proposed by Radcliffe et al. (2005) resulted in a decrease in post-surgical complications, notably fewer and less severe adhesions, together with less leakage of digesta around the cannulae. The surgery for this technique is more complex than the PVTC method, but it appears to be less prone to blockages.

In comparison to the simple T cannula, Zhang et al. (2004) concluded that ileal digesta samples collected using the SICV-cannula were more homogenous.

17.4.6 Simple Ileo-Ileal Re-entrant Cannula

Although re-entrant fistulae have been commonly used to study the digestive process in ruminants (Phillipson 1952), Cunningham et al. (1962, 1963) used a similar re-entrant cannula to compare the digestibility and rate of passage of a variety of purified and natural feed materials in the gut of the pig (Fig. 17.4). Blockages would often occur in the cannulae, particularly, if coarsely ground feeds were administered, but the cannulae were well tolerated by the pigs. Kowalik et al. (2004) studying the digestion of starch and crude fibre in segments of the digestive tract of sheep reported no problems with blockages.

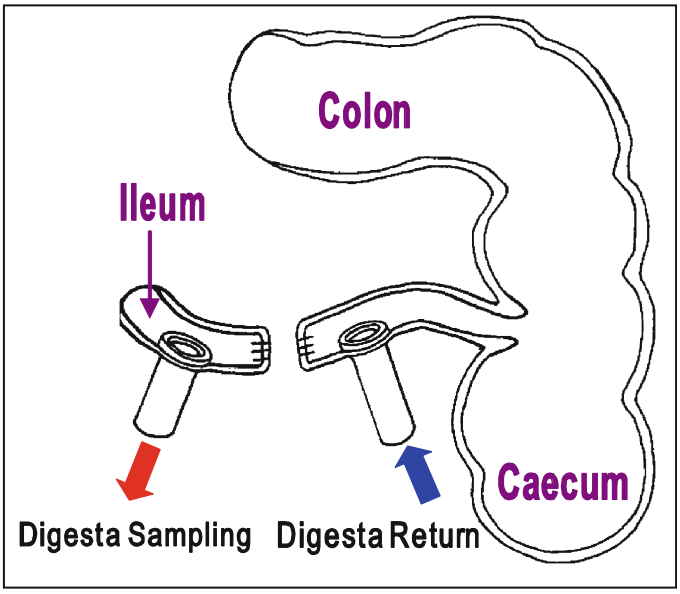

Fig. 17.4 Simple ileo-ileal re-entrant cannula

17.4.7 Ileo-Caecal Cannula

Both Cunningham et al. (1963) and later Cho et al. (1971) found that the flow of digesta through the externally joined ileo-ileal re-entrant cannulae was not uniform. Easter et al. (1972) and Easter and Tanksley (1973) believed that the surgically disrupted small intestine was not capable of forcing digesta through the externalised cannulae and the ileo-caecal valve. As the ileo-caecal valve was not essential to the digestion process (Davenport 1966), changing the configuration of the re-entrant cannula from ileo-ileal to ileo-caecal offered a solution to this resistance (Fig. 17.5). Although the use of single caecal cannulae in the digestion studies of pigs had been demonstrated by earlier researchers (Henderickx et al. 1964; Redman et al. 1964), this was the first time that a double, ileo-caecal re-entrant had been used. Although not widely used, ileo-caecal re-entrant cannulae are still employed particularly in studies involving dietary fibre (Bartelt et al. 1999; Rapp et al. 2001).

17.4.8 Ileo-Colic Post-valve Fistulation

First described by Darcy et al. (1980), Ileo-colic post-valve fistulation (ICPV), which they considered to be a reference method (Fig. 17.6), is surgically difficult and too time consuming for routine ileal digestibility measurement. Early

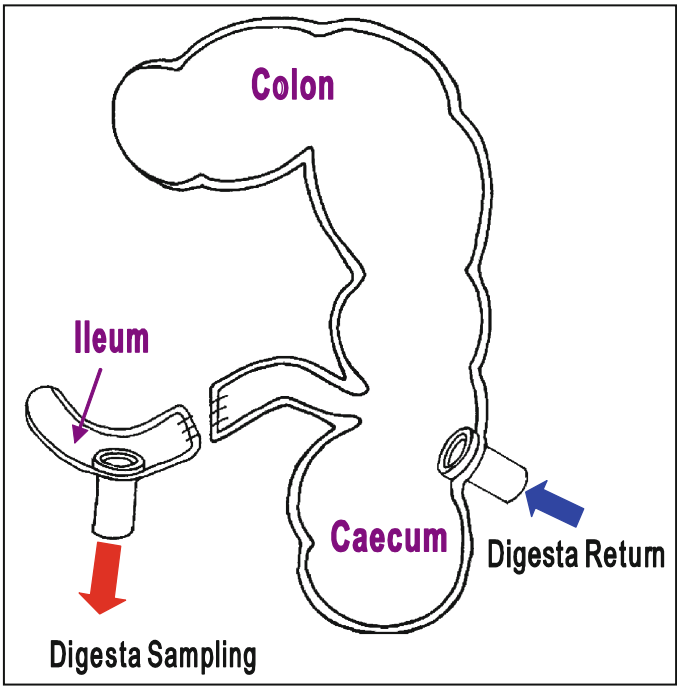

Fig. 17.5 Ileo-caecal cannula

suggestions that this method could be used in diets rich in fibre (Sauer and Ozimek 1986) were recanted by Fuller et al. (1994) who reported evidence to the contrary for diets rich in barley.

17.4.9 Comparative Summary of Ileal Digesta Collection Techniques

There is little agreement between researchers as to which digesta collection technique is the best, although lately the PVTC method has been popular. Variations in determined ileal amino acid digestibility values have been noted due to differences in:

- Collection techniques (Fuller et al. 1994).
- Basal diets (Viljoen et al. 2000; Yin and McCracken 1996).
- Dietary fibre (Darcy-Vrillon and Laplace 1985; Laplace et al. 1985).
- Laboratories (Radcliffe et al. 2005; Yin and McCracken 1996).

Indeed Fuller et al. (1994), state that each method has its strengths and limitations such that no single method is suited for all purposes, a point echoed

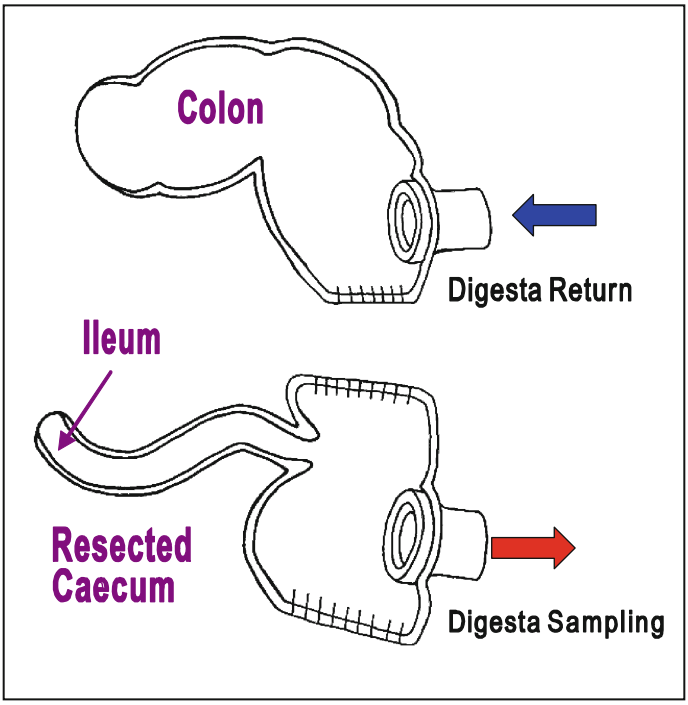

Fig. 17.6 Ileo-colic post-valve fistulation

more recently by Knudsen et al. (2006) when reviewing in vivo methods of studying the digestion of starch in pigs and poultry. Such variation is also compounded by the differing performance of indigestible markers necessary to quantify the total flow of digesta (Yin et al. 2000b). A comparative summary of the advantages and disadvantages of the various collection techniques is given in Tables 17.2 and 17.3.

Some digesta collection techniques are not suitable when the dietary fibre content is high. To avoid potentially fatal intestinal blockages some researchers have overcome this problem by regrinding the feed to a smaller mesh size (Fuller et al. 1994), but this of course may alter the digestibility of the feed.

Köhler et al. (1992a) question whether experimental animals with re-entrant cannulae or ileo-rectal anastomoses (IRA), compared to those with intact gastrointestinal tracts have similar metabolic rates. MacRae et al. (1982) found that sheep fitted with re-entrant cannulae had a higher metabolic rate than those of intact animals. With the IRA technique, Köhler et al. (1992a) argue that an increased metabolic rate would result in a lower live weight gain for the same metabolizable energy intake. Such differences in live weight gain were reported by Köhler et al. (1992a), with IRA-animals gaining only 47 % of the body mass gained by intact animals. In contrast, animals fitted with PVTC cannulae gained 93 % of the body weight gain achieved by intact animals.

Table 17.2 Advantages and disadvantages of different cannulation techniques

Ileal digesta collection method	Advantages	Disadvantages
Simple "T" cannula	Minimal disruption of normal gut activity	Indigestible marker required
	Minimal surgical trauma	Use of indigestible marker is likely to induce errors with heterogeneous digesta
	Appetite and rate of growth unaffected	
	Ileo-caecal valve remains intact	Possibility of unrepresentative sampling and marker recovery
		Blockages with high fibre diets
Post-valve T-caecum cannula	Semi-quantitative sampling	Involves the removal of gut tissue.
	Greater precision and less labour intensive than simple T-cannulation	High fibre diets cause blockages and interfere with the normal rate of digesta passing through the gut
	Greater recovery of indigestible marker	Variable recovery of digesta confirms the need for an indigestible marker
	No interference with gut wall, ileo-caecal valve or colon	
Steered ileo-caecal valve	Semi-quantitative sampling	Invasive, costly and complex surgery
	Normal flow through the gut when not sampling	Possibility of adhesions causing fatal gut obstructions
	Minimal blockages	
Simple ileo-ileal re-entrant cannula	Quantitative collection of digesta samples	Assumption of 100 % marker recovery incorrect
		Transection of the gut interrupts migrating myoelectric complex necessary for the normal passage of digesta through the gut
		Complex surgery and associated trauma
Ileo-caecal cannula	Quantitative collection of digesta samples	Assumption of 100 % marker recovery incorrect
	Placement of the distal cannula in the caecum reduces the number of blockages	Inhibition of the myoelectric complex
		Complex surgery and associated trauma
Ileo-colic post-valve fistulation	Fewer blockages	Surgical technique "too difficult and time consuming for routine measurements"
	Claims to be a reference method	
		Inhibition of the myoelectric complex
		Complex surgery and associated trauma

Table 17.3 Advantages and disadvantages of different ileal digesta collection techniques

Ileal digesta collection method	Advantages	Disadvantages
Cannulation	Simple T-cannulae cause minimal disturbance to the digestion of protein throughout the digestive tract	Use of an indigestible marker likely to introduce errors with heterogeneous digesta
	Surgery straightforward and less invasive for simple T-cannulae	Re-entrant cannulation reduces gut motility which can cause blockages
	Less adverse affects upon gut physiology for simple T-cannulae	Cannot be used for diets rich in fibre unless particle size is reduced
		Marker recovery less than 100 %
Ileorectal anastomosis	Quantitative digesta collection via the anus	Ethically less acceptable, prohibited in the Netherlands
	Use of an indigestible marker not required	Complex surgery with longer animal recovery time
	Minimal blockage and leakage	Colon has no digestive/absorption role, therefore need for electrolyte replacement
	No dietary restrictions	Colonic bypass may affect animal physiology which leads to some compensatory adaptation
		Increased microbial colonisation in gut
		Possibility of digesta reflux into colon and microbial fermentation
Slaughter technique	Technique straightforward	Possible sloughing of epithelial tissue, although this can be avoided with the correct technique
	Time required for study reduced	
	Ethically more acceptable	
	Particularly suitable for small lab animals and birds	Small volume of digesta recovered
	No dietary restrictions	Difficulty in determining the optimal time for slaughter of test animals
		One sample per animal

Using the diaminopimelic acid (DAPA), and volatile fatty acid (VFA) levels, as a measure of microbial activity in the gut, Köhler et al. (1992a) found the digesta concentration of VFA in IRA-animals was 3–8 times higher than in PVTC-animals and DAPA 1.3–2.3 times higher. Higher microbial digestive activity, he argued, might therefore affect the composition of digesta collected.

Although both re-entrant cannulae and ileo-rectal anastomoses are claimed to be quantitative methods of digesta collection, where the inclusion of an indigestible marker is unnecessary, both Köhler et al. (1990) and Fuller et al. (1994) reported that when a marker is included less than 100 % is recovered for both techniques. This may be a property of the marker itself, [e.g., the recovery of chromic III oxide (Cr_2O_3), appears to be less than that of titanium IV oxide (TiO_2)] or consequent to the digesta collection method used or the diet being studied. The percentage recovery of Cr_2O_3 is lower for T-cannulae than for PVTC cannulae and the

recovery of Cr_2O_3 is lower for diets high in fibre than for those with less fibre (Yin et al. 2000b).

For many researchers the chosen method of digesta collection is the PVTC cannula as it allows the semi-quantitative sampling of digesta (Köhler et al. 1992a) with greater precision and is less labour intensive than simple T-cannulation (Donkoh et al. 1994b). There is proportionally a greater recovery of indigestible marker (Sauer and Ozimek 1986) and there is no interference with the gut wall, ileo-caecal valve or colon (Köhler et al. 1992a). Finally, the PVTC cannula appears to interfere less with the determination of microbial activity in the GIT and the composition of the digest collected (Köhler et al. 1992a). It seems that the advantages of using the PVTC cannula far outweigh any disadvantages.

17.5 Conclusion

The digestibility of amino acids and proteins varies greatly among different pig food ingredients and between batches of a given food ingredient. Recognising and managing this variability, during the formulation of pig rations, has important economic implications. The development and practical application of true or standardised ileal amino acid digestibility (and for lysine, availability) bioassays has been one of the major technical successes in animal science of the twentieth century. All feed manufacturers should use standardised ileal amino acid digestible amino acid contents, when formulating pig feeds. Similar gains in efficiency can also be made by using an ileal as opposed to faecal (TAAA) amino acid digestibility system with poultry.

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Chapter 18
Measurement of Synthesis and Degradation of Proteins

Dingfu Xiao, Fugui Yin, Yulong Yin, Yangxiao Jiang, Jun Fang, Tiejun Li, Ruilin Huang, and Martin Nyachoti

Contents

D. Xiao • J. Fang
College of Animal Sciences and Technology, Hunan Agricultural University, Changsha, Hunan 410128, People's Republic of China

F. Yin • Y. Yin (✉) • Y. Jiang • T. Li • R. Huang
Observation and Experiment Station of Animal Nutrition and Feed Science in South-Central China, Ministry of Agriculture; Hunan Provincial Engineering Research Center for Healthy Livestock and Poultry Production; Key Laboratory of Agro-Ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, Hunan, 410125, People's Republic of China
e-mail: yinyulong@isa.ac.cn

M. Nyachoti
Department of Animal science, University of Manitoba, Winnipeg, MB, R3T 2N2, Canada

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_18,

18.1 Introduction

Continuous synthesis and breakdown or remodeling of proteins (also called protein turnover) is a principal characteristic of protein metabolism. During animal production, the net differences between synthesis and breakdown represent the actual marketable muscle foods. Because protein synthesis is highly endergonic and protein breakdown is metabolically energy dependent, the efficiency of production can be markedly enhanced by lower muscle protein breakdown rates. Various methodological approaches to study protein synthesis and breakdown, with particular emphasis toward food-producing animals, are presented. These include whole-animal tracer amino acid infusion in vivo, quantifying marker amino acid release from muscle proteins, and in vitro amino acid release-based methodologies. From such methods, protein synthesis rates and protein breakdown rates (mass units/time) may be obtained (Fouillet et al. 2002; Deng et al. 2010; Tan et al. 2010; Kong et al. 2012). The paper briefly reviews the methodology developed over the past 30 years to study protein turnover.

18.2 Marker Methods

18.2.1 Whole-Body Steady-State Tracer Method Application

The flooding dose technique for the measurement of fractional protein synthesis rates (FSR) was first described by Henshaw et al. (1971) and since modified by McNurlan et al. (1979) and Garlick et al. (1980). Stable isotope-labeled tracers have been used in the research of protein (or amino acids), lipid, and carbohydrate metabolism. The principles and practice of stable isotope tracer methodology have been described in detail (Wolfe 1992). Protein synthesis has been measured by using the luminal flooding dose technique (Adegoke et al. 1999). Whole-body tracer kinetic models assume that the amount of amino acids fed in the diet is the same as the amount of amino acids that is entering the metabolic pool from outside the body. Any removal of the intragastric tracer on the first pass through the gut (i.e., PDV) and liver will be reflected by a lower (systemic) plasma enrichment of the intra-gastric tracer compared with that achieved with the intravenous tracer.

The ^{15}N-glycine labeling (Waterlow et al. 1978) in either a continuous infusion or a single oral dose was developed as the end-products method (Fern et al. 1981), followed by the measurement of ^{15}N in urine and blood urea. The use of L-[^{15}N]-Phenylalanine provided an alternative tracer to radiolabeled amino acids for measuring in vivo rates of protein synthesis in fish using the "flooding dose" technique and also allows studies of fish protein metabolism to be carried out in environmentally sensitive (field and laboratory) conditions where the use of radiolabels is restricted (Owen et al. 1999). This method is based on several assumptions, which include the existence of a single metabolic nitrogen pool and the possibility of labeling it through the administration of an amino acid tracer. However, this

statement has proven to be inaccurate (Matthews et al. 1981). Although the end-product method has been the subject of methodological criticisms and is little used today, in some situations it represents a practical noninvasive way of estimating nitrogen kinetics, e.g., in frail or sick populations. The ^{15}N-glycine end-product method has gradually been replaced by the ^{13}C-leucine technique for measuring whole-body protein kinetics. The most commonly used isotopic method is the precursor method, based on the continuous infusion of L-[1-^{13}C]-leucine (Matthews et al. 1980). The rate of appearance (R_a) of leucine is measured in terms of tracer dilution when enrichment has reached a plateau. Achievement of the plateau is accelerated by the infusion of a primed dose of the tracer. A better estimate of intracellular leucine enrichment is obtained when its α-ketoacid (ketoisocaproate) is considered. The leucine flux from endogenous protein breakdown is calculated from the difference between R_a and intake, whereas the synthesis flux is indirectly assessed from the difference between the rate of disappearance (R_a in the steady state) and oxidative losses of leucine. Leucine oxidation is measured by monitoring $^{13}CO_2$ excretion. These classical scenario for ^{13}C-based flux analysis relies on labeling enrichments observed when the isotopic labeling has become equilibrated (Blank and Kuepfer 2010; Zamboni 2011). Metabolic flux analysis (MFA) aims at the quantification of intracellular metabolic fluxes in the biochemical networks and has evolved into several model-based approaches to assess in vivo fluxes within cell populations that cannot be measured directly (Nöh and Wiechert 2011). Using the isotopic nonstationary metabolic flux analysis (INST ^{13}C-MFA) method, time profiles of metabolite labeling patterns are measured and the transient label information along with the metabolites steady-state concentrations are used to determine the in vivo fluxes (Hasunuma et al. 2010; Noack et al. 2010). Dai et al. (2011) investigated the metabolism of selected amino acids in bacterial strains (*Streptococcus* sp., *Escherichia coli*, and *Klebsiella* sp.) and mixed bacterial cultures derived from the jejunum and ileum of pigs by using [U-(14)C]-labeled tracers.

L-[ring $^2H_5$]-phenylalanine was used as a tracer by continuous infusion to assess the rate of oxidation by enrichment at the plateau of the product of phenylalanine hydroxylation and its subsequent loss, i.e., L-[$^2H_4$]-tyrosine (Thompson et al. 1989). The total production of tyrosine can concomitantly be monitored by an infusion of L-[$^2H_2$]-tyrosine (Marchini 1993). Adegoke et al. (2003) developed an in situ experimental system that allows controlled exposure of intestinal mucosa to nutrients systemically, luminally, or both and examined the effects of systemic glucose and amino acid infusion in overnight-fasted piglets. Tissue protein synthesis was measured using a flooding dose of L-[4-(3)H]phenylalanine in neonatal pigs (Davis et al. 2002).

Tissue protein synthesis has been measured in vivo using a modification of the flooding dose technique (Fan et al. 2006). At 60 min after feeding, pigs were injected via the jugular vein catheter with 10 ml/kg body weight of a flooding dose of phenylalanine, which provided 1.5 mmol phenylalanine/kg body weight and 1 m Ci of L-[4-^{3}H]phenylalanine/kg body weight. Samples of whole blood were taken 5, 15, and 30 min after the injection of [^{3}H]phenylalanine for measurement of the specific radioactivity of the extracellular free pool of phenylalanine. Immediately

after the 30-min, blood samples were taken and pigs were given a lethal injection of sodium pentobarbital (50 mg/kg body weight). Fractional rates of protein synthesis (K_s, percentage of protein mass synthesized in a day) for each tissue were calculated as K_s (% per day) = $[S_b/S_a]\cdot(T/t)\cdot 100$, where S_b (dpm/min) is the specific radioactivity of the protein-bound phenylalanine, S_a (dpm/min) is the specific radioactivity of the tissue-free phenylalanine at the time of tissue collection, T is a constant equal to 1,440 min per day, and t is the time of labeling (in minutes) of the specific tissue. The specific radioactivity of the tissue-free phenylalanine after a flooding dose of phenylalanine is in equilibrium with the aminoacyl-tRNA-specific radioactivity. Hence, the tissue-free phenylalanine reflects the specific radioactivity of the tissue precursor pool (Frank et al. 2007).

Leucine is unique among the amino acids in its ability to promote protein synthesis by activating translation initiation via the mammalian target of rapamycin (mTOR) pathway (Yin et al. 2010; Li et al. 2011). The fractional rate of protein synthesis was measured with a flooding dose of 1.5 mmol/kg body weight of L [4-^{3}H]phenylalanine injected 30 min prior to the end of the infusion, using the denaturing gradient gel electrophoresis analysis (Dai et al. 2010; Escobar et al. 2010; Torrazza et al. 2010; Wilson et al. 2011). A continuously perfused gut loop model and intraluminal flooding dose technique including a 30-min ^{3}H-phenylalanine flooding dose was used to study the acute effects of varying luminal availability of threonine on intestinal protein and mucin synthesis in young and well-nourished pigs. This technique was shown to be acutely sensitive to luminal threonine concentration (Nichols and Bertolo 2008). Fractional rates of protein synthesis were measured in muscle and liver using the [(3)H]phenylalanine flooding-dose technique, and the results suggested that dietary arginine supplementation increased the mammalian target of rapamycin (mTOR) signaling activity in skeletal muscle, providing a molecular mechanism for explaining the previous observation that increased circulating arginine stimulated muscle protein synthesis and promoted weight gain in neonatal pigs (Yao et al. 2008; Hou et al. 2011).

Routes of tracer delivery, including comparison between the intragastric (i.g.) and the intravenous (i.v.) route, are an important methodological consideration for application of the flooding dose technique for the measurement of FSR (Davis and Reeds 2001; Fan et al. 2006). Measurements of FSR are widely carried out by both an i.p. route (Martinez 1987; Danicke 2001) and an i.v. route (Burrin et al. 1999; Nyachoti et al. 2000; Davis et al. 2002; Hunter et al. 2004). Time courses of isotopic enrichments of free and bound tracer pools and corresponding FSR in plasma, visceral organs, cardiac muscle, and skeletal muscle after an i.p. route of injection of a flooding dose of containing L-[$^2H_5$]Phenylalanine was investigated between 15 and 75 min of post-tracer injection in studies with pigs (Bregendahl 2004). A "flooding dose" of the stable isotope l-[(2)H(5)]-phenylalanine (125 mg/kg live weight) was given and frequent blood samples (permanent catheter) were collected over a 60-min period, and the results obtained showed that the flooding dose technique appeared to be suitable for distinguishing deoxynivalenol (DON)-related effects on the protein synthesis (Goyarts et al. 2006). Dänicke et al. (2006) measured porcine tissue protein synthesis employing the so-called flooding dose technique using [(2)H(5)]-phenylalanine as tracer in pig. Bregendahl et al. (2008) concluded

that FSR can be measured by the flooding dose through an i.p. or an i.v. route and that the i.p. route might underestimate FSR by the flooding dose for plasma, cardiac muscle, and skeletal muscles. This concern might be addressed by a fast regimen of sampling to be completed within 12–20 min after an i.p. route of tracer injection.

Protein synthesis was determined using the [^{2}H] phenylalanine flooding-dose technique (Yin et al. 2010). Three days after the catheter insertion and at 1 h after the last meal, all piglets were injected, through the jugular catheter, with a flooding dose of L-phenylalanine (1.50 mmol/kg body weight) containing L-[ring-^{2}H5] phenylalanine at 40 mol% (0.60 mmol/kg BW) in sterile saline. The intravenous administration of phenylalanine was completed in 5–10 s. The exact time (min) of tracer labeling was recorded from the end of the intravenous injection of phenylalanine to the time of placing tissue samples in liquid nitrogen (Deng et al. 2009). The isotopic enrichment of L-[^{2}H5]phenylalanine in the tissue-free pool was measured according to the procedures of Wang et al. (2007). Ions with mass-to-charge ratios of 91 and 96 were monitored and converted to percentage of molar enrichment (mol%) using calibration curves. The isotopic enrichment of L-[^{2}H5]phenylalanine in the free pool of tissue is in equilibrium with that in the aminoacyl-tRNA pool and is therefore an appropriate measure of fractional synthesis rate (Bregendahl et al. 2004).

Protein turnover, the result of protein synthesis and degradation, represents a dynamic process, which is of equal importance to understand physiological processes. Methods employing isotopic tracers have been developed to measure protein turnover. However, applying these methods to live animals is often complicated by the fact that an assessment of precursor pool relative to isotope abundance is required. Also, data analysis becomes difficult in case of low label incorporation, which results in a complex convolution of labeled and unlabeled peptide mass spectrometry signals. Zhang et al. (2011) presented a protein turnover analysis method that circumvents this problem using a ^{15}N-labeled diet as an isotopic tracer and developed the ProTurnyzer software that allows the determination of protein fractional synthesis rates without the need of precursor relative isotope abundance information.

Protein translation has even been dissected in single-molecule studies, albeit at lower than physiological concentrations. So what has been lacking is the ability to study the timing of translation at micromolar concentrations of components, relates partly to the conditions in cells that enable this fast and efficient process. Joseph Puglisi perfected a technology for single-molecule, real-time studies in tiny (10–21 l) reaction chambers (Kaganman 2010). The enzyme is immobilized in chambers, and the system is called zero-mode waveguides.

However, use of classical approaches to assess protein synthesis and breakdown in food animals is expensive (e.g., reagents, isotopes, and animals) and require suitable experimental animal facilities and skilled technical human resources. Thus, any of the classical isotope and marker amino acid-based approaches to follow protein breakdown are not applicable to studies involving large numbers of animals required to assess (a) genotype variations in protein metabolism efficiency for selection purposes and (b) genotype by performance enhancer interactions in growth trials.

18.2.2 Biomarker Methods

To date, none of the methods is completely satisfactory to generate comprehensive data on amino acid requirements of animals or humans. Because of many influencing factors, amino acid requirements remain a complex and controversial issue in nutrition that warrants further investigations. Benefits from the rapid advances in the emerging omics technologies and bioinformatics/biomarker discovery show great potential for obtaining in-depth understanding of regulatory networks in protein metabolism (Lin et al. 2011). The amino acid signaling pathway leading to the stimulation of protein synthesis has not been fully elucidated. Among the amino acids, leucine is considered to be a principal anabolic agent that regulates protein synthesis. Biomarker methods were used to study the role of amino acids in enhancing muscle protein synthesis in vivo. To examine the effects of enteral protein and carbohydrate on protein synthesis, biomarkers of mRNA translation were also assessed (Frank et al. 2006).

18.3 Arteriovenous Balance Methods

The arteriovenous catheterization of regions (e.g., splanchnic bed, forearm, and leg) or organs (e.g., gut and liver) allows study of their net balance in total amino acids (i.e., dietary plus endogenous amino acids) under various nutritional conditions. Such studies emphasize the importance of substrate supply to tissue metabolism, and the specialized roles by various organs in amino acid homeostasis, as well as the possible roles of specific amino acids in regional protein metabolism (e.g., Tessari et al. 2000). Organ-balance studies have shown that, in the postabsorptive state, the skeletal muscle exhibits a net negative α-amino nitrogen balance, releasing principally alanine and glutamine that are taken up by the tissues of the splanchnic bed, which has a net positive α-amino nitrogen balance. After a protein meal, the splanchnic area switches from basal net amino acid uptake to net amino acid release. The splanchnic bed extracts alanine and glutamine but releases to the periphery many other amino acids, such as BCAA, which account for the majority of muscle uptake and play a key role in postprandial muscle protein repletion. The exchange of most amino acids, such as leucine, reverts from the basal net negative balance of the postabsorptive state to a net uptake after ingestion of a mixed meal, despite a continuous net release of alanine and glutamine.

18.4 Methods Based on the Incorporation of a Tracer

Tracer incorporation studies have been widely used to measure protein synthesis in tissues and specific proteins. During such studies, isotopic tracers in the form of labeled amino acid are administered either by constant infusion or using a flooding dose method, and their incorporation into a protein pool is then quantified

(Balagopal et al. 1998). The constant infusion method requires steady-state conditions and is particularly well suited to measure the synthesis of proteins with slow turnover rates, such as those in the muscles. This method involves the infusion of a labeled tracer amino acid at a constant rate until steady-state labeling of the precursor pool for protein synthesis is achieved. However, due to the technical difficulties of measuring enrichment in the true precursor pool, i.e., the intracellular amino acyl-tRNA pool, other alternatives have been proposed, which are largely discussed elsewhere. For instance, one alternative to directly measuring the labeling of aminoacyl-tRNA is to measure the labeling of proteins with very rapid turnover in condition of isotopic enrichment quite similar to that of the synthetic precursor pool. This approach has been applied to the liver from the enrichment of apolipoprotein B-100 (Stoll et al. 1998, 1999) and to the mucosa by using the labeling of the precursor forms of sucrase and lactase (Dudley et al. 1998). A variant of this method was proposed to determine the albumin fractional synthesis rate under non-steady-state conditions by adjusting the doses of the tracer infused to produce steady state enrichment of the precursor pool (de Meer et al. 2000). *For the determination of protein synthesis rate and level, incorporation of [^{14}C]-leucine into translational products in a cell-free* system was measured on isolated pig hearts (Kassauskas et al. 2004). Stable isotope-labeled amino acid also enable the determination of the metabolic fate of individual amino acid (Stoll et al. 2006).

The flooding dose method, which involves injection of a large dose of the trace amino acid together with the tracer amino acid, may avoid the problem of sampling the true precursor pool, because it minimizes the differences in isotopic enrichment between the extracellular, intracellular, and amino acyl-tRNA pools (Davis et al. 1999). Furthermore, the flooding dose method enables rapid measurements because it rapidly increases the labeling of the intracellular amino acid pool (Davis et al. 2001), thus allowing the determination of tissue protein synthesis under acute, non-steady-state conditions of feeding (Davis et al. 1996) and hormone infusion (Bark et al. 1998). However, the large dose of the trace amino acid that needs to be injected with this method is thought to alter protein synthesis, as extensively discussed elsewhere (Rennie et al. 1994). Despite the fact that protein synthesis rates of liver-exported plasma proteins have frequently been studied in humans using either the constant infusion method or the flooding-dose technique (Caso et al. 2000), the protein synthesis of splanchnic constitutive proteins has been the subject of little investigation, particularly in the fed state (Barle et al. 1999). The studies that have been performed revealed the broad differences between protein synthesis rates in different tissues and specific proteins, which are slower in skeletal muscle and much more rapid in the gut and liver. They also emphasized the variability in the response of the different proteins pools to the ingestion of a mixed meal. Overall, quantitative aspects of protein breakdown and synthesis can be determined with isotope-tracer kinetics methodologies. Whole body protein turnover has been of interest to relate to amino acid needs, whereas in animal agriculture applications, the focus is more often on synthesis and degradation in tissues, particularly in skeletal muscles containing desirable cuts of meat (Bergen et al. 2007). Eden et al. (2011) developed a method called “bleach-chase” that was applicable to fluorescently tagged proteins to

measure protein degradation and proposed that this approach opened a way to understand proteome half-life dynamics in living cells.

18.5 Combined Methods

18.5.1 Arteriovenous Catheterization with Isotopic Tracer

The organ-balance tracer technique (sometimes applied in conjunction with sampling of the tissue under study for direct measurements of amino acid labeling) has been applied to the study of protein turnover in the intestine and liver of animals (Stoll et al. 1997; Deutz et al. 1998). The organ-balance tracer technique, which involves arteriovenous catheterization with the concomitant continuous infusion of an isotopic tracer, enables a distinction between amino acid uptake and release in a net amino acid balance; as well as the measurement of the protein turnover under steady-state conditions. It is achieved by combining repeated measurements of the tracee and tracer balances across the tissues under investigation; the first being proportional to protein synthesis whereas the second measures the difference between protein synthesis and degradation. Compared with the tracer incorporation method, the organ-balance tracer approach has the advantage of providing information on the relative rates of both protein synthesis and degradation in specific organs and tissues. Another advantage of the tracer balance technique is its dynamic nature, because it allows measurement of the changes in amino acid uptake and protein turnover over relatively short periods of time (Reeds and Davis 1999). This is a highly significant advantage to the design of studies focusing on acute nutritional or hormonal effects (Biolo et al. 1997; Volpi et al. 1999). However, the invasive tracer balance approach shares the same problem when determining the isotopic enrichment of the protein synthetic precursor pool as the continuous infusion technique. Moreover, some technical problems have been encountered using the tracer balance approach. For instance, although the accurate measurement of blood flow is of crucial importance for determining the net balance of both the tracee and the tracer, there is no consensus in the literature as to whether plasma or whole-blood measurements are preferable. In addition, the siting of the venous catheter is particularly critical when measuring muscle protein turnover by catheterization of the forearm or leg (Reeds and Davis 1999).

Compartmental analysis of organ-balance tracer data has provided new estimates of protein and amino acid kinetics across the muscle under steady-state conditions. Thus, a six-compartment model for the intracellular muscle kinetics of leucine and KIC in the human forearm was developed (Tessari et al. 1995) and used to evaluate muscle protein synthesis and degradation under different nutritional conditions (Zanetti et al. 1999). These models have also enabled calculations of the rate of inward (from artery to muscle) and outward (from muscle to vein) amino acid transport and have demonstrated that infused or ingested amino acids are capable of stimulating muscle protein anabolism by enhancing intracellular amino acid

transport and protein synthesis. They have also been able to take account of the reutilization of amino acids derived from proteolysis for protein synthesis, thus overcoming a classical limitation to the organ-balance tracer technique (i.e., recycling of the tracer).

18.5.2 Modified ELISA

Bioinformatics has been incorporated into a competitive enzyme-linked immunosorbent assay (ELISA) and was shown to be able to detect the heat-processed parent protein after digestion with either pepsin or trypsin (Reece et al. 2009). Standard indirect ELISA screenings (with immobilization of hapten–protein conjugate and use of enzyme-labeled anti-mouse IgG as tracer) was compared with direct ELISA (with antibody immobilization and use of a hapten–enzyme conjugate as tracer) for the detection of high-affinity hapten-specific monoclonal antibodies (Cervino et al. 2008). The antibodies raised against recombinant Ail protein could specifically identify pathogenic *Y. enterocolitica* strains both by indirect plate ELISA and Western blot immunoassay (Balakrishna et al. 2010). These methods have not yet been widely applied to protein breakdown and synthesis in animal nutrition studies.

18.6 Contemporary Genomic Regulation Strategies

Biochemical mechanisms and molecular regulation of protein biosynthesis and protein breakdown have been extensively documented. Proteolysis is dependent in part on the extent of expression of genes for components of cellular proteolytic machinery during skeletal muscle atrophy. It was proposed that high throughput methods, based on emerging understanding about protein breakdown, may be useful in enhancing production efficiency.

On the contrary, during skeletal muscle atrophy and sepsis, expression of genes for the components of the intracellular protein breakdown mechanism is elevated (Lecker et al. 2004; Sacheck et al. 2007). Upon induction of sepsis or muscle atrophy, a parallel increase in gene regulation of inflammation-related transcription factors and expression of several genes associated with proteolytic mechanisms has been noted, but a direct relationship between upregulation of these genes and increased proteolytic activity has not been demonstrated (Hasselgren et al. 2005). Genomic tools are becoming more available and specific swine and beef cDNA microarrays have been developed and validated (Collier and Largaespada 2007). Although transcriptional profiling is experimentally well defined, changes in mRNA abundance for any gene do not necessarily imply regulation at the level of gene expression but reflect potential capacity for synthesis of a given protein (Carey 2000). Gene expression profiles of skeletal muscle of nursing pigs was investigated using Affymetrix Porcine Gene Chip (Jiang et al. 2010).

Application of proteomics may also be useful in studying proteolytic mechanisms in breeding animals. Assessing products of protein degradation (i.e., the degradome) is another proteomic approach (Schilling 2007) that may be fruitful in quantifying

degradation rates in breeding animals. Proteomic approaches have not been widely used or validated to evaluate protein turnover in skeletal muscle. Clearly, new data and new information on regulatory and quantitative aspects of protein breakdown may be expected in the future. The combination of muscle biopsies and laboratory assessment of protein synthesis and breakdown status will be a strategy to explore many unanswered questions in breeding animal protein metabolism.

18.7 Metabonomics

In the post-genomic era, biology of systems is central to the biological sciences. Functional genomics such as transcriptomics and proteomics can simultaneously determine marked gene or protein expression changes following amino acid intervention. Metabonomics and its many pseudonyms (metabolomics, metabolic profiling, etc.) has exploded onto the scientific scene in the past few years. Metabonomics, "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modifications," combines metabolic profiling and multivariate data analysis to facilitate the high-throughput analysis of metabolites in biological samples, providing a system approach to understand global metabolic regulations of organisms (Rezzi et al. 2007). As a sensitive and powerful method, metabonomics can quantitatively measure subtle dynamic perturbations of metabolic pathways in organisms due to changes in nutritional and epigenetic states. Therefore, metabonomics holds great promise to enhance our understanding of the complex relationship between amino acids and metabolism to define the roles for dietary amino acids in maintaining health and the development of disease. Such a technique also aids in the studies of functions, metabolic regulation, safety, and individualized requirements of amino acids (He et al. 2011). Metabonomics is a rapidly growing research area and a system approach for comprehensive and quantitative analysis of the global metabolites in a biological matrix. Analytical chemistry approach is necessary for the development of comprehensive metabonomics investigations. Fundamentally, there are two types of metabonomics approaches: mass-spectrometry based and nuclear magnetic resonance methodologies. Metabonomics measurements provide a wealth of data information and interpretation of these data relies mainly on chemometrics approaches to perform large-scale data analysis and data visualization, such as principal and independent component analysis, multidimensional scaling, a variety of clustering techniques, and discriminant function analysis, among many others (Li et al. 2008).

18.8 Conclusion

Most of the methods currently used to determine protein metabolism in different dietary scenarios are tracer based. Steady-state whole-body studies in humans and animals have provided important data, but these data have been somewhat limited

in interpretation. Regional approaches to protein metabolism have enabled a clearer understanding of the different responses of splanchnic and peripheral protein metabolism to nutrient ingestion. The splanchnic zone, which undergoes very rapid turnover, seems to respond to almost all nutritional perturbations, whereas the peripheral muscle protein mass, which is slowly renewed, appears to be only slightly affected by acute dietary factors. However, these pictures become more complicated when dietary and recycled sources of amino acids or nitrogen are taken into account (Tomé et al. 2000). The techniques used at present need to be improved to address directly the still unresolved questions. Organ-balance studies allow determination of the net organ balance of amino acids, without discrimination between dietary and endogenous amino acid metabolism, whereas multiple tracer methods address the splanchnic extraction of dietary amino acid without making a distinction between the proportion used for protein synthesis and that utilized (deaminated) by the splanchnic bed (Dangin et al. 2001). The difficulties encountered in establishing the underlying mechanisms of sarcopenia illustrate well the limitations of the current approaches in the context of understanding regional protein metabolism. The widespread use of leucine in whole-body studies to quantify protein kinetics in response to dietary modulations might be called into question in view of the limited metabolism of this amino acid in the splanchnic zone. This latter metabolism is of crucial importance for postprandial protein accretion. The measurement of fluxes at the whole-body, organ or tissue levels is probably insufficiently specific to accurately detect any chronic slight dysfunction in the normal pattern of daily protein depletion–repletion cycling.

Newer, well-developed methods based on modifications of traditional approaches have emerged during the last 10 years. For example, the fluorescein isothiocyanate-labeled peptide technique is able to detect the synthesis of porcine reproductive and respiratory syndrome virus (PRRSV) (Xu et al. 2011). However, most new technologies have not been widely applied to protein breakdown and synthesis in farm animal research. Because protein breakdown and synthesis are energy-dependent processes, these processes are directly related to the efficiency of animal production. High-throughput methods are needed to study molecular regulation of protein turnover, and such understanding should be applied to regulate protein turnover during animal production.

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Chapter 19
Methods for Measuring Amino Acids of Endogenous Origin in Intestines

Yulong Yin, Zhang Yuzhe, Feng Zemeng, Ziwei Xu, Jie Feng, Bo Deng, Zhou Chuanshe, and Ruin Huang

Contents

Abbreviations

AA	Amino acids
CaR	Ca^{2+}-sensing receptor
ENL	Endogenous nitrogen loss

Y. Yin (✉) • Z. Yuzhe • F. Zemeng • Z. Chuanshe • R. Huang
Chinese Academy of Sciences, Institute of Subtropical Agriculture, Research Center for Healthy Breeding of Livestock and Poultry, Hunan Engineering and Research Center of Animal and Poultry Science, Key Laboratory for Agro-ecological Processes in Subtropical Region, Scientific Observing and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Changsha, Hunan 410125, Peoples Republic of China
e-mail: yinyulong@isa.ac.cn

Z. Xu • B. Deng
Institute of Animal Husbandry and Veterinary Science, Zhejiang Academy of Agricultural Sciences, Hang Zhou, Zhe Jiang Province 310021, Peoples Republic of China

J. Feng
The Key Laboratory of Molecular Animal Nutrition of Ministry of Education, College of Animal Science, Zhejiang University, Hangzhou, Peoples Republic of China

F. Blachier et al. (eds.), *Nutritional and Physiological Functions of Amino Acids in Pigs*,
DOI 10.1007/978-3-7091-1328-8_19,

GPRC6A	G-protein-coupled receptor family C member 6A
HA	Homoarginine
mGlu	Metabotropic glutamate
TCA	Trichloroacetic acid
VFT	Venus Fly Trap

19.1 Introduction

Evaluation of the true ileal digestibility of dietary proteins and amino acids (AA) in pigs is essential to improve growth and reduce production costs and nitrogen excretion in the environment. Several methods have been used to quantify endogenous nitrogen flow at the distal ileum of the pig. Earlier studies made use of conventional methods. These include feeding protein-free diets, feeding diets containing protein sources with an assumed 100 % digestibility, and mathematical regression techniques. More recently, isotope dilution, homoarginine, and peptide alimentation ultrafiltration techniques have been used. They all have their difficulties and limitations, as well be demonstrated in the following sections.

19.2 Feeding Protein-Free Diets Method

The classical method for measuring endogenous loss along the gastrointestinal tract in to measure fecal N excretion after feeding with N-free diet. When protein-free diets are given, all nitrogen-containing compounds found in the ileal digesta are assumed to be of endogenous origin. The main criticism of this method is its nonphysiological nature (Low 1980) that may affect the normal body protein metabolism and in turn may reduce secretion of nitrogenous compounds into the gut lumen and affect the efficiency of reabsorption (Darragh et al. 1990; Schulze 1994). The fact that animals are in a negative protein balance appears to have minor effects on endogenous loss of essential amino acids at the distal ileum of the pig (de Lange et al. 1989). In this study, only the recovery of endogenous proline, glycine, and total N was affected being reduced when amino acids rather than saline (control) were administered intravenously to animals fed a protein-free diet. As some research indicated, a protein-free diet may lack the stimulatory effect on endogenous gut protein secretions (Butts et al. 1993; Donkoh et al. 1995). This may lead to an underestimation of endogenous nitrogen loss (ENL) at the distal ileum and in feces. In addition, dietary constituents, such as fibers and other compounds, that are associated with dietary proteins, may enhance endogenous nitrogen loss. A further limitation of this approach is that it only yields information under the specific feeding conditions applied. Thus, for instance the effects of specific feed ingredients on endogenous losses, like proteins, cannot be measured.

19.3 Homoarginine Method

This method is based on the transformation of dietary lysine to homoarginine (HA) through a guanidination process (a reaction with *O*-methylisourea), which was first suggested by Hagemeister and Erbersdobler in 1985 and allows a differentiation between dietary lysine (converted to HA) and endogenous lysine (Hagemeister and Erbersdobler 1985). Homoarginine does not occur naturally in protein because it is not a substrate for protein synthesis and hence a return into the intestine of absorbed label in endogenous protein can be excluded a priori (Libao-Mercado et al. 2006).

Prerequisites for an effective use of this method include (1) uniform, or almost complete, guanidination of lysine in test protein, (2) guanidination must not affect protein digestibility, (3) HA must not be hydrolyzed to urea and lysine in the gut, (4) HA must be absorbed and metabolized like other amino acids, (5) HA must not re-enter the digestive tract, i.e., must not be used for protein synthesis, and (6) the presence of HA in the plasma or in the gut, per se, must not affect endogenous nitrogen loss (Nyachoti et al. 1997).

The suitability of this method for direct estimation of endogenous lysine flow and true lysine digestibility has been demonstrated in a number of studies (Rutherfurd and Moughan 1990) (Schmitz et al. 1991; Marty et al. 1994). However, since homoarginine is not used for protein synthesis, this technique may only be used for a short time to avoid lysine deficiency. Tews and Harper in a study in rats showed that the addition of an amino acid analogue (e.g., homoarginine) to a diet that is limiting in a natural amino acid and that has similar characteristics to that analogue (e.g., lysine) can induce an amino acid imbalance (Tews and Harper 1986). However, if HA would be rapidly hydrolyzed by arginase in the liver to lysine and urea and then if HA would serve as an effective source of lysine to the animal, then this concern would be unjustified. The fact that optimum guanidination conditions may vary for individual proteins and that near complete guanidination is not always achieved may impose some limitations on this method (Rutherfurd and Moughan 1990; De Vrese et al. 1994). So far this method has largely been used with diets having just one, purified protein source. A key requirement, however, for any method is that it has to be applied to a wide variety of feedstuffs. It needs to be determined whether this method can be equally employed to measure endogenous nitrogen flow when feeding a practical diet containing more than one protein source, and in particularly when plant proteins are evaluated. With this method, the true digestibility of amino acids other than lysine is not directly determined. Instead, they are estimated based on an assumed constant relationship between lysine and other amino acids in endogenous nitrogen (De Lange et al. 1990; Marty et al. 1994).

The main advantage with the method is that it allows the endogenous ileal excretion of lysine to be determined with the animal being fed a near-normal diet. A disadvantage is that it provides direct information about the endogenous flow of lysine only, and the flow of other amino acid are estimated by assuming a

constant ratio of lysine to the other amino acids in the endogenous protein. In addition, another disadvantage of this technique is that homoarginine, although partially transformed to lysine, accumulates in the liver over time and has toxic effect on the animal, thus restricting the duration of experiments, especially when the direct method is used. Whereas the direct method does not rely on all of the assumptions that are inherent with the indirect method, there are often difficulties in achieving 100 % homoarginine of dietary origin as required for the direct method.

Overall, the homoarginine method appears to offer considerable promise for the determination of endogenous ileal amino acid flows. Endogenous flows can be determined in animals that are in a physiologically normal state, after ingestion of a wide range of dietary protein sources. Further studies are required to fully establish the validity of some of the assumptions inherent in the method. Comparisons of endogenous ileal lysine flows determined using the homoarginine and protein-free diet approaches have shown a lower lysine flow with the protein-free diet method (Moughan et al. 1990).

19.4 Enzymatically Hydrolyzed Casein Method

In this method (also referred to as Peptide Alimentation Ultrafiltration Method), first suggested by Moughan and Rutherfurd, animals are fed a diet in which the sole nitrogen source is enzymatically hydrolyzed casein (Moughan et al. 1990). Hydrolyzed casein contains approximately 56 % free amino acids and 41 % di- and tri-peptides with molecular weights of less than 5,000 Da (Leterme et al. 1996; Yin et al. 2004, 2008).

It is assumed that all endogenous nitrogen is present in fractions with molecular weights greater that 10,000 Da. Therefore, by separating low (<5,000 Da) and high (>10,000 Da) molecular weight nitrogen compounds in the ileal digesta, a distinction is made between any unabsorbed exogenous nitrogen and nitrogen of endogenous origin (Moughan et al. 1990).

In order to achieve this separation, protein-precipitating agents like perchloric acid and trichloroacetic acid have been tested but found to be unsuitable (Darragh et al. 1990).

As an alternative, Moughan et al. suggested separation of protein in ileal digesta immediately after collection by centrifugation and ultrafiltration in which low molecular fractions (amino acids and peptides) are removed with the supernatant. Any free amino acids and small peptides of endogenous origin present in ileal digesta are discarded as well (Moughan et al. 1990).

However, according to Moughan and Schuttert free and peptide-bound amino acids are only a small portion (11 %) of ENL (Moughan and Schuttert 1991). This suggests that any endogenous nitrogen loss in the supernatant is likely to result in slight underestimations of ENL. However, the exact proportion of these components needs to be clearly determined to enhance the reliability of this method. Some research reported values of 21 % and 9.2 %, respectively, suggesting the presence of considerable variation in the contribution of free and peptide-bound amino acids

to ENL (Butts et al. 1992; Marty et al. 1994). Loss of material during sample preparation (centrifugation and ultrafiltration) may also lead to lower estimates with this method (Butts et al. 1993). A major restriction of the peptide alimentation ultrafiltration method is the fact that it cannot be used to estimate endogenous protein secretion since there is a direct association between protein and dietary factors that are known to influence endogenous secretions and ENL (e.g., in diets containing practical ingredients and/or with high levels of ANFs). Donkoh indicated that this method can be applied to feeds containing animal protein sources such as meat and bone meal (Donkoh et al. 1995).

A limitation of the method is that low molecular weight fractions of endogenous or endogenous origin, like peptides, free amino acids, urea, and ammonia (which account for 10–20 % of total digesta) are discarded (Butts et al. 1992, 1993).

19.5 Stable Isotopes Labeled Endogenous Nitrogen Methods

The use of stable isotopes allows the determination of endogenous protein flows while the animal is fed a diet that contains protein. The use of ^{15}N and ^{13}C seems most appropriate for this purpose. What can be used is the labeling of the dietary (Leterme et al. 1994) or animal nitrogen pool (Schulze 1994) with distinction between endogenous nitrogen and the proportion of endogenous nitrogen in the digesta and calculation from dilution of the isotope.

The disadvantage of labeling the dietary compounds is that contamination of endogenous secretions starts almost immediately after feeding (Schulze et al. 1995), though sequential slaughtering of animals shortly after the introduction of the labeled dietary is possible in rats (Moughan et al. 1992). However, with farm animals this approach may not be applicable because of the expensive cost and thus difficulty to repeat measurements. So labeling the animal or at least the precursor pool for endogenous secretions is commonly used.

Labeling the animal's precursor pool usually involves continuous intravenous infusion of labeled amino acids and then measurement of the amount of labeled amino acids present in the ileal digesta relative to the labeling in the precursor pool for the measurement of the endogenous gut protein synthesis. The deproteinized fraction of blood plasma is generally considered to be the precursor pool (Moughan et al. 1992). ^{15}N-labeled leucine is the most commonly used amino acid as it is presumed to be readily metabolized resulting in labeling of other amino acid by transamination. However, ^{15}N-labeled leucine is not transferred to lysine or threonine, and the isotopic enrichment of other amino acids is less than that of the labeled leucine (Lien et al. 1997). Alternatively, the use of mixtures of ^{15}N-labeled amino acids may solve the problem, but the cost is very expensive (Yin et al. 2000).

It is very important to choose an appropriate precursor pool (Moughan et al. 1992). The TCA-soluble blood plasma fraction is commonly used to indicate ^{15}N enrichment. It is assumed that the free amino acid pool in the TCA-soluble fraction is used for the synthesis of body protein and thus for most of the endogenous secretions into the digestive tract. The assumption is not completely true, however,

for small intestinal mucosa secretions, in which case absorbed dietary amino acids are used directly for protein synthesis (Leterme et al. 1994). Therefore, plasma enrichment underestimates the enrichment of endogenous nitrogen secreted into the intestinal lumen which leads to an overestimation of endogenous protein excretion (Lien et al. 1997). More work is required to isolate the actual precursor pool for endogenous secretion.

A limitation with the ^{15}N method is that only endogenous nitrogen is directly measured, not the amino acid. Thus to calculate the endogenous amino acid flows, it must be assumed that there is a constant ratio in the endogenous material between nitrogen and the amino acid.

The ^{15}N-isotope dilution technique is a valuable method for studying factors that influence the recovery of endogenous protein in digesta, such as antinutritional factors and plant fiber, but requires modification to improve accuracy.

The stable carbon isotope ^{13}C has also been investigated for the determination of endogenous protein flows. When infused intravenously or given orally to the animals, ^{13}C is incorporated into body proteins (Minson et al. 1975; Kong et al. 2012).

Atmospheric carbon dioxide contains approximately 1.1 % of the heavier isotope ^{13}C and 98.9 % of the lighter ^{12}C. Plants discriminate between ^{12}C and ^{13}C during photosynthesis in ways that reflect plant metabolism (O'Leary 1981). Plants that fix carbon dioxide via the dicarboxylic acid pathway (C4 pathway) show less isotopic discrimination than plants that fix carbon dioxide via the Calvin cycle(Minson et al. 1975). In plant physiology, ^{13}C abundances are commonly expressed as a delta ^{13}C value in parts per thousand or parts per mil (‰). Absolute values are difficult to obtain, and for most purposes, it is adequate to give delta ^{13}C values relative to carbon from a standard. Therefore, feedstuffs that are normally used to formulate diets differ in the degree of labeling with ^{13}C, so that diets can be prepared with different abundances of ^{13}C. The endogenous ileal protein flows in pigs by feeding ^{13}C-labeled dietaries has been studied and the results obtained demonstrate that the endogenous flows are similar to those previously obtained using ^{15}N (Arentson and Zimmerman 1995). Leterme et al. compared endogenous protein flows determined following intravenous infusion of ^{13}C-leucine with infusion of ^{15}N-leucine in the pig and found lower values for endogenous ileal nitrogen flows with the ^{13}C method i.e. 1.4 vs. 5.7 mg/g dry matter intake for the ^{13}C and ^{15}N method, respectively. This difference in endogenous flows between the two labels was suggested to be due to an overestimation of endogenous N with ^{15}N due to underestimation of the enrichment of the endogenous N which occurs when the TCA-soluble blood plasma fraction is used as the precursor pool, as described above. The use of ^{13}C for the determination of endogenous protein flows has the same disadvantages as ^{15}N in terms of recycling of the label and the determination of an appropriate precursor pool, but the advantage of using ^{13}C over ^{15}N is the lower cost of labeling the experimental diets. When ^{13}C-leucine is intravenously administered to the animal, however, the ^{13}C cannot be transferred to other amino acids, so that only the endogenous flow of leucine can be determined (Leterme et al. 1998).

19.6 Model of Association Between Amino Acid-Sensing Genes and the Amino Acids in Plasma

A group of extracellular amino acid sensors has recently been identified within class 3 of the G-protein-coupled receptor superfamily (Feng et al. 2012). These sensors control G-protein-regulated signaling pathways and are widely distributed. They seem to be designed for the detection of subgroups of amino acids or even specific amino acids (including the acidic amino acid, L-glutamate and the metabotropic glutamate (mGlu) receptors). They also include several broad-spectrum amino acid sensors; the extracellular Ca^{2+}-sensing receptor (CaR), which responds to aromatic, aliphatic, and polar amino acids (but not to positively charged or branched-chain amino acids) (Conigrave et al. 2000); a heterodimeric taste receptor composed of the T1R1 and T1R3 receptors (which respond to aliphatic, polar, charged, and branched-chain amino acids but not to aromatic amino acids) (Nelson et al. 2002); and finally the goldfish 5.24 receptor (Speca et al. 1999; Kuang et al. 2003) and its putative mammalian ortholog, G-protein-coupled receptor family Cmember 6A (GPRC6A) (Wellendorph and Bräuner-Osborne 2004; Kuang et al. 2005; Wellendorph et al. 2005), which respond to basic, aliphatic, and polar amino acids.

The binding of amino acids to these receptors, including CaR, GPRC6A, and the T1R taste receptors provide mechanisms by which changes in the intestinal and plasma levels of amino acids regulate digestion, absorption, growth and metabolism; and mechanisms by which oral amino acids mediate the sensation of umami taste. Amino acid binding depends on highly conserved residues in the N-terminal VFT domains that bind the a-amino and a-carboxylate functional groups.

The VFT domains mediate amino acid binding in the class 3 GPCRs. These bilobed structural motifs have an ancient lineage providing the basis of nutrient sensing by the periplasmic binding proteins of Gram-negative bacteria (Felder et al. 1999).

The CaR shares pluripotency along with 3 GPCRs that mediate taste (Conigrave and Brown 2006). With various signals to process and numerous signaling pathways to access, the CaR acts as a multichannel switch box, providing ligand selective control of physiological responses. The CaR's response to amino acids is dependent not only on variations in amino acid concentration to which the receptor is exposed but also on the prevailing Ca^{2+}concentration. Indeed, the different amino acids exhibit differential Ca^{2+} concentration thresholds for receptor activation.

The CaR is widely expressed in mammalian tissues including cells with recognized roles in amino acid sensing. These include acid-, enzyme-, and hormone-secreting cells of the stomach and small intestine, the liver, exocrine and endocrine cells of the pancreas, and endocrine cells of the anterior pituitary and parathyroid (for a review, see Hofer and Brown 2003). It is also expressed in glia and neurons in the brain (Ruat et al. 1995; Chattopadhyay et al. 1997, 1998).

CaR is selective for aromatic amino acids because these amino acids are its most potent activators (Conigrave et al. 2000). However, when the plasma concentrations of different amino acids are taken into consideration (aliphatic and polar amino acids $>$ aromatic amino acids), CaR can be recognized as a broad-spectrum amino

acid sensor that is "tuned" to respond to comparable relative changes in 6–10 of the genetically encoded amino acids including L-Phe, L-Trp, L-Tyr, L-His, L-Thr, L-Ser, L-Ala, L-Gln, L-Asn, and Gly (Conigrave et al. 2004).

Although CaR is a broad-spectrum amino acid sensor, it is not a universal amino acid sensor. Thus, it is significantly less sensitive to the acidic amino acids aspartate and glutamate and is essentially unresponsive to basic and branched-chain amino acids. This means that universal amino acid sensing would require coexpression of the CaR with one or more additional amino acid sensors such as GPRC6A or the amino acid-sensing T1R1/T1R3 taste receptor (Nelson et al. 2002).

Activated CaR markedly stimulate gastric acid production (Busque et al. 2005). It seems likely that amino acid dependent activation of CaR also modulates the secretion of hormones involved in the control of calcium metabolism, growth, nutrient disposition, and intestinal digestion and absorption of nutrients. GPRC6A was classified as an orphan class 3 GPCR until the recent demonstration that both the mouse and human orthologs are activated by amino acids (Kuang et al. 2005; Wellendorph et al. 2005).

CaR can integrate signals arising from distinct classes of nutrients: mineral ions and amino acids.

The extracellular CaR is activated by a variety of divalent and polyvalent cations that act as receptor agonists (Hofer and Brown 2003). In addition, recent work indicates that the CaR is allosterically activated by L-amino acids (Conigrave et al. 2000). These effects are stereoselective for natural (i.e., L-) isomers of CaR activated by amino acids including aromatic amino acids such as Phe, Trp, and His as well as aliphatic and polar amino acids. Branched-chain amino acids are almost inactive, and charged amino acids, including arginine and lysine, are much less effective than aromatic and other amino acids (Conigrave et al. 2000). The CaR Venus flytrap domain, but none of its other domains, is required for L-amino acid sensing (Mun et al. 2004). Ca^{2+} and L-amino acid-sensing function of CaR can be dissociated (Mun et al. 2005). Mutational analysis of conserved binding residues in the CaR Venus flytrap domain has led to the identification of two mutants, T145A and S170T, which selectively impair L-amino acid sensing, leaving extracellular Ca^{2+} sensing intact. Furthermore, the double mutant T145A/S170T selectively disables amino acid sensing (Mun et al. 2005).

The CaR is, however, the only member of this receptor family identified thus far to select for aromatic L-amino acids and to respond more generally to a large number of L-amino acids.

The CaR is widely expressed in mammalian tissues and is expressed at high levels in endocrine cells such as parathyroid chief cells, thyroid C cells, and anterior pituitary cells (Brown and MacLeod 2001). In the brain, the CaR is expressed in the ionic strength-sensing subfornical organ that provides inputs to hypothalamic centers that control antidiuretic hormone secretion, various other organs including the hippocampus and myelin-producing oligodendrocytes.

In the GI tract, the CaR is expressed at high levels on the basolateral membranes of gastric parietal cells (Cheng et al. 1999). It provides an explanation for the recognized phenomena of Ca^{2+}-dependent and amino acid-dependent activation of

acid secretion (Busque et al. 2005), and the CaR's selectivity for aromatic amino acids provides an explanation for the recognized selectivity of gastric acid secretion for aromatic amino acids. The CaR is also expressed in epithelial cells and enteroendocrine cells of the small intestine (Ray et al. 1997). It seems plausible that the CaR mediates the known effects of aromatic amino acids, on cholecystokinin release and thus bile flow and pancreatic enzyme secretion (Conigrave and Brown 2006).

GPRC6A shows the highest degree of amino acid sequence identity to the goldfish 5.24 chemosensory receptor (41 %), which has a broad spectrum of amino acid sensing and an apparent preference for the basic amino acids L-Arg and L-Lys (Speca et al. 1999). The 5.24 receptor is expressed in the olfactory epithelium and is thought to have a role in feeding-related navigation by sensing gradients of amino acids in water.

GPRC6A is widely expressed in mammalian tissues including brain, skeletal muscles, heart, lung, spleen, kidney, and liver (Kuang et al. 2005), although its roles in these tissues are currently unknown.

Three alternatively spliced forms of GPRC6A are present in both the mouse and the human; two of these are short forms that have inframe deletions of all, or parts, of exons 3 and 4, which code for sequences in the Venus Fly Trap (VFT) domain. Surprisingly, whereas the mouse long protein isoform is readily expressed on the cell surface of transfected HEK293 cells, the corresponding human isoform is expressed at much lower levels (Kuang et al. 2005; Wellendorph et al. 2005).

Both GPRC6A and the goldfish 5.24 chemosensory receptor have broad-spectrum amino acid sensing with greatest sensitivities to the basic amino acids L-Arg and L-Lys.

GPRC6A also responds sensitively to polar and aliphatic, but not other, amino acids (Kuang et al. 2005; Wellendorph et al. 2005). These findings seem to indicate that the 5.24 receptor is more tolerant of variations in the size, shape, and even charge of the amino acid side chain than is GPRC6A.

Much less is known about the expression and function in enteroendocrine cells of heterodimers of T1R1 and T1R3, thought to act as broadly tuned amino acid sensors in the mouse (Nelson et al. 2002; Wellendorph et al. 2009).

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