Advances in Experimental Medicine and Biology 1332

Guoyao Wu *Editor*

Amino Acids in Nutrition and Health

Amino Acids in Gene Expression, Metabolic Regulation, and Exercising Performance

Springer

Advances in Experimental Medicine and Biology

Volume 1332

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2019 Impact Factor: 2.450 5 Year Impact Factor: 2.324

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-030-74179-2 ISBN 978-3-030-74180-8 (eBook)
https://doi.org/10.1007/978-3-030-74180-8

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

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Regulation of Gene Expression by Amino Acids in Animal Cells

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Nirvay Sah, Guoyao Wu, and Fuller W. Bazer

Abstract

Amino acids have pleiotropic roles in animal biology including protein and glucose synthesis, cellular metabolism, antioxidant reactions, immune enhancers, and inducers or suppressors of gene expression. Recent studies have revealed important roles of amino acids in the regulation of gene expression in animals. Discoveries of cellular amino acid sensors and their mechanistic pathways have broadened our understanding of how the body responds to the deprivation of nutrients and amino acids in particular. Alterations in concentrations of extracellular amino acids can modulate transcription, translation, posttranscriptional modifications, and epigenetic regulation of genes and proteins. Cells have intracellular amino acid sensors, for example, Sestrin2 for leucine and CASTOR2 for arginine, that respond to sufficiency or deficiency in amino acids, thereby inhibiting or activating downstream signals for gene expression, respectively. The sufficiency of an amino acid in cells ensures its binding to cognate sensors and suppression of inhibitors of MTOR, leading to increased global protein synthesis. On the other hand, deprivation of amino acids activates the amino acid response pathway (GCN2-eIF2a-ATF4), leading to increased selective translation of the activating transcription factor 4 (ATF4). Deficiency of an amino acid itself or via the action of ATF4 suppression of MTORC1 activity limits global protein synthesis. ATF4, in response to low concentrations of cellular amino acids, mediates the transcription of groups of genes such as those for amino acid transport and biosynthesis (*ASNS, CAT-1, SNAT2*), autophagy (*ATG3, ATG10, ATG12*), and serine–glycine synthesis (*PHGDH, PSAT1, PSPH, MTHFD2*). Long-term amino acid starvation has a pronounced effect on cells: suppressed expression and translation of genes required for normal cell growth and metabolism and enhanced expression of genes required for cell adaptation and survival. Levels of amino acids also affect the posttranslational modifications of proteins through mechanisms such as acetylation, ADP-ribosylation, disulfide bond formation, glutamylation, and hydroxylation.

Keywords

Amino acids · ATF4 · Gene expression · MTOR · Protein synthesis

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1332,
https://doi.org/10.1007/978-3-030-74180-8_1

Abbreviations

4EBP1	EIF4E-binding protein 1
AA	Amino acid
AAR	Amino acid response
ADPr	ADP-ribose molecules
ARTs	ADP-ribosyltransferases
ASNS	Asparagine synthetase
ATF4	Activating transcription factor 4
CARE	C/EBP-ATF4 response element
CASTOR1	Cytosolic arginine sensor of MTOR
CAT	Cationic amino acid transporter
CHOP	C/EBP homology protein
CREB	CAMP-response element-binding protein
C/EBP	CCAAT/enhancer-binding protein
eIF2a	Eukaryotic translation initiation factor 2A
GAP	Guanosine triphosphatase-activating protein
GATOR	GAP activity toward Rags
GCN2	General control nonderepressible 2
MTOR	Mechanistic target of rapamycin
PEPCK1	Phosphoenolpyruvate carboxykinase-1
PTMs	Posttranslational modifications
RagA/B	Ras-related GTP-binding proteins A and B
RPS6	Ribosomal protein S6
RPS6K1	Ribosomal protein S6 kinase-1
SIRT1	Sirtuin 1
SNAT	Sodium-dependent neutral amino acid transporter

1.1 Introduction

Amino acids (AAs) organic substances have both amino and acid groups. The importance of AAs for the growth, development, and maintenance of an organism cannot be overemphasized. For decades, AAs have been recognized as an indispensable nutrient for growth, development, maintenance, and function of animals (Wu 2018). They have been most valued because of their role in protein synthesis. In addition, AAs have several other important functions in the body. For example, Ala, Asn, Arg, Val, Met, Gln, and Asp can be used for the synthesis of glucose during starvation, while Lys and Leu can be used to make ketone bodies as an alternative energy source for the body (Wu 2013). The AAs themselves or metabolites of Glu, Gly, Trp, and Tyr are used directly as neurotransmitters or indirectly in their synthesis. Ammonia detoxification in the body is facilitated by Asp, Arg, Orn, Asn, and Glu. Amino acids (e.g., Cys, β-Ala, Pro, Cit, Cys, His, Met, and Trp) or their metabolites (e.g., glutathione, creatine, and melanin) protect cells from oxidative damage. Leu, Arg, and Thr modulate immune responses in the body, with Arg being a mitogen for T-cells (Ren et al. 2018). The synthesis of purines and pyrimidines required for DNA duplication is carried out in the presence of Asp, Gln, Gly, Pro, and Ser. These AAs are abundant in animal-sourced products (Li and Wu 2020). Furthermore, Arg, Asn, Gln, Met, and Pro or their metabolites regulate expression of genes required for normal body functioning (Wu 2009).

Our understanding of the role of AAs in the regulation of gene expression has been increasing over the past decades, and it is now well known that AAs can modulate gene expression at several steps including chromatin modification, transcription, translation, and posttranslational modifications to enhance protein stability and expression (Averous et al. 2016; Manjarín et al. 2020; Wu 2009). Normal concentrations of both essential and so-called non-essential AAs are required for proper gene expression to support physiological functions of the body. However, when cells in the body are exposed to imbalanced or abnormal levels of AAs, they respond by modulating the normal biosynthetic process and switching from cell growth and development to cell survival. First, the cell activates the general AA control (GAAC) pathway leading to the translation of activating transcription factor 4 (ATF4) mRNA. The ATF4 then selectively upregulates the expression of genes for AA transport and enzymes for de novo synthesis of AAs in a cell-specific manner (Wu 2013). The cells have increased apoptosis to recycle the non-essential cellular contents (Chen et al. 2014).

Long-term starvation of AAs leads to a global decrease in protein synthesis via inhibition of the mammalian target of rapamycin complex 1 (MTORC1) and increased transcription of genes for cell survival. This review aims to summarize how AAs regulate gene expression by regulating gene transcription, mRNA translation, post-translational modifications of proteins, and epigenetic modifications at the molecular level.

1.2 Amino Acids and DNA Transcription

Amino acids can act as a nutrient signal to regulate gene transcription. While the limitation of some AAs can stimulate the transcription of downstream genes, a deficiency in other AAs can negatively impact mRNA synthesis. The cell has nutrient-sensing mechanisms that respond to AA sufficiency and deficiency. In general, adequate levels of cellular AAs lead to activation of the MTOR cell signaling pathway, which results in increased DNA transcription, mRNA translation, and protein synthesis and maturation. By contrast, the cell also senses the deficiency or insufficiency of an essential AA (required for a particular cell type, for example, asparagine for acute lymphoblastic leukemia) and activates the AA response (AAR) pathway, leading to a decrease in global protein synthesis (Kilberg et al. 2005).

1.2.1 Transcription Factor Assembly

With the deprivation of essential or so-called non-essential AAs to maintain normal cellular functions, the cells respond by increasing the transcription of unique adaptive genes. One of such several genes is ATF4, which remains minimally translated at normal nutritional levels. ATF4 is a transcription factor that codes for the cAMP-response element-binding protein (CREB). A human liver cell line cultured with complete AA deprivation or a histidine deficiency alone increased ATF4 mRNA by two to threefold within 12 h of culture (Siu et al. 2002). This transcriptional increase in ATF4 further promotes the transcription of several other genes in response to a cellular nutrient deficiency, such as AA transporters (Siu et al. 2002). Furthermore, the transcriptional response to the limitation of individual AAs is different for different AAs. For example, His limitation promotes the synthesis of C-X-C motif chemokine ligand 10 (*Cxcl10*) gene, whereas a Leu or Lys insufficiency induces CCAAT/enhancer-binding protein (C/EBP) homology protein (*Chop*) mRNA transcription (Palii et al. 2009). The transcriptional change in ATF4 with AA deprivation may be transient, and gene expression can be normalized with the re-supply of AAs. Abrogation of ATF4 expression in tumor cells fails to activate the upstream GCN2 (general control nonderepressible 2; a serine/threonine-protein kinase) that phosphorylates eIF2a (eukaryotic translation initiation factor 2A) and inhibits mRNA translation (Ye et al. 2010). Therefore, the GCN2-eIF2a-ATF4 is an important component of the AA response pathway (Fig. 1.1).

1.2.2 Promoter Activity

An AA deficiency triggers the transcription of a wide array of genes, downstream of ATF4, involved in AA transport, intermediary metabolism, oxidative stress, and energy metabolism to cope with AA starvation. Some major genes for which expression is stimulated by ATF4 (reviewed by Kilberg et al. 2005) include asparagine synthetase (ASNS), cationic AA transporter (CAT-1), CHOP, and sodium-dependent neutral amino acid transporter-2 (SNAT2). These and other AAR genes have a C/EBP-ATF4 response element (CARE) sequence. Following AA deprivation, upregulation of *ASNS*, *CAT-1,* and *SNAT2* transcription occurs with ATF4 binding to the CARE region to induce recruitment of general transcriptional machinery. Interestingly, transcription of CHOP is activated upon binding to phosphorylated ATF4, and then CHOP may further stimulate or inhibit gene transcription (Kilberg et al. 2005). ATF4 can also lead to increased expression of

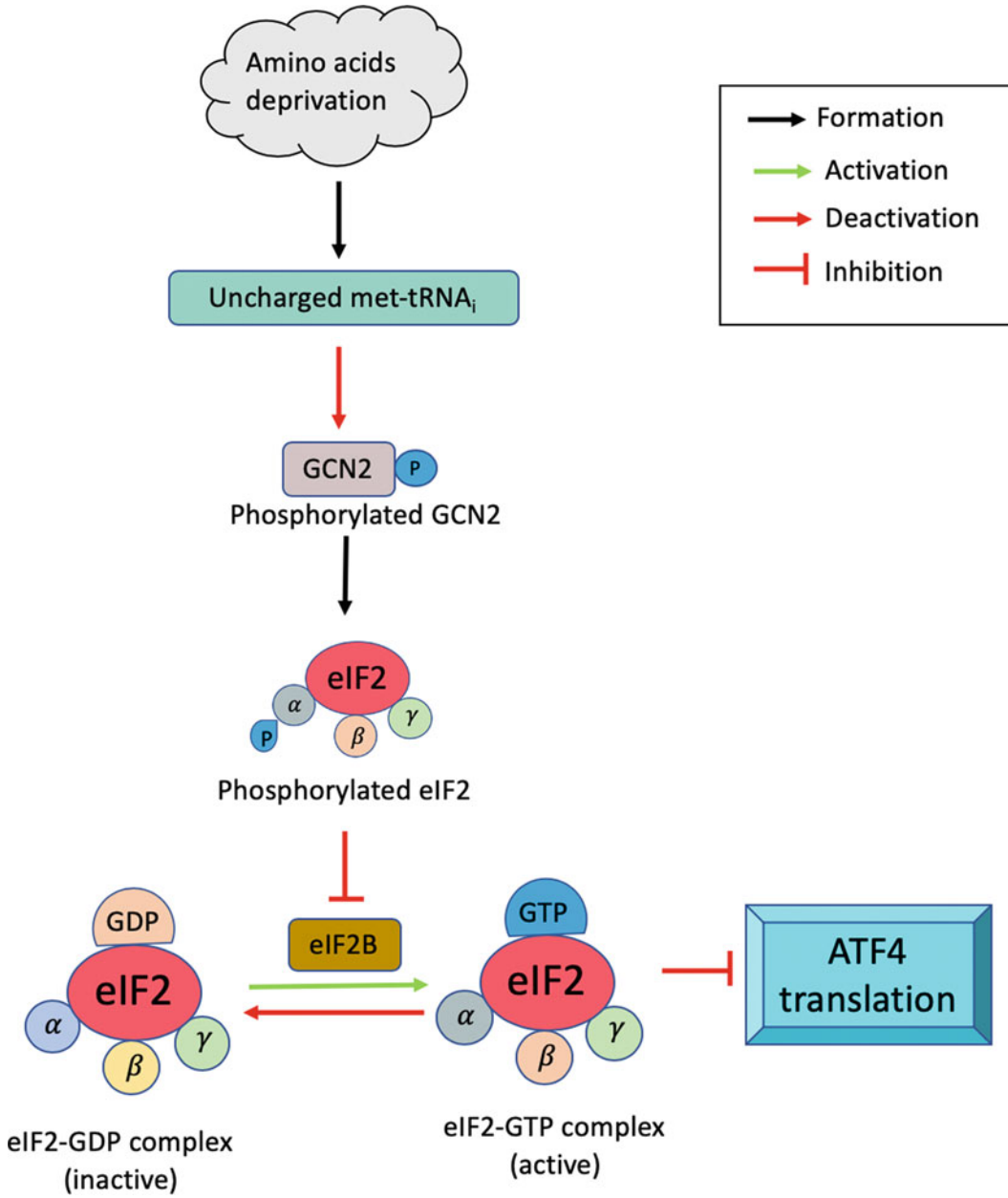

Fig. 1.1 Amino acid deprivation induces translation of ATF4 via the amino acid response pathway

enzymes involved in serinogenesis to produce serine and glycine, as seen in fibroblasts (Selvarajah et al. 2019). Both serine and glycine are critical substrates for one-carbon metabolism to regulate cell survival (Bazer et al. 2021; Seo et al. 2021), but are relatively deficient in plant-sourced foods (Hou et al. 2019; Wu 2020). Also, ATF4-induced increases in expression of MTHFD2 (methylenetetrahydrofolate dehydrogenase 2) for purine biosynthesis in mouse embryonic fibroblast (MEF) cells have been reported; however, ATF4 was expressed in response to growth factors with signals downstream of MTORC1, unlike eIF2a induction

resulting from AA deprivation (Ben-Sahra et al. 2016). ATF4 increases the expression of regulated in development and DNA damage responses 1 (REDD1) and Sestrin2 in cases of a Leu deficiency for suppression of MTORC1 in rat liver (Xu et al. 2020). The AAR pathway induced by AA deprivation in vitro also leads to transcriptional activation of many autophagy-related genes such as *p62* (nucleoporin 62), *Atg10* (autophagy-related 10), *Atg3*, and *Atg12* (B'chir et al. 2013; Chen et al. 2014) (See Fig. 1.2).

1.3 Amino Acids and mRNA Translation

The process of mRNA translation involves three basic stages for completion: initiation (leading to the formation of translationally active 80S initiation complex), elongation (addition of AA residues to the peptide chain), and termination (encountering a stop codon and completion of peptide elongation followed by dissociation of the 80S ribosome) (Wu 2013). The initiation stage is critical for translation, and therefore, it is

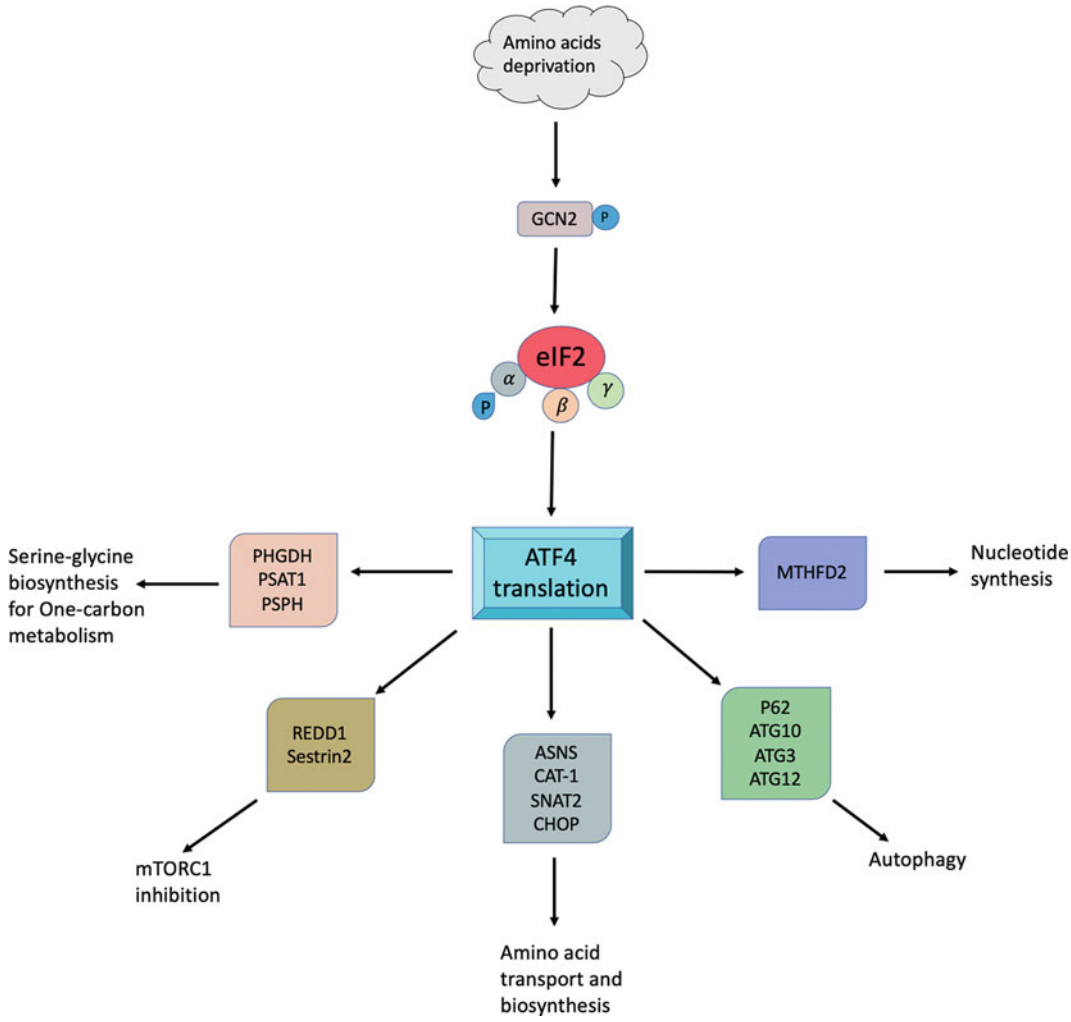

Fig. 1.2 Amino acid response pathway via GCN2-eIF2-ATF4 mediates transcriptional activation for cell survival

the most-studied stage with several known regulatory mechanisms involved. For initiation of translation, the initiator methionyl-tRNA (met-$tRNA_i$) must bind to the 40S ribosomal unit forming the 43S preinitiation complex, and then the ternary complex eIF4F (eIF4A, eIF4E, eIF4G) binds with the mRNA followed by association with the 43S preinitiation complex to form the 48S initiation complex at the start codon. The next step is the recruitment of ribosomal protein S6 (RPS6) to the 48S initiation complex followed by joining of the 60S ribosome to form the translationally competent 80S initiation complex for elongation.

When concentrations of nutrients in plasma are normal, cells have normal protein synthesis that is regulated by AA sufficiency (see Fig. 1.3). There are different mechanisms to sense different AAs, at least for Leu and Arg (discussed later in this review); however, protein synthesis is regulated at three checkpoints of translation, namely hypophosphorylation of eIF2a, hyperphosphorylation of 4EBP1 (eIF4E-binding protein 1), and hyperphosphorylation of RPS6. The activated eIF2-GTP enables the binding of initiator methionyl-tRNA (met-$tRNA_i$) to the ribosome complex for translation. The generation of eIF2-GTP from eIF2-GDP is facilitated by eIF2B; however, the phosphorylated eIF2a subunit is an inhibitor of eIF2B activity. Therefore, for normal protein synthesis to occur, a continuous hypophosphorylated form of eIF2a is required. The initiation of translation is also mediated by the phosphorylation of 4EBP1. In essence, eIF4E is bound to unphosphorylated 4EBP1 and thus remains in a dormant state as an eIF4E-4EBP1 binary complex. Phosphorylation inactivates 4EBP1 and prevents binding to eIF4E allowing eIF4E the opportunity to bind with eIF4G forming the active eIF4F complex that mediates mRNA binding. Formation of the translationally competent 80S initiation complex is mediated by RPS6. RPS6 is inactive in its phosphorylated state that requires the RPS6 kinase-1 (RPS6K1). Therefore, hypophosphorylation leads to the activation of RPS6 and its binding to form the 80S initiation complex.

1.3.1 Amino Acid Regulation of eIF2a Phosphorylation

The role of AAs in the regulation of mRNA translation has been studied by using AA deprivations in cell culture, tissue perfusion, and whole-animal models. In response to a specific or complete AA limitation, the cells alter their normal pathways of protein synthesis via modulation of translation initiation and elongation factors resulting in a decrease in global protein synthesis with the selective translation of specific proteins or families of proteins. AA deprivation leads to the elevated concentrations of uncharged tRNAs, which bind to and activate GCN2. The GCN2 in turn phosphorylates eIF2α at Serine 51 and inactivates eIF2α and thus inhibits the initiation of translation, leading to decreased global protein synthesis (Palii et al. 2009; Zhang et al. 2002) (see Fig. 1.3). Fibroblast cells devoid of Leu or Arg, for example, increase the phosphorylation of eIF2α mediated by GCN2 (Averous et al. 2016). However, cellular stress such as insufficiency of AAs can selectively lead to increased translation of unique mRNAs (such as ATF4) that may be the mammalian master regulator of nutrient sensing and the transcriptional regulation of genes in response to cellular nutritional stress (Vattem and Wek 2004, Palii et al. 2009). Translation of ATF4 is repressed when cellular levels of eIF2-GTP are normal. This repression is lifted when CGN2 phosphorylates eIF2a, which deactivates eIF2B, a transcription factor that ensures the formation of the eIF2-GTP complex.

1.3.2 Amino Acids Regulate mRNA Translation via an MTOR Dependent Pathway

The MTOR, composed of MTOCR1 and MTORC2, is a protein kinase with a central role in metabolism, growth, and disease (reviewed by Saxton and Sabatini 2017). The MTORC1 regulates cellular metabolism and growth, while MTORC2 is required for cellular proliferation

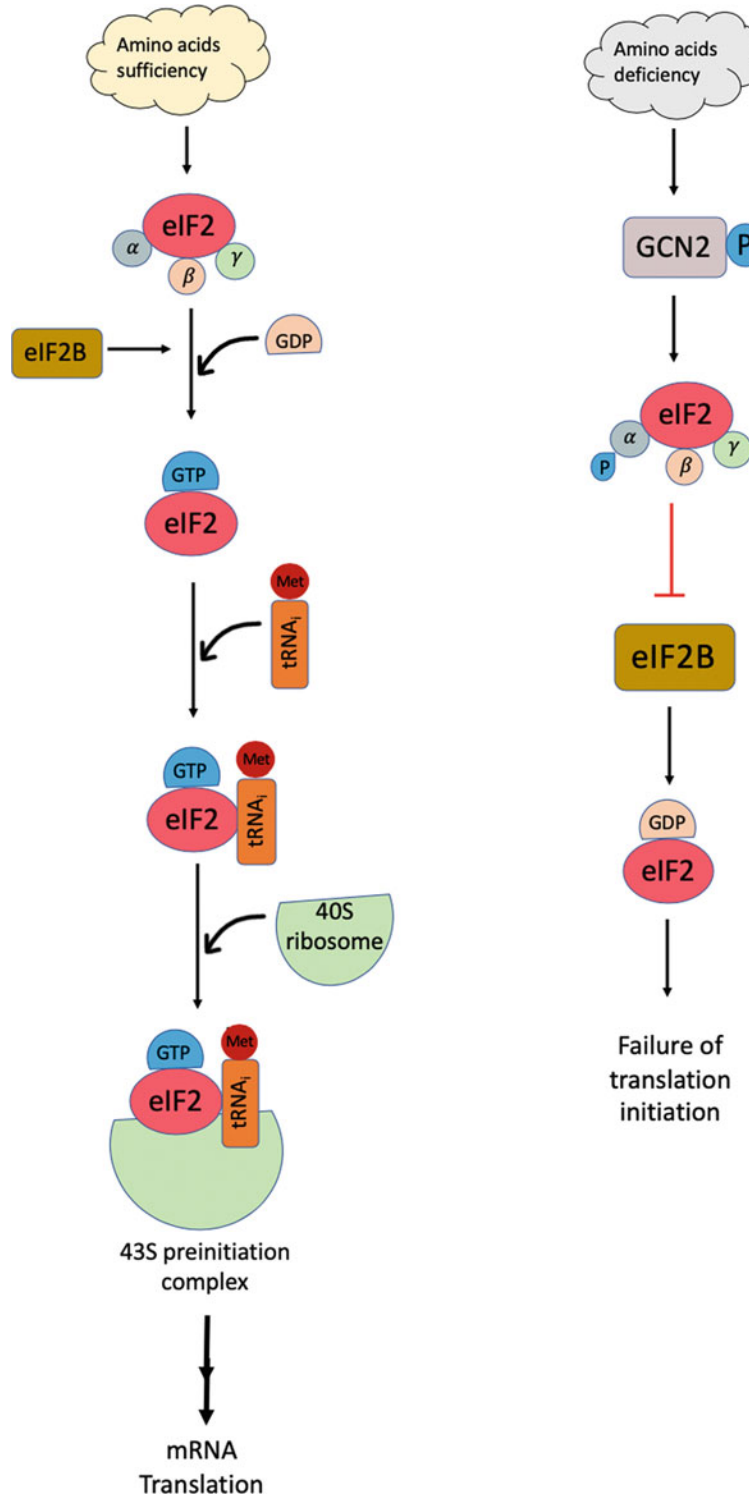

Fig. 1.3 Amino acid regulation of initiation of translation via eIF2a phosphorylation

and survival. MTORC1 enhances protein synthesis by phosphorylation of RPS6 and 4EPBP1. MTORC1 directly phosphorylates and deactivates 4EBP1 protein, thereby freeing eIF4E from the eIF4E-4EBP1 complex. The free eIF4E then recruits other transcription factors such as eIF4A and eIF4G to form the eIF4F complex which can then bind to mRNA and the 43S pre-initiation complex for initiation of translation. Phosphorylation of RPS6K1 stimulated by MTORC1 leads to phosphorylation of RPS6, which activates eIF4B required for circularization and activation of the mRNA. RPS6 kinase also phosphorylates the programmed cell death protein-4 (PDCD4), an inhibitor of eIF4B, resulting in activation of eIF4B (Fig. 1.4).

Balanced and/or sufficient abundances of AAs in the body lead to MTORC1 activation and an increase in protein synthesis. Leu and Arg are two AAs that are well known to activate the MTORC1 pathway for protein synthesis and cell proliferation (reviewed by Bazer et al. 2015). The cellular sufficiency of Leu is sensed by a cytosolic leucine sensor called Sestrin2. Sesterin2 is a physiological regulator of the GATOR [guanosine triphosphatase activating protein (GAP) activity toward Rags] complex, which consists of GATOR1 and GATOR2. GATOR1 is an inhibitor of RagA/B (ras-related GTP-binding proteins A and B) required for the activation of MTORC1, whereas GATOR2 suppresses the Rag GTPase-activating protein activity of the GATOR1 sub-complex to promote MTORC1 activity. By binding with GATOR2, Sestrin2 releases GATOR1 from the GATOR2-mediated inhibition. Thereafter, GATOR1 inhibits RagB GTPase, thereby preventing MTORC1 activation by amino acids. The addition of Leu to myotubes and human embryonic kidney 293 (HEK) cells activates MTORC1 (Gran and Cameron-Smith 2011; Wolfson et al. 2017). Therefore, when cells have sufficient AAs, especially Leu, the MTORC1 inhibitor Sestrin2 interacts with Leu, allowing free GATOR2 that ultimately leads to the activation of MTORC1 for initiation of translation (Kim et al. 2015; Wolfson et al. 2017). Similarly, cells have an Arg sensor known as cytosolic arginine sensor of MTOR (CASTOR1). CASTOR1 interacts with GATOR2, forming the CASTOR1-GATOR2 complex (Chantranupong et al. 2015). Arginine sufficiency in cells disrupts the CASTOR1-GATOR2 complex and drives the formation of the CASTOR2-Arginine complex required for the Arg-mediated activation of MTORC1 (Chantranupong et al. 2015; See Fig. 1.4).

With the deprivation of AAs, such as Leu in culture medium, MTORC1 is inhibited in HEK cells while re-supplementation of Leu reverses the inhibition (Wolfson et al. 2017). Leu deprivation also deactivated MTORC1 as indicated by hypophosphorylated RPS6 in HepG2 (liver hepatocellular carcinoma) cells in vitro and in rat liver in vivo (Xiao et al. 2011). Removal of Leu from culture media inhibits MTORC1 activity due to decreased downstream phosphorylation of RPS6K1 and 4EBP1 in MEF cells (Averous et al. 2016). Deprivation of Gln in rat kidney cells reduces MTORC1 activity leading to increased autophagy as a GCN2 pathway (Chen et al. 2014). All these studies suggest that MTORC1 is a central molecule in the regulation of gene expression by AAs. While Leu deprivation mediates the inhibitory effect on MTORC1 by the GCN2-mediated phosphorylation of eIF2a, the activation of GCN2 alone does not mandate decreased MTORC1 activity, suggesting that there are additional mechanisms for deactivation of the MTOR pathway (Averous et al. 2016). Also, GCN2 may be responsible for Leu- or Arg-mediated inhibition of translation during the early stages of AA deprivation that is independent of ATF4 (Averous et al. 2016).

A recent report indicated an ATF4-dependent triphasic response of MTORC1 activity following Leu starvation (Xu et al. 2020). In that study, Leu deprivation resulted in an initial inhibition of MTORC1 due to the activation of the Sesterin2-GATOR2 complex that further stimulates autophagy. This initial inhibition stage is followed by transient activation of MTORC1 by AAs generated in lysosomes from autophagy. The third stage is re-suppression of MTORC1 due to increased expression of REDD1 and Sestrin2 initiated by ATF4. Indeed, the final re-suppression of MTORC1 is plausible, since this

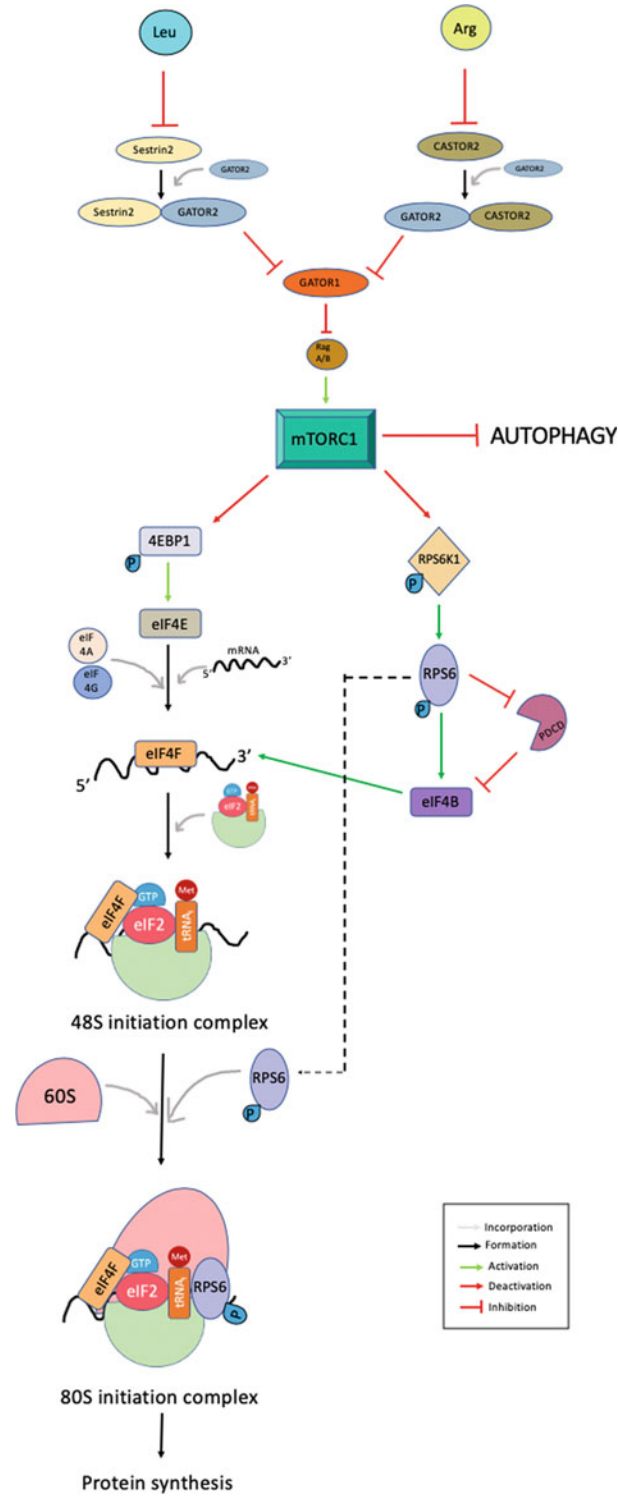

Fig. 1.4 Amino acid regulation of the initiation of mRNA translation via mTOR

would be a mechanism for cells to prevent further cellular stress, possibly resulting from increased MTORC1 activity in an AA-deficient condition.

1.4 Amino Acids and Posttranslational Modification

After the mRNAs have been translated into proteins, additional changes occur in some proteins to make them stable and biologically active. Such changes can result due to alterations of the C- or N-terminus of the proteins or modification of the functional groups in the AA side chains, known as posttranslational modifications (PTMs). The modifications in translated proteins are very important for their maturation and action. PTMs include acetylation, ADP-ribosylation, biotinylation, γ-carboxylation, disulfide linkage, flavin attachment, glutamylation, glycation, glycosylation, glycylation, heme attachment, hydroxylation, methylation, myristoylation, nitrosylation, oxidation, phosphorylation, palmitoylation, proteolytic cleavage, racemization, selenoylation, sulfation, and ubiquitination (Wu 2009). One or more of the PTMs is involved in protein maturation; however, this review focuses on acetylation, ADP-ribosylation, disulfide bonding, and glutamylation as examples how AAs regulate protein expression.

1.4.1 Acetylation

Most of the intermediate enzymes in major metabolic pathways, such as those required for glycolysis, gluconeogenesis, Krebs cycle, and urea cycle, are reversibly acetylated or deacetylated to regulate metabolic homeostasis. The acetylation of metabolic enzymes is controlled by the availability of extracellular AAs. Extracellular limitations in nutrients dictate the metabolism of nutrients by rendering the biological activity or stability of intermediate metabolic enzymes. For example, the addition of extra AAs in cell culture media decreases the acetylation of Lys (Lys288) in the urea cycle enzyme argininosuccinate lyase. The reduced acetylation of argininosuccinate lyase increases its enzymatic activity and, thus, improves the detoxification of ammonia via the urea cycle (Zhao et al. 2010). Also, glucose-deprived mammalian hepatocytes treated with AAs have less acetylation of lysine residues in the gluconeogenic enzyme phosphoenolpyruvate carboxykinase 1 (PEPCK1). The AA-induced deacetylation of Lys in PEPCK1 likely enhances stability of the enzyme and hence its activity (Zhao et al. 2010). Therefore, mammalian hepatocytes exposed to sufficient glucose have inactive PEPCK1, and gluconeogenesis is inhibited. However, with the limitation of cellular glucose, PEPCK1 is activated in these cells through lysine deacetylation in the presence of AAs to increase glucose production. This demonstrates the role of AAs in the regulation of metabolic activities by PTMs of metabolic enzymes. The molecular pathway of AA-induced lysine deacetylation is unclear; however, Yu and Auwerx (2010) proposed Sirtuin 1 (SIRT1; an NAD^+-dependent class III histone deacetylase) as a novel deacetylase for posttranslational modification of several metabolic enzymes and transcription factors. Extracellular nutrient availability regulates activation of SIRT1 which in turn deacetylates enzymes to maintain metabolic homeostasis. Nutrient deprivation may activate SIRT1, which stimulates the transfer of the acetyl group from lysine residues in the enzymes to nicotinamide adenine dinucleotide (NAD^+). Loss of the acetyl group from lysine residues makes the enzymes both more stable and more active. Taken together, it can be hypothesized that under conditions of limitations in available cellular glucose, the addition of AAs to media activates SIRT1 in animal cells to increase the removal of an acetyl group from lysine residues for the activation of enzymatic activity.

1.4.2 ADP-Ribosylation

ADP-ribosylation is the reversible transfer of either monomeric or polymeric ADP-ribose

molecules (ADPr) to a protein that may increase its biological function or provide a scaffold for protein interactions, respectively (Kaufmann et al. 2014). The transfer of ADPr is provided from the NAD^+ molecule by the catalytic action of ADP-ribosyltransferases (ARTs). According to the most widely used nomenclature system, there are two forms of ARTs: The first type of ART is homologous to bacterial diphtheria toxins, and the second type of ART shares homology to clostridial toxins. The ARTD enzymes are localized intracellularly and transfer either mono- or poly-ADPr to proteins. The ART enzymes can transfer only mono-ADPr and are mostly membrane-bound, secretory, or present extracellularly. Amino acid residues play a critical role in the biological activity of ARTs. The catalytic domain of all ARTs contains an NAD^+ binding triad motif with three essential amino acid residues, His, Tyr, and Glu, each with a specialized role. The positively charged His and negatively charged Tyr residues interact with NAD^+ to facilitate its correct binding to the A-ribose and N-ribose moieties of NAD^+, respectively. The Glu residue of the triad motif is critical for the actual transfer of ADPr. The substitution of any one or all of the critical AA residue(s) in the triad motif modifies the biological function of ARTs (Liu and Yu 2015). The ARTs with all the three conserved AA residues (e.g., ARTD1 and ARTD2) are usually capable of transferring the polymers of ADPr. The replacement of Glu, for example, by either a Ile, Leu, Thr, Val, or Tyr residue in the triad motif of ARTD10 limits the transferase activity to mono-ADPr only (Kaufmann et al. 2014). In addition, loss of both His and Glu residues of the motif in ARTD9 and ARTD13 (replaced as Gln-Tyr-Thr, and Tyr-Tyr-Val, respectively) prevents NAD^+ binding and thus completely inhibits the transfer of any ADPr (Kaufmann et al. 2014; Liu and Yu 2015).

1.4.3 Disulfide Bonding

The formation of disulfide bonds in some newly synthesized proteins is very important for their structural stability and biological activity. Cysteine is the only amino acid that allows the formation of a disulfide bridge between the thiol groups of two cysteine residues in proteins. The covalent bonding between two sulfur atoms of the thiol groups in cysteine residues is catalyzed by protein disulfide isomerases (Kozlov et al. 2010). This covalent bonding primarily occurs in the rough endoplasmic reticulum or intermembrane space of mitochondria (Hell and Neupert 2010). Besides the classical sites of oxidative folding, disulfide cross-linkages also occur in some nuclear and cytoplasmic proteins by mechanisms that remain elusive (Butera et al. 2014). The disulfide linkage can occur within a peptide chain, for example, Cys18-Cys138 linkage in angiotensinogen (known as intra-protein disulfide bridge) or between polypeptide chains of multi-subunit proteins such as the light and heavy chains of immunoglobulins (known as inter-protein disulfide bonding). Disulfide cross-linkages regulate tertiary and quaternary structures of proteins by reducing the entropy of the unfolded state (Feige and Buchner 2010). Oxidative folding of proteins via a disulfide bridge is critical especially for secreted and surface proteins. Because such proteins are present extracellularly, they must be synthesized, folded, and assembled in a protective way for transport across the cell and to function in an extracellular environment. Integrins, for example, are surface receptor proteins that mediate cell–cell interactions. The thiol group of different cysteine residues in integrin αIIbβ3 has varying roles in ensuring stability, expression, and function (Mor-Cohen et al. 2008). Substitution of some cysteine residues with serine reduces the expression of integrin αIIbβ3 implying a functional role of disulfide bonds in protein stability, while mutations of the Cys567-Cys581 disulfide bridge confirmed its critical role inactivation of integrin αIIbβ3 (Mor-Cohen et al. 2008). Also, results of the same study suggested the unique role of free thiol groups of Cys583 and Cys608 residues in activation of integrin αIIbβ3. Disruption of four unique disulfide bonds of the integrins aIIbß3 and αvß3 significantly decreases expression of those integrins on cellular membranes (Mor-Cohen et al. 2012). Therefore, posttranslational

modifications by induction of disulfide bridges play an important role in the expression of extracellular proteins.

1.4.4 Glutamylation

Glutamylation, a form of protein PTMs, involves the addition of free glutamate to the γ-carboxy residues of glutamate in proteins. The addition process can add up to six glutamate residues to existing glutamate residues (polyglutamylation). Glutamylation occurs primarily in the α- and β-tubulins of the microtubules that modulate binding and interactions among microtubule proteins. Glutamylation is critical for several cytoskeletal processes, such as neurite extension, motility of cilia, and formation of mitotic spindles during cell division (Hammond et al. 2008). The addition of free glutamate is essential to the maturation of tubulin proteins for the maintenance of structural integrity and shape of cells. The reversible reaction of glutamylation is regulated by polyglutamylases from the tubulin–tyrosine ligase-like family and deglutamylase (a cytosolic carboxypeptidases) critical for the long axonal projections of neurons. Hyperglutamylation caused by dysfunction of deglutamylase may interfere the axonal transport and can cause neurodegenerative diseases (Magiera et al. 2018). Therefore, Glu is a critical component for normal neuronal functioning.

1.5 Amino Acids and Epigenetic Regulation

The expression of genes is largely governed by the methylation status of DNA, RNA, and histone proteins. In fact, about 70% of the biological reactions in the body require methylation for activation. The active methyl group in all those reactions is provided by AAs such as Ser, Gly, His, and Met. Several developmental disorders and diseases have been linked with abnormal DNA methylation caused by a deficiency in one-carbon units in both human and animal models (Burgoon et al. 2002; Safi et al. 2012; Kim et al. 2015). Generally, DNA methylation suppresses gene transcription, while demethylation of DNA enhances gene expression (Busslinger et al. 1983; Bhutani et al. 2011). The dynamic changes in the methylation status of DNA regulate time-, tissue-, cell-, and species-specific expression of genes. The paternal genome is demethylated earlier in the zygote (at the one-cell stage) compared to the maternal genome which maintains methylation up to the two-cell stage of embryonic development (Mayer et al. 2000) which represents time-specific paternal regulation of early embryonic development. Insulin, the major regulator of concentrations of glucose in plasma, has three unique cytosine–guanosine dinucleotides (CpG) sites that are demethylated only in the pancreatic beta cells to allow for expression of the *Ins2* gene. Indeed, methylation of the insulin promoter sequence decreased transcription of *Ins2* in both human and mice models (Kuroda et al. 2009). At the blastocyst stage, bovine conceptuses (embryo and associated extra-embryonic membranes) have methylated genes in cells of the inner cell mass and trophectoderm, while there is hypomethylation of genes in mouse trophectoderm and human inner cell mass based on results of anti-5-MeC staining (Fulka et al. 2004). Moreover, for DNA in the pronucleus of most mammalian sperm, there is rapid removal of methyl groups from CpG before the first cell division. By contrast, the ovine zygote does not undergo demethylation at similar times and very minimal demethylation of the embryonic genome occurs at the morula stage (Young and Beaujean 2004). Suppression or silencing of gene expression by addition of a methyl group, incorporated from AAs to the cytosine nucleotide, ensures the inactivation of the gene until a time- and tissue-specific demethylation occurs to initiate the transcription of the specific gene for the desired function (e.g., placental protein synthesis, water transport, and angiogenesis).

1.6 Conclusion

Recent studies have revealed molecular mechanisms of how AAs regulate gene expression at different stages including gene transcription, mRNA translation, PTMs, and epigenetics. Amino acids regulate gene expression via a complex mechanistic network with checkpoints at several stages to ensure cellular metabolism and growth during conditions of AA sufficiency or promote cell proliferation and survival when deficient in AAs. Deprivation of intracellular AAs triggers the cell to limit metabolism and decreased global protein synthesis to conserve substrates and energy for cell survival. On the other hand, an insufficiency of AAs stimulates translation of a unique transcription factor, ATF4, that leads to transcriptional activation of several genes for AA synthesis and transport, one-carbon unit synthesis, autophagy, and inhibition of MTORC1, a holistic approach to cell survival. Understanding the mechanisms whereby AAs regulate gene expression at the cellular and molecular levels is warranted in health (e.g., conceptus survival, growth, and development) and disease (e.g., insulin insensitivity, cardiovascular disorders, and cancer).

Acknowledgements Work in our laboratories was supported by Agriculture and Food Research Initiative Competitive Grants (Nos. 2015-67015-23276, 2016-67015-24958, and 2018-67015-28093) from the USDA National Institute of Food and Agriculture.

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Amino Acids in Cell Signaling: Regulation and Function

2

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Abstract

Amino acids are the main building blocks for life. Aside from their roles in composing proteins, functional amino acids and their metabolites play regulatory roles in key metabolic cascades, gene expressions, and cell-to-cell communication via a variety of cell signaling pathways. These metabolic networks are necessary for maintenance, growth, reproduction, and immunity in humans and animals. These amino acids include, but are not limited to, arginine, glutamine, glutamate, glycine, leucine, proline, and tryptophan. We will discuss these functional amino acids in cell signaling pathways in mammals with a particular emphasis on mTORC1, AMPK, and MAPK pathways for protein synthesis, nutrient sensing, and anti-inflammatory responses, as well as cell survival, growth, and development.

Keywords

Functional amino acids · Transceptor · Sensor · mTORC1 · AMPK · MAPK

Abbreviations

AMPK	AMP-activated protein kinase
Akt	Protein kinase B
EAA	Nutritionally essential amino acids
IGF	Insulin-like growth factor
ERK	Extracellular signal-regulated kinases
JNK	c-Jun NH(2)-terminal kinase
LEL	Late endosome and lysosome
MAPK	Mitogen-activated protein kinase
mTOR	Mechanistic target of rapamycin
NEAA	Nutritionally nonessential amino acids
PI3K	Phosphatidylinositol-3-kinase
Tr	Trophectoderm

2.1 Introduction

Amino acids (AAs), defined as organic compounds containing both amino and acid groups, are major components of cells and physiological fluids (Wu 2009). Among more than 300 AA in nature, only 20 of them (α-AAs) serve as the building blocks of proteins in animals. These AAs are histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp), valine (Val), alanine (Ala), arginine (Arg), asparagine (Asn), aspartate (Asp), cysteine (Cys), glutamate (Glu), glutamine (Gln), glycine (Gly),

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1332,
https://doi.org/10.1007/978-3-030-74180-8_2

proline (Pro), serine (Ser), and tyrosine (Tyr). Other AAs, such as non-proteinogenic α-AA (e.g., ornithine, citrulline, and homocysteine) and non-α AAs (e.g., taurine and β-alanine) also have physiological importance in cell metabolism (Katane et al. 2008; Manna et al. 2009). Taurine and β-alanine are abundant in animal tissues and animal-sourced foodstuffs (Li and Wu 2020; Wu 2020), whereas taurine is absent from plant-sourced foodstuffs (Hou et al. 2019).

Based on the dietary needs for nitrogen balance or growth, AAs have been traditionally classified as nutritionally essential (EAA; indispensable) or non-essential (NEAA; dispensable) for mammalian species (Wu 2009). EAAs are defined as those AA for which carbon skeletons cannot be synthesized or those that are inadequately synthesized de novo by the body to meet metabolic need, and must be acquired from the diet to meet requirements. By contrast, NEAAs are those AA which can be synthesized de novo in adequate amounts by the body to meet requirements (Wu 2009). Even though EAAs and NEAAs had been described for over a century, there are no compelling data to substantiate the assumption that NEAAs are synthesized sufficiently in animals and humans to meet the needs for maximal growth, optimum survival, and physiological function (e.g., reproduction and disease prevention). In fact, some NEAAs (e.g., arginine, glycine, proline, and glutamine) act as conditionally EAAs in which they normally can be synthesized de novo by the organism, but must be acquired from the diet to meet optimal needs under conditions (e.g., in young animal, during pregnancy and lactation, and in response to immunological challenges) where rates of the utilization of the AAs are greater than the rates of their synthesis (Hou et al. 2015; Kim et al. 2009; Wu et al. 2017). NEAAs and conditionally EAAs are now collectively referred to as AAs that are synthesized de novo in animal cells (AASAs). Besides serving as substrates for protein synthesis, AAs per se as well as their metabolites [e.g., glutathione, creatine, H_2S, nitric oxide (NO), and polyamines] indeed play regulatory roles in key metabolic pathways, gene expression, and cell-to-cell communication via a variety of cell signaling pathways that are necessary for maintenance, growth, reproduction, and immunity in humans and animals (Kong et al. 2014; Manjarín et al. 2020; Rider et al. 2007; Wang et al. 2014a, b, c, 2015c; Wu et al. 2009). These functional AAs can be either EAAs or AASAs, which include but are not limited to arginine, glutamine, glutamate, glycine, leucine, and tryptophan (Li et al. 2009; Tan et al. 2009). Thus, this review focuses on functional AAs in cell signaling pathways in mammals with particular emphasis on mTOR (mainly mTORC1), AMPK, and MAPK pathways.

2.2 Functional Amino Acids in Growth, Reproduction, and Immunity

Arginine is one of the most abundant AAs deposited in fetal tissue proteins and conditionally essential for young and adult mammals (Wu et al. 2014). Arg represents 14% of total N in body proteins and is synthesized from glutamine, glutamate, and proline via the intestinal-renal axis in humans and most other mammals (including cattle, pigs, sheep, and rats) (Wu et al. 2009). Concentrations of arginine in uterine histotroph (molecules secreted or selectively transported from uterine epithelia into the uterine lumen) increase by 13-fold during the peri-implantation period of pregnancy (Gao et al. 2009; Wu et al. 2004). Arginine is the common substrate for the production of NO via NO synthases (NOSs) and for the biosynthesis of polyamines (putrescine, spermidine, and spermine) via arginase followed by ornithine decarboxylase 1 (ODC1). Arginine also stimulates the secretion of growth hormone and insulin that play a vital role in stimulating protein synthesis and inhibiting protein degradation (Oh et al. 2017; Zajac et al. 2010). In addition, supplementation of the diet with arginine increases embryonic and conceptus (embryonic/fetus and its extra-embryonic membranes) survival and growth in gilts, cattle, sheep, and rats (Mateo et al. 2007; Zeng et al. 2008), which suggests that dietary arginine (in the case of nonruminant mammals) or citrulline (in the case

of ruminants) is important for fetal growth and development during pregnancy. Arginine is an allosteric activator of acetylglutamate (NAG) synthase, a mitochondrial enzyme that converts glutamate and acetyl-CoA into NAG (an allosteric activator of CPS-I). Thus, arginine, along with glutamate, is required for maintaining hepatic and intestinal urea synthesis in an active state for ammonia detoxification (Wu et al. 2018). Thus, arginine, together with its metabolites (ornithine, polyamines, proline, glutamate, agmatine, creatine, and nitric oxide), has enormous biological importance (Wang et al. 2014a, b, c, 2015c; Wu et al. 2013).

Unlike arginine, leucine is not synthesized de novo in any animal cells. It was one of the three branched-chain AAs (BCAAs) abundant in both plant proteins and most animal proteins (Hou et al. 2019; Li and Wu 2020). Leucine allosterically activates glutamate dehydrogenase (GDH), a major enzyme that directly produces ammonia from AA catabolism in animal cells, which also has important implications for the regulation of glutamate metabolism and hormone secretion (Rhoads and Wu 2009). In the pancreas, this effect of leucine results in enhanced secretion of insulin from β-cells via a series of biological responses (Yang et al. 2012). Specifically, a leucine-induced increase in GDH activity stimulates glutamate oxidation, leading to an elevated ratio of intracellular [ATP]/[ADP]. This, in turn, inhibits the plasma membrane-bound ATP-gated K^+ channel, resulting in membrane depolarization, the influx of extracellular Ca^{2+} into cells, and the activation of the exocytosis of insulin granules from pancreatic β-cells. Leucine also induces the expression of genes such as insulin-like growth factor 2 (IGF2) (Kimball et al. 1999). In mice, leucine is required for the expansion of blastocysts to exhibit motility and outgrowth of trophectoderm (Tr), which is essential for conceptus implantation (Martin et al. 2003). In obese animals, elevated concentrations of leucine and other BCAAs in plasma can be reduced and whole-body insulin sensitivity through the oral administration of α-ketoglutarate (Tekwe et al. 2019) and interferon-tau (Tekwe et al. 2013) to stimulate BCAA catabolism and improve mitochondrial function.

Glutamine is a major physiological precursor of ornithine and arginine in most mammals (including humans, cattle, sheep, and pigs; Wu et al. 2011), and an essential substrate for the synthesis of purine and pyrimidine nucleotides, as well as amino sugars and NAD^+ for cell division in all animals, including ungulates and rodents (Krebs et al. 1980; Wu et al. 2009). Also, glutamine serves as an energy source for rapidly dividing cells (e.g., enterocytes and immunologically activated lymphocytes) and macrophages (Wu et al. 1991a, b, c, d). In sheep and swine, the maternal to fetal flux of glutamine was the greatest among all amino acids, particularly between Days 13 and 16 of pregnancy when conceptuses are undergoing rapid elongation (Gao et al. 2009). Transfer of glutamine from ewes to their fetuses is also the greatest among all amino acids measured during mid-gestation (Bell et al. 1989; Gao et al. 2009). Glutamine modulates the expression of genes that beneficially regulate nutrient metabolism and cell survival, including ODC1, heat-shock proteins, and NOSs in multiple cell types. In activated macrophages, glutamine is indispensable for the expression of NOS that is critical for the killing of pathogens (e.g., bacteria, fungi, viruses, and parasites) by these phagocytes (Wu and Meininger 2002). Glutamine also regulates ion and nutrient transport, as well as protein turnover (e.g., stimulation of protein synthesis and inhibition of protein degradation in enterocytes), therefore preventing intestinal atrophy and enhancing growth in animals (e.g., weanling pigs) with intestinal damage and dysfunction (Haynes et al. 2009; Liu et al. 2018; Wang et al. 2008, 2015a).

2.3 Amino Acid Transporters

Amino acids do not directly diffuse across cell membranes to participate in signaling and/or acting as building blocks for protein synthesis. Instead, extracellular (cell membrane) and intracellular (membranes of subcellular organelles including lysosome, Golgi apparatus, and mitochondria) AA transport require membrane-spanning transporter proteins (Broer 2008; Verrey et al. 2004), which belongs to solute carrier superfamily (SLC) (reviewed in Broer and Broer 2017; Fan and Goberdhan 2018; Zhuang et al. 20fv19). Over 25% of SLCs transport amino acids as their primary substrates with various specificities. For instance, CD98 heterodimeric transporters (SLC3A2-SLC7A5, also termed LAT1 composed of a 4F2hc/CD98 heavy chain encoded by *SLC3A2* gene and a CD98 light chain encoded by *SLC7A5* gene) and SLC6A14 have broad AA specificity (Deves and Boyd 1998; Sikder et al. 2017). Others are highly selective in which (1) proton-assisted AA transporter family (PAT or SLC36) are specific for transport of alanine, glycine, and proline; (2) the sodium-coupled neutral AA transporter (SNAT or SLC38) family co-transports small neutral AAs such as alanine, glutamine, serine, glycine, methionine, and threonine together with sodium ions; and (3) the monocarboxylate SLC16 family can transport aromatic amino acids including tyrosine, phenylalanine, and tryptophan (Halestrap and Meredith 2004). Transporters that undergo a cycle of allosteric changes during the transport of substrates are termed transceptors, in which they not only act as gate keepers at the surface of membranes, but also exhibit receptor function to convey signals. Thus, AA transceptors have dual functions as both AA transporters and AA receptors. Transporters of arginine (SLC7A1-4), glutamine (SLC1A5), and leucine (SLC7A5) on the cell membranes are identified as respective transceptors to directly influence mTORC1 activation (Nicklin et al. 2009; Yeramian et al. 2006). Transporters that exhibit higher amino acid affinity and lower transport capacity are also excellent candidates for being transceptors, including SLC38A2 (cell membrane and Golgi apparatus), SLC36A1 (cell membrane and lysosomal surface), SLC38A9 (lysosomal surface), and SLC36A4 (Golgi apparatus).

2.4 Amino Acids and mTOR Cell Signaling Pathways

2.4.1 Elements of mTORC1 and mTORC2

The highly conserved mechanistic serine/threonine protein kinase target of rapamycin (TOR), also known as FK506 binding protein 12-rapamycin associated protein 1, is a key integrator of environmental cues, including, but not restricted to, nutrient and growth factor availability, as well as diverse forms of stress (Chantranupong et al. 2015). Mammalian TOR (mTOR), now also known as mechanistic target of rapamycin, controls many biological processes, including cell proliferation via the regulation of protein, lipid, and nucleotide syntheses, ribosome, and production, the expression of metabolism-regulated genes, autophagy, and cytoskeletal reorganization (Wullschleger et al. 2006). Unlike *Saccharomyces cerevisiae* that encode two different TOR proteins (TOR1 and TOR2) (Helliwell et al. 1994), most eukaryotes and all mammals have only one gene that encodes mTOR. However, all eukaryotes have two mTOR complexes, that is, the mTOR complex 1 (mTORC1) and the mTOR complex 2 (mTORC2) (Guertin et al. 2006). In mammals, mTORC1 comprises mTOR itself and other components, including regulatory-associated protein of mTOR (RAPTOR), which aids in substrate recognition (Hara et al. 2002); mammalian lethal with SEC13 protein 8 (mLST8; also known as GβL), which is the positive regulator of mTORC1 (Kim et al. 2003); proline-rich AKT/PKB substrate 40 kDa (PRAS40; also known as AKT1S1) and DEP domain-containing mTOR-interacting protein (DEPTOR), both of which negatively regulate mTORC1 (Fonseca

et al. 2007; Zhao et al. 2011). The mTORC1 regulates cell proliferation in part by phosphorylating ribosomal protein S6 kinases (RPS6K1) and the eIF4E-binding protein (EIF4EBP1), well-known regulators of protein synthesis. The mTOR also controls cell survival and spatial aspects of growth, such as cytoskeletal organization and AKT/PKB phosphorylation (Sarbassov et al. 2005), through mTORC2 that contains mTOR itself and the following components: rapamycin-insensitive compassion of mTOR (RICTOR), mammalian stress-activated MAP kinase interacting protein (MSIN1), mLST8, DEPTOR, and protein observed with RICTOR (PROTOR) (Laplante and Sabatini 2009; Yang et al. 2006). RICTOR, MSIN1, mLST8, and mTOR are the essential core components of the complexes required for maintaining structural integrity. DEPTOR and PROTOR are not required for mTORC2 activity, but function as regulatory proteins. In fact, DEPTOR interacts with mTOR via its PDZ domain, thereby inhibiting mTORC1 and mTORC2. However, by inhibiting mTORC1, DEPTOR overexpression suppresses RPS6K1, which relieves mTORC1-mediated inhibition of PI3K, and in turn triggers the activation of PI3K and, paradoxically, an increase in mTORC2-dependent responses, such as the stimulation of AKT (Peterson et al. 2009). PROTOR interacts with RICTOR through a conserved N-terminal region, which may stabilize PROTOR. However, the function of PROTOR is unclear due to the lack of obvious functional domains. PROTOR may play a critical role in mediating the phosphorylation of SGK1 by mTORC2 in the kidney (Pearce et al. 2011). The mTORC2 controls actin cytoskeleton organization and AKT/PKB phosphorylation, which further regulates cell migration, growth, survival, and metabolism. Since mTORC2 is less sensitive to rapamycin than mTORC1, and the relationship between mTORC2 and AA-driven signaling is unclear, we will only discuss AAs and the mTORC1 cell signaling pathway hereafter.

2.4.2 Amino Acids and mTORC1 Signaling Pathway

Amino acids are the most crucial signals for mTORC1 activation as well as the prerequisite for other growth factors to efficiently activate mTOR (Hara et al. 1998; Sancak et al. 2008; Wang et al. 1998). Specifically, arginine, leucine, and glutamine participate in or regulate key metabolic mTORC1 pathways to improve growth, development, and reproduction of mammals (Fig. 2.1) (Duran and Hall 2012; Jewell et al. 2015; Wang et al. 2015b, 2017). Activation of mTORC1 occurs via sensing complex components at several different locations in cells, namely the cell membrane, acidic late endosomes and lysosomes (LELs), and the Golgi apparatus.

2.4.2.1 mTORC1 Activation Close to the Cell Membrane

Close to the cell membrane, mTORC1 can be activated via SLC38A2, a SNAT family member that transports neutral AAs such as alanine, glutamine, serine, glycine, methionine, and threonine into cells. In fact, SLC38A2 serves as a transceptor since it activates mTORC1 in the presence of the nonmetabolizable AA analog Me-AIB (for α-methylaminoisobutyrate) (Pinilla et al. 2011). SLC38A2 is thought to act at the cell membrane and to be stored in the Golgi apparatus when not required; however, the sensing complex components that activate mTORC1 are still unknown.

2.4.2.2 mTORC1 Activation at Lysosomal Surface

The classic location for mTORC1 activation is LELs. The Rags and Rheb are two different types of small GTPases that reside on the lysosomal surface and coordinate to accurately regulate mTORC1 activity in response to environmental cues, including arginine, leucine, and glutamine. The Rag GTPases are redundant heterodimers composed of RagA or RagB (RagA/B) bound to

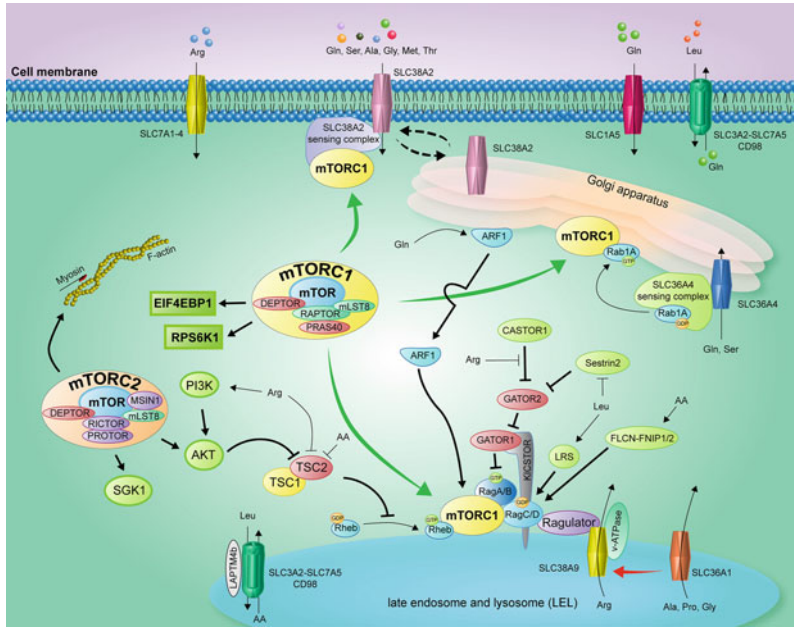

Fig. 2.1 Amino acid sensing in mTOR cell signaling pathways. Schematic models are shown in which amino acids act through their transporter/transceptors, amino acid sensors, and other key molecular players to activate mTOR, particularly mTORC1 at different subcellular locations. A green arrow indicates the translocation of mTORC1 for full activation, including the cell membrane, the late endosome and lysosome (LEL) complex, and the Golgi apparatus. A red arrow indicates the interaction between SLC36A1 and mTORC1 on the surface of LEL, which is less stable than for SLC38A9

RagC or RagD (RagC/D). When RagA/B is GTP loaded ($RagA/B^{GTP}$) and RagC/D is GDP loaded ($RagC/D^{GDP}$), mTORC1 is translocated to the lysosomal surface where it can come into contact with its kinases activator, Rheb, for full activation. In vitro studies with an established ovine trophectoderm (oTr1) cell line isolated from Day 15 ovine conceptuses demonstrated that arginine induces both proliferation and migration of oTr cells through stimulation of the TSC2-Rheb-mTORC1 signaling cascade (Kim et al. 2011a, b; Wang et al. 2015c). Further studies revealed that such activation of the TSC2-mTORC1 cell signaling pathway is induced by the production of NO and polyamines from arginine, but also by arginine itself (Kong et al. 2014; Wang et al. 2015c). Therefore, arginine per se, as well as its metabolites, activates mTORC1 partially via the TSC2-Rheb signaling pathway.

Aside from Rheb for the full activation of mTORC1, a number of other proteins have been identified as functional players to modulate the nucleotide loading state of the Rag GTPases or the localization of other components as the nutrient-sensing upstream of mTORC1. The necessary sensing components include Ragulator, folliculin (FLCN), FNIP1/2, GATOR1, GATOR2, and KICSTOR (Fig. 2.1) (Bar-Peled et al. 2012, 2013; Peng et al. 2017; Tsun et al. 2013; Wolfson et al. 2017; Wolfson and Sabatini 2017). Regulator is a pentameric complex (p18, p14, HBXIP, C7orf59, and MP1) that governs the lysosomal localization and nucleotide loading state of the Rag GTPases. Regulator also interacts with the vacuolar H^+-adenosine triphosphatase ATPases (v-ATPase), which act as a positive regulator of this pathway. FLCN and its binding partners, FNIP1/2, have GTPase-activating protein (GAP) activity toward RagC/D, resulting in the $RagC/D^{GDP}$ form. GATOR1 (for GAP activity toward the Rag GTPases 1) is also a GAP for RagA/B

($RagA/B^{GDP}$), thereby inhibiting mTORC1 translocation to the lysosomal surface. GATOR1 is a trimeric complex (DEPDC6, Nprl2, and Nprl3) that interacts with a pentameric complex GATOR2, a positive regulator of the nutrient-sensing branch of the pathway. The KICSTOR complex (KPTN, ITFG2, C12orf66, and SZT2-contaning regulator of mTORC1) was recently identified as a scaffold for GATOR1 on the lysosomal surface.

Recently, several AA sensors have been identified, including Leucyl-tRNA synthetase (LRS; a leucine sensor in the cytoplasm), Sestrin2 (a leucine sensor in the cytoplasm), CASTOR1 (a arginine sensor in the cytoplasm), SLC38A9 (a putative arginine sensor in the lysosomal surface), SLC36A1 (a alanine/proline/glycine sensor in the lysosomal surface), SLC36A4 (a glutamine/serine sensor in the Golgi Apparatus), and AFR1 (a glutamine sensor in the Golgi Apparatus). The LRS acts as a cytosolic leucine sensor that switches on RagD (Han et al. 2012). In vitro binding assays showed that LRS binds leucine with the affinity of 45 μM (Chen et al. 2011). Sestrin2 negatively regulates the mTORC1 upstream of GATOR2 in which Sestrin2 binds to GATOR2 under leucine deprivation and likely inhibits this process via unknown mechanisms. With the presence of leucine bound to Sestrin2 with the affinity of 20 μM, Sestrin2 dissociates from GATOR2 and likely relieves its inhibition of this positive regulator (Wolfson et al. 2016). Under arginine deprivation conditions, the homodimer of CASTOR1 (for cellular arginine sensor for mTORC1) interacts with GATOR2 to inhibit mTORC1 activation. Arginine-bound CASTOR1 (affinity of 30 μM) can lead to the dissociation of CASTOR1 form GATOR2, thereby removing the inhibition for mTORC1 activation on the lysosomal surface (Chantranupong et al. 2016; Saxton et al. 2016). SLC38A9 is a lysosomal transmembrane protein that interacts with the Rag GTPases-Ragulator complex via its cytosolic N-terminal tail. Knockout of SLC38A9 inhibits mTORC1 activation by arginine, and overexpression of SLC38A9 leads to mTORC1 activation more sensitive to arginine (Jung et al. 2015; Wang et al. 2015b). Thus, the transceptor SLC38A9 is considered as an arginine sensor in the lysosomal surface for mTORC1 activation. SLC36A1 is another transceptor located in the lysosomal surface that transports alanine, proline, and glycine from the acidic lumens of LELs into the cytosol. Likewise, SLC36A1 activates mTORC1 via interaction with Rag GTPases-Ragulator complex, which is less stable than for SLC38A9. In addition, the lysosomal protein LAPTM4b (for lysosomal protein transmembrane 4β) serves as a binding partner to recruit CD98 heterodimer (SLC7A5-SLAC3A2) to lysosomes, leading to the uptake of leucine and other EAAs into lysosomes, and is required for mTORC1 activation via v-ATPase following leucine or EAA stimulation (Milkereit et al. 2015).

2.4.2.3 mTORC1 Activation at Golgi Apparatus

The Golgi apparatus provides a third site from which mTORC1 can be activated (Goberdhan et al. 2016; Jewell et al. 2015). As a transporter of glutamine and serine, SLC36A4 has recently been shown to be predominantly localized on the trans-Golgi network where it interacts with Rab1A (a GTPase) to activate mTORC1, as also shown by a proximity ligation assay that detects closely apposed antigens in situ in whole-mount cells (Fan et al. 2016a; Weibrecht et al. 2010). SLC36A4 levels control the resistance of mTORC1 activation at the Golgi apparatus to the deficiency of either glutamine or serine. ARF1 (for adenosine diphosphate ribosylation factor-1) is another GTPase that is typically found on Golgi membranes but serves as a cytosolic glutamine sensor required for lysosomal mTORC1 activation by glutamine. Unlike the SLC38A9- and SLC36A1-regulated LEL-localized sensing complexes, the modulation of mTORC1 by SLC36A4 or ARF1 appears to be Rag-independent and, therefore, is not under the control of cytosolic sensors mentioned above. However, it remains unclear whether there is any functional relationship between the SLC36A4-regulated mTORC1 and the lysosomal form of the Rag-independent, glutamine-sensitive, and ARF1-regulated mTORC1.

2.5 Amino Acids and AMPK Signaling Pathway

AMP-activated protein kinase (AMPK) is a heterotrimeric enzyme consisting of three subunits: a catalytic α subunit as well as regulatory β and γ units (Hardie and Carling 1997). Specifically, the γ subunit includes four particular cystathionine β-synthase (CBS) domains that create two binding sites (i.e., Bateman domains) for AMPs, empowering AMPK to sensitively detect shifts in the [AMP]/[ATP] ratio. As a sensor for cellular energy, AMPK is activated by an increased [AMP]/[ATP] ratio whereby AMP binds both Bateman domains to trigger a conformational change of the γ subunit that exposes the catalytic α subunit, particularly at threonine-172 (T-172) site for phosphorylation. Activation of AMPK occurs via phosphorylation by at least three established upstream AMPK kinases (AMPKKs). The known AMPKKs are liver kinase B1 (LKB1, also known as STK11) which works in a complex with STRAD and MO25 (LKB1-STRAD-MO25 complex, originally identified genetically as a tumor suppressor); calcium/calmodulin-dependent protein kinase kinase II (CAMKK2); and TGFβ-activated kinase 1 (TAK1) (Jeon 2016). The overall effect of AMPK activation is to switch off the ATP-consuming pathways such as lipogenesis, gluconeogenesis, glycogenesis, and protein synthesis, while switching on the ATP-producing pathways such as glycolysis as well as fatty acid and glucose oxidation (Hardie et al. 2012). Short-term (2 h) incubation of human umbilical vein endothelial cells (HUVEC) with 100 μM Arg triggers the activation of AMPK predominantly via the NO-CAMKK2-AMPK axis (Mount et al. 2008; Stahmann et al. 2006) instead of the NO-guanylyl cyclase-AMPK axis (Hwang et al. 2003; Wohlfart et al. 1999); however, long-term (7 days) incubation of HUVEC with 100 μM Arg results in the accumulation of $ONOO^-$ derived from the reaction between NO and $O_2^{\bullet-}$, can also impose the regulation on AMPK activation by impairing guanylyl cyclase, thereby causing oxidative stress in vitro (Mohan et al. 2013; Zhang et al. 2008). Likewise, in juvenile blunt snout bream, dietary Arg levels (1.62 and 1.96%) could improve antioxidant capacity and attenuate tissue inflammation, while high Arg level (2.70%) results in oxidative stress (Liang et al. 2018). Both are involved with the AMPK signaling pathway. It should be borne in mind that the proper interpretation of results from feeding experiments must depend on our sufficient knowledge of dietary intakes of all nutrients, particularly basic AAs that share the same transporters with Arg on cell membranes (Wu 2018).

In vitro and in vivo administrations of exceedingly large (physiologically irrelevant) doses of L-alanine activate hepatic AMPK and AMPK-dependent downstream pathways, increasing phosphorylation of adenylyl cyclase (p-ACC, inactive) and inhibiting phosphorylation of MTOR target RPS6K1 (Adachi et al. 2018). Ala activation of AMPK requires alanine transaminase 1 (ALT1)-mediated metabolism, in which Ala is primarily metabolized to pyruvate and in return, α ketoglutarate (αKG) is converted to glutamate, thereby increasing the glutamate/glutamine (Glu/Gln) ratio and reducing TCA cycle intermediates succinate, malate, and αKG. In addition, Ala per se allosterically inhibits glutamine synthase (Haussinger et al. 1989; Tate and Meister 1971), which indirectly increases the Glu level. Elevated Glu/Gln ratio requires the deamination of Glu by glutamate dehydrogenase (GDH) that yields ammonia (NH_3). Excessive ammonia switches the urea cycle into an active state where a total of 6.5 mol of ATP are required to convert 2 mol of ammonia into 1 mol of urea in the mammalian liver (Davis and Wu 1998; Wu 1995). Therefore, an increased [AMP]/[ATP] ratio is detected which activates AMPK. However, which AMPKK that Ala triggered to activate AMPK is still unknown.

2.6 Amino Acids and MAPK Signaling Pathway

Mitogen-activated protein kinases (MAPKs) are a group of serine/threonine-specific protein kinases that are specific to serine and threonine. MAPKs are activated within the protein kinase cascades that transmit the signals from many extracellular stimuli (including mitogens, stress, and proinflammatory cytokines) to intracellular organelles, thereby regulating cell proliferation, differentiation, and death (Morrison 2012; Sun et al. 2015). MAPKs are catalytically inactive in their dephosphorylated form and require phosphorylation events in their activation loop, in which the specific extracellular cue leads to the successive activation of a MAPK kinase kinase (MAPKKK or MAP3K) (Cuevas et al. 2007), a MAPK kinase (MAPKK or MAP2K) (Bardwell and Thorner 1996), and eventually the MAPK (Tanoue and Nishida 2003). In mammals, members of the MAPK family include extracellular signal-regulated kinases (ERK 1 and 2), c-Jun NH(2)-terminal kinase (JNK), p38 MAPK, and ERK5 (Fig. 2.2) (Morrison 2012). The classic ERKs (ERK1/2) possess a TEY motif in the activation segment that consists mainly of a kinases domain (Zhang and Dong 2007). This classic ERK1/2 module responds primarily to growth factors and mitogens [including epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF)] to induce cell proliferation, cell cycle progression, and cell division and differentiation (McKay and Morrison 2007; Shaul and Seger 2007). Top upstream regulators of this pathway include cell surface receptors, such as receptor tyrosine kinases (RTKs), G-protein-coupled receptors (GPCRs), and integrins, as well as the small GTPases Ras and Rap. MAPKKKs for this classic ERK1/2 module are members of the Raf family (A-Raf, B-Raf, c-Raf), c-Mos, and tumor-promoting locus 2 (TPL2). These enzymes phosphorylate and thus activate the MAPKKs including MEK1 (MKK1) and MEK2 (MKK2) that are highly specific activators for ERK1 and ERK2, respectively.

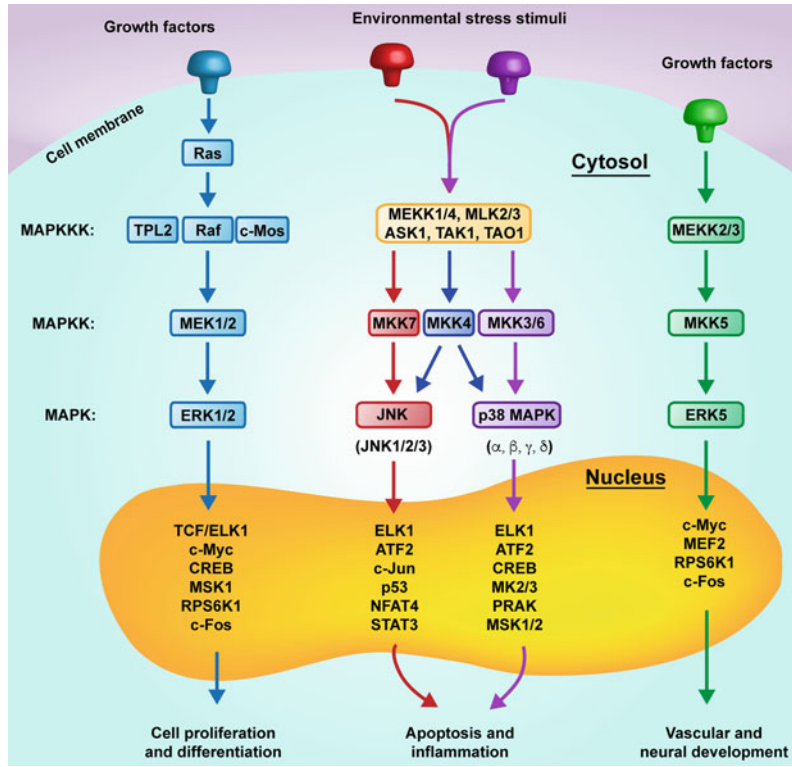

Fig. 2.2 Schematic detailing of the key molecules in MAPK cell signaling cascades. Four members of the MAPK family are extracellular signal-regulated kinases (ERK 1 and 2), c-Jun NH(2)-terminal kinase (JNK), p38 MAPK, and ERK5

JNK and p38 MAPK contain a TPY and a TGY motif, respectively, in the activation segment (Zhang and Dong 2007). JNK family members include JNK1, JNK2, and JNK3, whilst p38 family members include p38α, p38β, p38γ, and p38δ. Both JNK and p38 MAPK modules are activated by environmental stresses (e.g., ionizing radiation, heat, oxidative stress, and DNA damage), inflammatory cytokines, and growth factors, thereby phosphorylating a number of substrates important for apoptosis and inflammation (Cuadrado and Nebreda 2010; Huang et al. 2009). Moreover, the JNK pathway plays an important role in cytokine production and metabolism, and the biochemical signaling to JNK often involves the Rho family GTPase Cdc42 and Rac (Johnson and Nakamura 2007). In addition, p38 MAPK activation also contributes to cell differentiation and cell cycle regulation (Cuadrado and Nebreda 2010; Huang et al. 2009). Unlike the ERK1/2 module, JNK and p38 MAPK pathways have most of their upstream regulators shared at the MAPKKK level, including MEKK1, MEKK4, MLK2, MLK3, ASK1, TAK1, and TAO1. At the MAPKK level, MKK4 can activate both JNK and p38 MAPK; whereas MKK7 and MKK3/6 are specific to JNK and p38 MAPK, respectively. Therefore, JNK activation is often coupled with simultaneous p38 MAPK activation and vice versa. As downstream substrates, c-Jun and NFAT4 are specifically phosphorylated by JNK, while MK2/3, PRAK, MSK1/2, and various transcription factors are dedicated targets by p38 MAPK.

ERK5 is a larger ERK that also possesses a TEY motif in the activation segment like ERK1/2 but contains a much more extended sequence carboxyl-terminal to the kinase domain (Zhang and Dong 2007). ERK5 has its sole specific upstream MAPKKK (MEKK2 and MEKK3) and MAPKK (MKK5) due to their unique architecture, whereby all MEKK2, MEKK3, and MKK5 contain an N-terminal PB1 domain (Nakamura and Johnson 2003). The PB1 domains not only allow heterodimerization between MEKK2/3 and MKK5, but also enable the MKK5-ERK5 interaction (via PB1 domain of MKK5), thereby activating the ERK5 signaling pathway (Glatz et al. 2013). ERK5 activation contributes to physiological functions (including cardiac morphogenesis, neural differentiation, and myocyte fusion), as well as pathological processes (including carcinogenesis, cardiac hypertrophy, and atherosclerosis) of vascular and neural development (Nithianandarajah-Jones et al. 2012).

There are several AAs that regulate MAPK signaling pathways, including Arg, Leu, and Gln. Arg sufficiency is required for TLR4-dependent activation of MAPKs in lipopolysaccharide (LPS)-stimulated macrophages (Mieulet et al. 2010). In particular, Arg (0.4 mM) facilitates the activation of ERK1/2 and consequently cytokine production by preventing the dephosphorylation and inactivation of the MAPKKK (TPL2 in particular) in vitro. Supplementation of starved mice with Arg (2.17 g/kg of body weight) promotes the subsequent activation of ERK1/2 by LPS. Activation of MAPKs (p38 MAPK and ERK1/2) results in the production of cytokines (e.g., IL-6 and IL-8) by NCI-H292 cells (Fan et al. 2016b) ERK1/2 activity is also implicated in regulating maximal Arg transport in LPS-stimulated macrophages (Caivano 1998). This result suggests that Arg sufficiency might promote further uptake of Arg into macrophages and subsequently MAPK activity. In rat small bowel intestinal epithelial cells, excessive and toxic levels of Arg (10 mM or 60 mM) decreases peroxisome proliferator-activated receptor gamma (PPARγ) transcriptional activity via activation of c-Jun, a dedicated substrate that JNK phosphorylates, thereby enhancing inflammation (Ban et al. 2012; Sato et al. 2006). These results must be interpreted very carefully not to implicate nutritional and physiological roles of Arg. However, dietary Arg increases the mRNA level of PPARγ in the porcine intestine treated with LPS, which is coupled with a parallel decrease in apoptosis and inflammatory response (Liu et al. 2008). Leucine can stimulate (at 5 mM) procollagen a1(I) translation in hepatic stellate cells (Perez de Obanos et al. 2006), enhance (at 7.5 mM) SLC1A5 amino acid transporter expression in porcine jejunal epithelial cells (Zhang et al. 2014), and induce (at

60 mM) growth arrest in glioma cells (Takeuchi et al. 2012) via the phosphorylation and activation of ERK1/2 instead of p38 MAPK and JNK. However, the excessive Leu used in these studies may cause dysfunction of normal tissues, likely via an effect on the transport of other AAs (particularly antagonisms among BCAAs), glucose, or other nutrients. Glutamine displays anti-inflammatory activity via regulation of MAPK (ERK1/2, p38, and JNK) signaling pathways in macrophages (Ayush et al. 2016). Briefly, Gln (40 mM) increases the intracellular Ca^{2+} level that initiates the phosphorylation cascade Ras/c-Raf/MEK, thereby activation of ERK1/2. Among the targets of ERK1/2 is MAPK phosphatase-1 (MKP-1) which functions as a critical negative regulator of inflammation. Thus, ERK1/2 phosphorylates MKP-1 on two carboxyl-terminal serine residues S359 and S364, which stabilizes MKP-1 by preventing the degradation from ubiquitin/proteasome pathway (Brondello et al. 1999). MKP-1, in turn, deactivates p38 and JNK which reduces the production of inflammatory cytokines (Kracht and Saklatvala 2002; Kumar et al. 2003). Therefore, Gln inhibits p38 MAPK and JNK pathways via ERK1/2-derived upregulation of MKP-1 (Ayush et al. 2013; Ma et al. 2019). In the intestinal cells, addition of Gln (10 mM) also stimulates cell proliferation and differentiation via activation of JNK and ERK1/2 signaling pathways, respectively (Brasse-Lagnel et al. 2010; Rhoads et al. 2000). Gln activates JNK activity that increases the phosphorylation of c-Jun transcription factor, leading to the subsequent formation of activating protein 1 (AP-1; DNA-binding homodimers or heterodimers), thereby stimulating the transcription of *c-jun* gene. On the other hand, ERK1/2 activation by Gln results in the subsequent phosphorylation of the transcription factor ELK1, which enhances the mRNA level of *c-myc* gene (Rhoads et al. 1997).

Emerging evidence shows that MAPK can negatively regulate AA signaling via the Girdin/SLC3A2 complex (Weng et al. 2018). Girders of actin filaments (Girdin; also known as Gα-interacting vesicle-associated protein) is a multifunctional protein that is involved in cell migration, endocytosis, cell-size control, as well as the PI3K-AKT pathway (Weng et al. 2014). Direct phosphorylation of Girdin by ERK1/2 is required for the formation of the Girdin/SLC3A2 complex, which in turn translocates SLC3A2 from the cell membrane to the lysosome, thereby reducing the intracellular concentrations of AAs (e.g., Gln and Leu). This negative regulation of AA signaling eventually inactivates the mTORC1 pathway and activates autophagy to degrade intracellular proteins.

2.7 Summary

A clear picture has emerged of the functional roles of AAs in regulating growth, reproduction, and immunity in humans and animals via various cell signaling cascades. Aside from their roles in synthesizing proteins, some functional AAs (e.g., arginine, glutamine, glutamate, glycine, leucine, proline, and tryptophan) act via their transceptors and sensors coupled with other sensing complex components to activate mTORC1 near the cell membrane, the lysosomal surface, and the Golgi apparatus, thereby controlling cell metabolism and growth. These AAs also govern the AMPK and MAPK signaling pathways to regulate cell proliferation, cell cycle progression, as well as cell division and differentiation. Moving forward, we will see whether other proteinogenic and nonproteinogenic AAs serve as functional AAs and whether other AA sensors exist across subcellular compartments. Reliable methods are needed to analyze amino acid levels within the subcellular compartments, such as the lysosome and Golgi apparatus. Moreover, the molecular mechanisms responsible for mTORC2 and AA sensing in animal cells require further investigation.

Acknowledgements This work is supported by the Hatch Project 1020014 from the USDA National Institute of Food Agriculture, Faculty Research and Professional Development Award 2020-2571, and Research and Innovation Seed Funding Award 2021-1946 from North Carolina State University, and Texas A&M AgriLife Research H-8200. We apologize to those authors whose articles were not cited because of space limitations.

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3 Amino Acids in Endoplasmic Reticulum Stress and Redox Signaling

Ying Yang, Yu He, Yuhang Jin, Guoyao Wu, and Zhenlong Wu

Abstract

Proteins are the chains of amino acids linked via peptide bonds. In cells, newly synthesized proteins are modified and folded in the endoplasmic reticulum (ER) and matured to be functional proteins before they are transported to other tissues or organs. In addition to protein synthesis, the ER is also a stress-sensing organelle for diverse biological functions, such as calcium storage, lipid synthesis, and cellular metabolism. Nutrient deprivation, accumulation of reactive oxygen species, and other intracellular insults can activate ER stress and unfolded protein response (UPR) to restore homeostasis. Dysfunction of the ER influences cellular physiology and metabolism, and contributes to the pathogenesis of various diseases. Amino acids are the building blocks for proteins of eukaryotic organisms. Both in vivo and in vitro studies have found that amino acids can function as signaling molecules to regulate gene expression, cell proliferation and apoptosis, immune response, and antioxidant capacity in numerous biological processes. Importantly, several lines of studies have indicated that amino acids regulate the abundances of proteins implicated in UPR and the redox state, therefore restoring the intracellular homeostasis. Amino acids play an important role in regulating ER stress and redox homeostasis in animal cells for their survival, growth, and development.

Keywords

Amino acid · Endoplasmic reticulum stress · Oxidative stress · Redox signaling

Abbreviations

AARE	Amino acid response element
Arg	L-arginine
ARE	Antioxidant responsive element
ATF6	Activating transcription factor 6
ATF4	Activating transcription factor 4
BCAA	Branched-chain amino acid
BiP	Binding immunoglobulin protein
CHOP	CCAAT/enhancer-binding protein homologous protein
Cys	L-cysteine
ER	Endoplasmic reticulum
ERS	Endoplasmic reticulum stress
ERAD	ER-associated degradation
Gly	Glycine
Gln	L-glutamine
GPX	Glutathione peroxidases
GR	Glutathione reductase
GSH	Glutathione

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1332,
https://doi.org/10.1007/978-3-030-74180-8_3

IRE1	Inositol-requiring kinase/endonuclease 1
Keap1	Kelch-like ECH-associating protein 1
Leu	L-leucine
Met	L-methionine
NO	Nitric oxide
Nfr2	Nuclear factor erythroid 2-related factor 2
PERK	Protein kinase (PKR)-like endoplasmic reticulum kinase
p-eIF2α	Phosphorylated eukaryotic initiation factor 2α
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Ser	L-serine
SOD	Superoxide dismutase
Trp	L-tryptophan
UPR	Unfolded protein response
XBP1	X-box binding protein-1

3.1 Introduction

The endoplasmic reticulum (ER) is a specialized intracellular organelle with multiple cellular functions, such as protein biosynthesis, folding, and modification, as well as calcium storage, lipid and phospholipid biosynthesis, hormone synthesis, and the detoxification of chemical compounds (Braakman and Bulleid 2011). Free proteinogenic amino acids are linked together via peptide bonds to form proteins in the ER, where they are folded and modified before being transported to other tissues and organs. It has been reported that the high concentration of calcium ions and the oxidizing properties of the contents of the ER cavities act together and contribute to the proper synthesis and folding of proteins. A characteristic of ER is its dynamic capacity to accommodate increases in the demand for protein folding in specific tissues or conditions. In cells with high rates of cellular protein turnover and energy metabolism, such as enterocytes and activated immune cells, a proper function of the ER is required to maintain the functions of secreted proteins (Schwarz and Blower 2016). However, a variety of factors, such as nutrient deficiency, disturbances in cellular redox regulation, and aberrations in calcium regulation, have been reported to disrupt the ER homeostasis and lead to the accumulation of unfolded or misfolded proteins in the ER lumen, namely the endoplasmic reticulum stress (ER stress) (Vincenz-Donnelly and Hipp 2017). Mammalian cells have evolved to possess a protective or adaptive response known as the unfolded protein response (UPR) to restore the ER homeostasis by either enhancing the capacity for protein folding and modification, or eliminating misfolded proteins by the ER-associated degradation (ERAD) signaling pathway. However, if the ER stress is severe or prolonged, it triggers the cell death pathways to remove the damaged cells (Hetz 2012; Tabas and Ron 2011). In addition, ER stress has been reported to be associated with alterations in metabolism, inflammation, and the autophagy signaling pathway (Hassler et al. 2012). Therefore, activation of UPR is a critical event for cell fate decision and needs to be well controlled, considering that it can trigger both pro-survival and pro-apoptotic components in the presence of misfolded or unfolded proteins (Gardner and Walter 2011; Yoshida et al. 2001). In this review, we highlighted recent studies on functional roles of amino acids in ER stress and redox homeostasis, especially how ER stress is activated in the context of amino acid deprivation and redox status alteration, as well as the influence of UPR on intracellular homeostasis. Amino acids and their metabolites with an ability to regulate UPR signaling and antioxidant activity were reviewed and discussed.

3.2 ER Stress Signaling Pathways

The ER is responsible for folding, processing, quality control of newly synthesized proteins, including secreted, membrane, and lysosomal proteins. To coordinate the complicated processes in the limited biological microenvironments, the cells are equipped with the unfolded protein response (UPR) in response to various stresses that challenged the ER. The adaptive UPR (Fig. 3.1) is comprised of signal transduction pathways initiated by three protein sensors

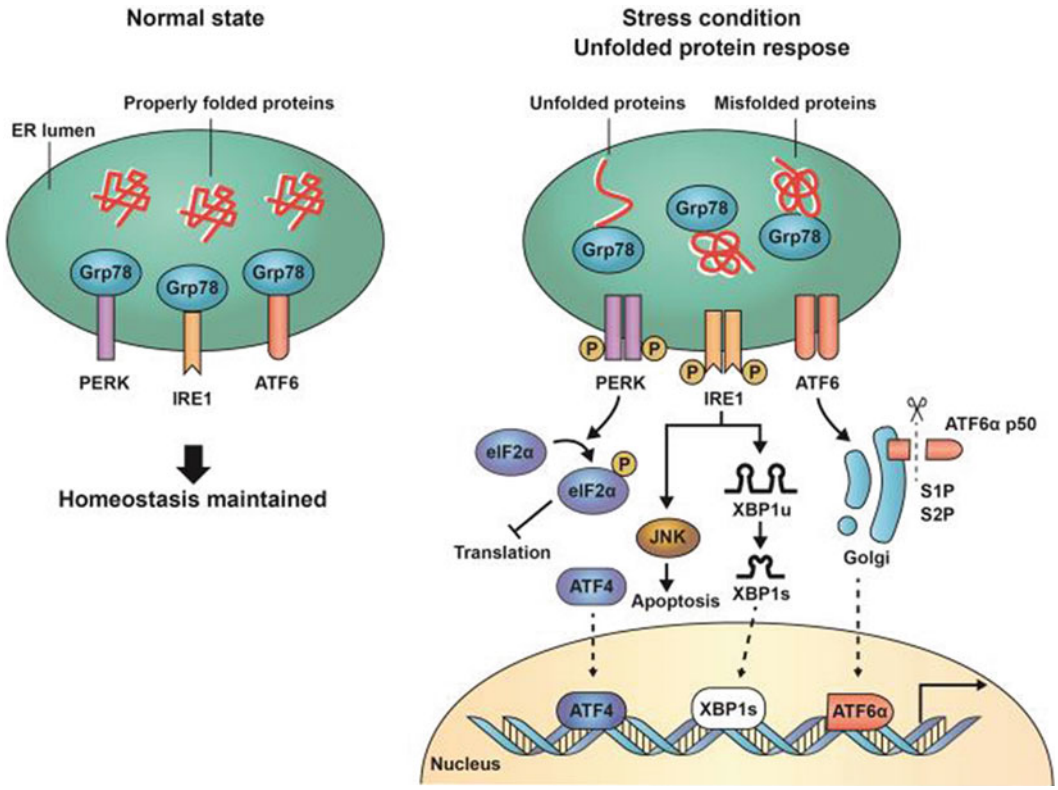

Fig. 3.1 Unfold protein response (UPR) in animal cells. Under non-stress conditions, the transmembrane stress sensor proteins, including PERK, IRE1, and ATF6, are bound by the ER chaperone binding immunoglobulin protein (BiP), therefore maintaining in an inactive state. Upon accumulation of misfolded or unfolded proteins in the ER, UPR signal transducers PERK, IRE1, and ATF6 are released by BiP, leading to the initiation of the UPR and downstream cascade signaling to restore intracellular homeostasis

on the ER membrane, including inositol-requiring kinase/endonuclease 1 (IRE1), double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6), in an attempt to restore homeostasis and normal ER function (Schroder and Kaufman 2005). These transducer proteins are expressed in the cells, but are tightly bounded by the ER chaperone binding immunoglobulin protein (BiP, also known as glucose-regulated protein 78) under non-stressed conditions (Bertolotti et al. 2000). In response to an increase in the concentrations of unfolded or misfolded proteins in the ER, BiP dissociates from the luminal domains of the three protein sensors to locate and bind the misfolded proteins, therefore activating IRE1, PERK, or ATF6, and initiating UPR, as well as the downstream cascade signaling (;Ron and Walter 2007; Wang and Kaufman 2012; Figs. 3.2 and 3.3).

IRE1 is an endoribonuclease located in the ER membrane and the most evolutionarily conserved branch of ER stress transducer. Up to now, two homologs, IRE1α and IRE1β, have been identified in mammalian cells. IRE1α is ubiquitously expressed in a variety of tissues, while IRE1β is mainly expressed in the intestinal epithelium of the gut and the respiratory tract (Iwawaki et al. 2009; Tsuru et al. 2013). IRE1 is activated through autophosphorylation of the C-terminal kinase domain at S724 following release from BiP-IRE1 complex (Adams et al. 2019; Gardner and Walter 2011). The endoribonuclease activity of activated IRE1 removes a 26 bp intron from an unspliced mRNA of a specific basic leucine

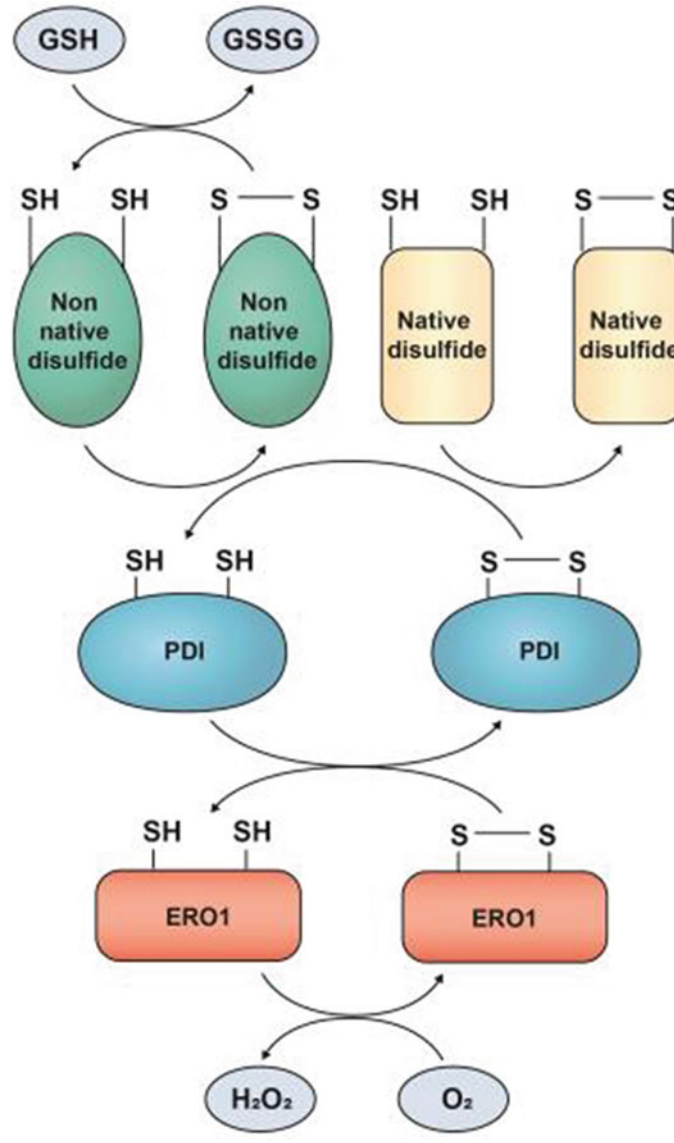

Fig. 3.2 Generation of ROS during the protein folding process in the ER. In response to ER stress, the formation of the non-native disulfide bonds results in the depletion of glutathione (GSH) and generation of H_2O_2

zipper-containing transcription factor, also known as X-box binding protein-1 (XBP1), leading to the generation of XBP1s, the activated forms of the XBP1 proteins (Calfon et al. 2002; Yoshida et al. 2001). XBP1s binds to the promoter region of targets and activates the expression of genes implicated in protein folding, secretion, maturation, ERAD signaling, and phospholipid synthesis (Lee et al. 2003).

PERK is a type I transmembrane protein, composed of an ER luminal stress sensor and a cytosolic protein kinase domain. Dissociation of BiP from the N terminus of PERK initiates the dimerization and autophosphorylation of the kinase domain, leading to PERK activation (Wek and Cavener 2007). Activated PERK can phosphorylate eukaryotic initiation factor 2α (p-eIF2α) and activating transcription factor 4 (ATF4) to reduce global protein synthesis and alleviate the overloading of misfolded or unfolded proteins in the ER. The transcription factor C/EBP homologous protein (CHOP) is a pro-

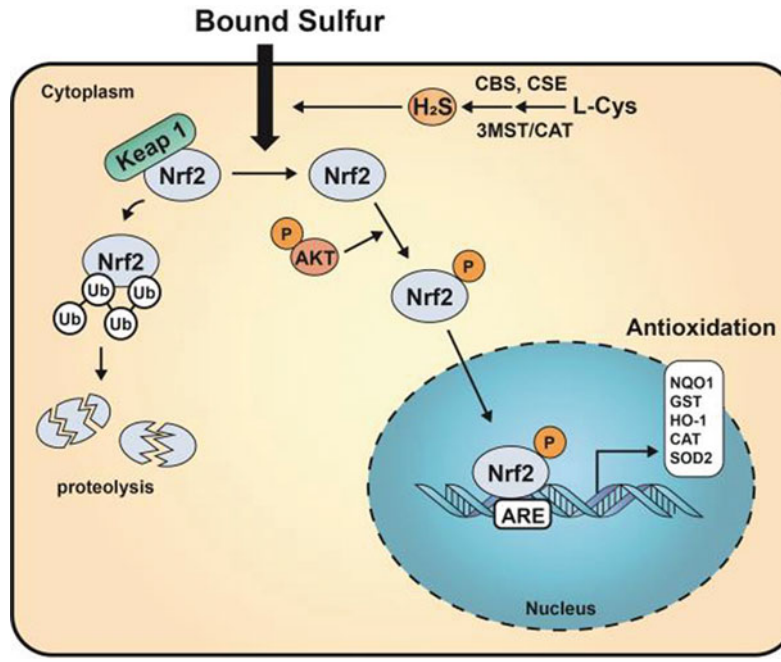

Fig. 3.3 Activation of the nuclear factor erythroid-related factor 2 (Nrf2) signaling. Under non-stress conditions, the Nrf2 protein is degraded via a ubiquitin-dependent mechanism. In animal cells, reactive species, including ROS and RNS, stimulate the translocation of Nrf2 from the cytoplasm to the nuclei, where it binds to the promoter of target genes and activates the expression of the corresponding proteins

apoptotic gene activated by ATF4 and plays a critical role in ER-related apoptosis by regulating the release of Bcl-2 family proteins from the mitochondria to the cytosol in various cells (Han et al. 2013; Lu et al. 2004). By contrast, depletion of CHOP in mice has been reported to abrogate ER stress-induced apoptosis (Hu et al. 2018).

Of particular note, the transcription factor CHOP can be activated by three ER-resident proteins, ATF6, IRE1, or PERK, while PERK-eIF2α is regarded as the strongest inducer in the epithelial cells (Scull and Tabas 2011). It remains unknown whether this effect of PERK is a tissue-specific or a general effect. More studies are required to address this question in both in vitro and in vivo experiments. In addition, ATF4 can also enhance the transcription of GADD34 (growth arrest and DNA damage-inducible protein) and can induce the protein phosphatase 1-mediated dephosphorylation of PERK, establishing a negative feedback regulation on the PERK signaling (Ghemrawi et al. 2018).

ATF6 is the third UPR transducer located in the ER with a molecular weight of 90 kDa. There are two distinct ATF6 isoforms, ATF6α and ATF6β, in mammalian cells (Yoshida et al. 2000). ATF6 activation involves a complex series of translation and irreversible proteolytic processing steps, ultimately leading to the upregulation of a pro-survival transcriptional program, in the presence of unfolded or misfolded proteins (Chen et al. 2002; Haze et al. 1999). Unlike IRE1 and PERK, immature ATF6 is transported to the Golgi apparatus, where it is cleaved by resident site-1 protease (S1P) and site-2 protease (S2P) and then released into the cytosol (Okada et al. 2003; Shen et al. 2002). Cleaved ATF6 translocates to the nucleus where it interacts with other transcription factors and forms a homodimer or a heterodimer to regulate the expression of genes for proteins involved in protein folding or ERAD to restore ER homeostasis, or induce cell death in response to severe or prolonged ER stress (Bailey and O'Hare 2007; Yamamoto et al. 2007).

3.3 Functions of UPR and the Pathogenesis of ER-Related Diseases

Due to the critical role of ER in maintaining proteostasis network in almost all the tissues of the body, the UPR signaling is inhibited under physiological conditions. Importantly, modest ER stress can be resolved by a dynamically adaptive UPR response through the above-mentioned mechanisms. For secretory cells, such as hepatocytes, pancreatic β-cells, and intestinal secretory cells with a high rate of protein synthesis, an appropriate function of ER is critical for the survival and biological functions of cells. Genetic depletion of the ER stress sensor proteins (IRE1α, PERK, or ATF6α) or its downstream targets leads to a decreased number of goblet cells, dysregulated epithelial barrier function, and increased susceptibility to chemically induced colitis in mice, indicating a critical role of UPR in maintaining the intestinal barrier integrity and functions (Brandl et al. 2009; Cao et al. 2014; Zhang et al. 2015).

The gastrointestinal tract is the habitat for 100 trillion of different microbial organisms, including bacteria, viruses, fungi, and protozoans. Recent studies have shown that the interaction between host and intestinal flora plays an important role in the intestinal mucosal barrier function (Zhang et al. 2017). A compromised mucosal barrier has been reported to be associated with a decreased number of commensal bacteria and the increased colonization of pathogenic bacteria onto intestinal epithelial cells (Ahmed et al. 2016). Intestinal dysbiosis is associated with the elevated generation of ROS or alterations in bacterial metabolites (Baumler and Sperandio 2016). ROS and metabolites interact with host cells, including epithelial cells, secretory cells, and immune cells in the gastrointestinal tract, resulting in increases in mucosal permeability, immune cell infiltration, and ER stress (Molloy et al. 2013; Sartor 2008). In vitro study shows that toll-like receptors (TLRs) in macrophages promote the splicing of XBP1 to optimize the production of proinflammatory cytokines, indicating a role of XBP1 in maintaining mammalian host defenses (Martinon et al. 2010). The goblet cells and Paneth cells can produce and secrete mucin glycoproteins, defensins, lysozymes, antimicrobial lectins, collectins, and smaller amounts of MUC2 to separate the luminal microbial flora from the intestinal epithelium (Harrison et al. 2013). These secretory cells highly depend on the ER function of the epithelial cells (Ma et al. 2017). Activation of TLRs following bacterial infection leads to ER stress and impairs the function of the secretory cells, therefore contributing to mucosal barrier dysfunction. By contrast, chemical molecules, such as tauroursodeoxycholate and 4-phenylbutyrate, have been found to reduce ER stress and improve intestinal mucosal barrier function in the spontaneous or the chemical-induced colitis of mice (Cao et al. 2013; Luo and Cao 2015).

3.4 Amino Acids with an Ability to Modulate the UPR

Branched-chain amino acids (BCAAs), including leucine, isoleucine, and valine, are nutritionally essential amino acids with an ability to regulate mitochondrial biogenesis, cellular energy metabolism, protein turnover, and antioxidant systems (Lynch and Adams 2014; Manjarín et al. 2020). These amino acids are abundant in both plant and animal proteins (Hou et al. 2019; Li and Wu 2020). It has been reported that activation of ER stress by tunicamycin, a pharmacological molecule that inhibits glycosylation, results in a downregulation of genes involved in BCAA metabolism in 3T3-L1 adipocytes. Similar phenotype was observed in the adipose-specific overexpression of spliced XBP1 of mice (Burrill et al. 2015). By contrast, the intake of L-leucine (Leu), one of BCAAs, can rescue excessive ER stress in the fatty liver by downregulating the XBP1 mRNA splicing level (Yokota et al. 2016). The balance of the three BCAAs in diets affects the food intake of animals, however, it remains unknown whether

their balance influences the UPR signalling in the intestinal epithelial cells (Wu 2018).

As the most abundant amino acid in the plasma, L-glutamine (Gln) is critical for rapidly diving cells, such as enterocytes and immune cells. Administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) has been reported to upregulate ER stress markers, including CHOP, BiP, and caspase12 in the colon tissue of rodents (Crespo et al. 2012). Interestingly, these effects are significantly attenuated by Gln supplementation, indicating a protective effect on TNBS-induced colitis. In another study, the authors find that Gln deprivation induces ER stress-related autophagic response and the secretion of IL-8 through the IRE1-JNK signaling (Shanware et al. 2014a), as well as the activation of CHOP through the p-eIF2α signaling pathway (Shanware et al. 2014b).

Depletion of L-arginine (Arg) induces activation of IRE1 and production of matured XBP1, which in turn upregulates the expression of genes responsible for the ER-associated degradation of misfolded glycoproteins in T lymphocytes (Garcia-Navas et al. 2012). Gene expression array analysis has shown that amino acid deprivation leads to the activation of the eIF2α-ATF4 signaling and promotes the expression of genes implicated in amino acid transport and glutathione biosynthesis to maintain a normal redox state in cells (Harding et al. 2003). By contrast, gene silencing of ATF4 results in the accumulation of endogenous peroxides during ER stress, indicating a protective effect of ATF4 on cellular survival in response to amino acid insufficiency and oxidative stress. In another study, the authors demonstrated that both ATF4 and ATF2 are involved in regulating CHOP expression following leucine starvation (Averous et al. 2004). It remains unknown how these two proteins are induced and contribute to cell survival in response to specific stimuli under various conditions. Homocysteine exposure has been reported to activate UPR and induce the sterol regulatory element-binding proteins (SREBPs), a regulator related to triglyceride biosynthesis and uptake, as well as the intracellular accumulation of cholesterol in various cells (Werstuck et al. 2001). Interestingly, homocysteine-induced ER stress and upregulation of SREBPs can be attenuated by glycine or serine administration through yet unknown mechanisms (Fukada et al. 2006; Sim et al. 2016).

Weaning stress impairs intestinal mucosal barrier, as well as the transport and absorption of nutrients in neonates and piglets (Wijtten et al. 2011). In our recent study, we found that the abundances of ER stress signaling proteins, such as IRE1, ATF6, and PERK, are increased in the jejunum of piglets, as compared with these of suckling piglets (He et al. 2019). Interestingly, these effects were significantly reduced by Gln or Gly pre-administration. This is the first report showing a regulatory effect of amino acids on mitigating the weaning stress-induced ER stress and intestinal barrier dysfunction in piglets (Fan et al. 2019; He et al. 2019). Despite a beneficial effect of amino acids on the UPR, excessive L-cysteine induced the phosphorylation of eIF2α, the activation of CHOP, and elevated mRNA levels for spliced XBP1 in the intestinal porcine epithelial cells, suggesting the activation of PERK-eIF2α and IRE1-XBP1s (Ji et al. 2016). Moreover, excessive nitric oxide (NO) causes the upregulation of CHOP expression and the induction of cell death in macrophages and pancreatic β-cells (Mori 2007). These results indicate that an appropriate dose of amino acid supplemented to stressed animals might benefit the physiological function and metabolism of host cells.

3.5 Cross Talk Between ER Stress and Oxidative Stress

Oxidative stress is another type of response through which the cells adapt to and survive stress conditions caused by endogenous or exogenous stimuli. It is a defense network that protects cells from oxidative damage triggered by the reactive oxygen species (ROS) or reactive nitrogen species (RNS). ROS are a heterogeneous population of molecules, including free radicals, such as hydroxyl radical (OH^-), superoxide anion (O_2^-), peroxyl (RO_2), and

hydroperoxyl (HRO_2^-), and non-charged species, such as hydrogen peroxide (H_2O_2) and hydrochloric acid (HCl) (Fang et al. 2002). RNS refers to radicals like nitric oxide (NO) and nitric dioxide (NO_2), as well as non-radicals such as nitrous acid (HNO_2) and dinitrogen tetroxide (N_2O_4). The mitochondrial electron transport chain is the main site of reactive species formation during the aerobic metabolism. Mammalian tissue has an innate antioxidant capacity including enzymatic antioxidants (SOD, catalase, glutathione peroxidase, glutathione S-transferase) and nonenzymatic antioxidants (glutathione, vitamin A, vitamin C, vitamin E) to maintain a redox homeostasis for intracellular processes and functions (Moldogazieva et al. 2018b; Poljsak et al. 2013). An imbalance between the generation and elimination of free radicals leads to oxidative stress and causes irreversible damage to macromolecules, such as DNA, protein, and lipid, resulting in the dysfunction of cells, tissues, or organs in animals (Gonsette 2008). The production of free radicals can lead to vastly different cellular outcomes, depending on their subcellular location, the amount of free radicals, and the antioxidant defense systems (Birben et al. 2012).

The interplay between the production of oxidants and the antioxidant defense is highly regulated to maintain cellular redox homeostasis (Moldogazieva et al. 2018a). Growing evidence shows that oxidative stress is actively interacted with the UPR response under both physiological and pathological conditions. Perturbation of these two stress responses has been implicated in the pathogenesis of various diseases, such as IBD, metabolic syndromes, and inflammatory response (Dandekar et al. 2015).

Integration of the ER stress and oxidative stress responses is based on the following observations. First, the ER and the peroxisomes are significant contributors to ROS generation in the cell. The ROS are the by-products of normal cellular metabolism in the mitochondria (Brown and Borutaite 2012; Starkov 2008). However, increasing evidence over the last decade indicates that the ER and the peroxisomes produce as much or even more ROS than the mitochondria (Fransen et al. 2012). Second, proteins that traverse the secretory pathway typically depend on the disulfide bonds for their maturation and function (Dandekar et al. 2015). The formation of disulfide bonds is mainly catalyzed by the protein disulfide isomerase (PDI) and ER oxidase 1α (ERO1), which produce ROS. Generation of ROS from the ER results in the release of calcium, which is taken up by the mitochondria to cause calcium overloading as well as increases in mitochondrial metabolism and the generation of ROS. In addition, it is estimated that peroxisomes in rat liver may be responsible for as much as 20% of the oxygen consumption and 35% of H_2O_2 production (Boveris et al. 1972). In another study, the authors found that a long-term administration of peroxisome proliferators to rodents induces oxidative stress in hepatic cells (Kasai et al. 1989). These observations indicate that generation of ROS is also a by-product of peroxisomal metabolism (Fransen et al. 2012). Due to these findings, it is estimated that the ER and the peroxisomes produce a larger amount of ROS than the mitochondria (Fransen et al. 2012). Also, the ER and the peroxisomes possess intricate protective mechanisms to counteract oxidative stress and maintain redox balance.

Furthermore, oxidative stress triggered by various stimuli can increase intracellular calcium concentration, impair protein folding, and initiate ER stress. Hyperglycemia, free fatty acids, and oxidized low-density lipoprotein have been shown to enhance endothelial NADPH oxidase (NOX) activity, the excessive generation of ROS, and UPR signaling in the ER (Maamoun et al. 2019). Intestinal epithelial cells are constantly exposed to a variety of xenobiotic factors, including pathogenic microorganisms, dietary antigens, or toxic components. These substances have been reported to induce oxidative stress and ER stress in the host cells and contribute to the pathogenesis of IBD (Zhang et al. 2017). Deoxynivalenol, a mycotoxin produced by *Fusarium graminearum* or *Fusarium culmorum*, is one of the most prevalent toxins in contaminated grains and affects the health of humans and animals (Payros et al. 2016). Exposure to DON has been reported to be associated with the

impairment of gastrointestinal integrity (Kang et al. 2019), the reduced absorption of nutrients (Maresca et al. 2002), the increased production of ROS, and the activation of the UPR signaling in intestinal epithelial cells (Tang et al. 2015; Wu et al. 2014). In our recent study, we found that 3-acetyldeoxynivalenol, the acetylated derivative of deoxynivalenol, induced ER stress and apoptosis in macrophages (Liu et al. 2020). Activation of the UPR under the conditions of oxidative stress is an adaptive mechanism to preserve cell function and survival in response to harmful exogenous stimuli. Persistent stress response activates apoptotic cascades and contributes to the pathogenesis of multiple human diseases, including diabetes, gastrointestinal diseases, and neurodegenerative diseases (Malhotra and Kaufman 2007).

3.6 Regulation of the UPR Signaling by Amino Acids Through Restoring the Redox Homeostasis

In contrast to the cytosol, the ER lumen is an oxidizing environment which is required for the formation of proper native disulfide bonds in proteins (van der Vlies et al. 2003). Generation of ROS from the mitochondria, ER, and the peroxisomes is associated with the activation of stress response, including both oxidative stress and ER stress. Amino acids with an ability to enhance the production of GSH might be potential nutrients to mitigate ER stress and maintain intracellular homeostasis. GSH, a critical antioxidant in mammalian cells, is synthesized from cysteine, glutamate, and glycine sequentially by two cytosolic enzymes, glutamate-cysteine ligase (GCL) and glutathione synthase (GS) (Wu et al. 2004). Glycine and cysteine are particularly abundant in collagen and hemoglobin, respectively (Li and Wu 2018; Wu 2013). A limited availability of substrates or a reduced activity of these enzymes leads to decreased intracellular GSH and triggers ER stress-mediated cell death in epithelial cells (Chaveroux et al. 2011; Harding et al. 2003; Wu et al. 2011). Both in vitro and in vivo studies have shown that supplementation with Gln, Gly, glutamate, or Arg increases GSH concentration, regulates redox homeostasis, and reduces ER stress-related cell death in the enterocytes of neonates (Circu and Aw 2012a; b).

L-Tryptophan (Trp) is a substrate for the synthesis of certain bioactive molecules, such as melatonin, kynurenic acid, and NAPD. The presence of the pyrrole ring in Trp confers to its susceptibility to oxidants. Trp and its metabolites can act as effective antioxidants and remove the intracellular ROS or RNS, therefore protecting cells from oxidant-induced cellular damage (Bitzer-Quintero et al. 2010; Perez-Gonzalez et al. 2015). In a recent study, Xu et al. found that both Trp and 3-hydroxyanthranilic acid (a metabolite of tryptophan) activated the Nrf2 cell signaling pathway by uncoupling the interaction between Nrf2 and Keap1. Activated Nrf2 is translocated to the nuclei to upregulate the expression and activities of downstream antioxidant proteins, thereby enhancing cellular antioxidant capacity (Xu et al. 2018). Our recent study indicates that *N*-acetylserotonin, a metabolite of Trp, attenuates the oxidative stress-induced upregulation of apoptotic proteins, including Bax and cleaved caspase-3, thereby alleviating the reduction of tight junction proteins in enterocytes of piglets (Liang et al. 2020). These effects of *N*-acetylserotonin are reversed by inhibitors of Nrf2, indicating a critical role of Nrf2 in apoptosis and the maintenance of the intestinal mucosal barrier.

Availability of L-cysteine (Cys) can limit the synthesis of GSH, and the dietary supplementation of Cys results in the enhanced production of GSH to resist oxidative stress in humans and animals (Badaloo et al. 2002; Rezzi et al. 2007). Also, Cys is the major substrate to produce about 70% endogenous H_2S, which is a potent antioxidant at physiological concentrations by directly scavenging the ROS or RNS, therefore contributing to redox homeostasis (Kimura et al. 2010; McBean 2012; Szabo 2007; Yonezawa et al. 2007). Taurine, a sulfur-containing beta amino acid synthesized from Cys, has antioxidative and anti-inflammatory activities in

multiple systems, including neurological, muscular, retinal, immunological, and cardiovascular function (Choi and Jung 2017; Parvez et al. 2008; Wu 2020). Therefore, the anti-oxidative property of Cys is mainly mediated by GSH, H_2S, and taurine.

3.7 Regulation of Nrf2 Signaling by Amino Acids and Its Contribution to ER Homeostasis

Nrf2 is a transcriptional factor implicated in regulating cellular anti-oxidative stress by upregulating the expression of numerous genes, including GR, GPX, SOD, NAD(P)H: quinone oxidoreductase (NQO1), and HO-1, therefore enhancing the antioxidant capacity and contributing to reduced oxidative damage and UPR signaling (Li et al. 2004; Nguyen et al. 2009). Under normal conditions, Nrf2 localizes in the cytoplasm, where it interacts with the actin-binding protein, Kelch-like ECH-associating protein 1 (Keap1). Keap1 functions as an adaptor of Cul3-based E3 ubiquitin ligase and targets Nrf2 for rapid degradation by the ubiquitin–proteasome (Kobayashi et al. 2004). Dissociation of Nrf2 from Keap1 leads to the release of the free Nrf2. The latter is translocated to the nucleus, heteromerizes with Maf(s), and binds to a cis-acting element known as the antioxidant responsive element (ARE) or electrophile responsive element within the regulatory regions of the target genes. Supplementation with amino acids, such as Gln, Trp, Arg, or their metabolites, has been reported to enhance the antioxidant capacity in cells and tissues by upregulating the downstream targets. Even though the dissociation of the Nrf2-Keap1 complex is considered as a key step in regulating Nrf2 activity, recent study shows that H_2S can activate the Nrf2-ARE pathway through the inhibition of Keap1 via a process that involves Keap1 sulfhydration (McBean 2012), indicating another level of regulation in the Nrf2 cell signaling.

Arg is used for the synthesis of both protein and other substances (e.g., NO, creatine, and polyamines) with enormous biological importance (Wu et al. 2009). For example, NO plays an important role in regulating the antioxidant defense system (Dai et al. 2013; Lass et al. 2002; Wu et al. 2009). Several lines of evidence have shown that Arg supplementation increases the activities of antioxidant enzymes in patients with ischemic heart disease (Tripathi and Misra 2009; Tripathi and Pandey 2013). Importantly, dietary supplementation of Arg stimulates GSH synthesis and activates Nrf2 pathway, leading to the upregulation of ARE-driven antioxidant expressions via Nrf2-Keap1 pathway (Liang et al. 2018).

Sulfur-contending amino acids, such as cysteine and methionine, are more susceptible to be oxidized (Finkelstein et al. 1988; MacKay et al. 2012; Xu et al. 2017). Met restriction is associated with reductions in oxidative stress biomarkers, including plasma 8-hydoxydeoxyguanosine (8-OHdG) and 8-isoprostane through a GSH-dependent mechanism (Hosseini et al. 2012; Liu et al. 2017) or by inhibiting the mitochondrial ROS generation (Maddineni et al. 2013; Yang et al. 2015). In addition, H2S, a gas molecule produced by Cys catabolism, can inhibit Keap1, therefore activating the Nrf2 cell signaling (McBean 2012).

3.8 Conclusion

The ER is an organelle for the synthesis, folding, and modification of proteins in both humans and animals. A functional role of the ER in response to various stresses and its correlation with the pathogenesis of disease expands our understanding of ER in health and disease. Both in vivo and in vitro studies have shown that amino acids can regulate the abundances of proteins implicated in UPR and the redox signaling, therefore contributing to intracellular homeostasis. Despite these progresses, the underlying mechanisms responsible for these regulation are not clear. Nutrient deprivation has been reported to trigger ER stress and oxidative stress in epithelial cells. However, how these two critical stress responses interact and contribute to cellular survival or cell death is largely unknown. The intestinal microbiota has been reported to play an important role in regulating the physiology and metabolism of the intestinal

epithelial cells. The relevant bacterial effector proteins implicated in this cross talk are elusive. Mechanistic studies are warranted to answer these questions before nutritionally therapeutic strategies targeting ER stress signaling can be successfully developed for both humans and animals.

Acknowledgements This work was supported by the National Natural Science Foundation of China (No. 31572423 and 31625025), the "111" Project (B16044), Jinxinnong Animal Science Development Foundation, and Texas A&M AgriLife Research (H-8200). Z. W. and G. W. designed the review; Y. Y., Y. H., and Y. J. drafted the manuscript; Z. W., Y. Y., and G. W. revised and finalized the manuscript. Z. W. had primary responsibility for final content. All authors read and approved the final manuscript.

Competing Interests The authors had no conflicts of interest.

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Amino Acids in Autophagy: Regulation and Function

4

James Z. Shen, Guoyao Wu, and Shaodong Guo

Abstract

Autophagy is a dynamic process in which the eukaryotic cells break down intracellular components by lysosomal degradation. Under the normal condition, the basal level of autophagy removes damaged organelles, misfolded proteins, or protein aggregates to keep cells in a homeostatic condition. Deprivation of nutrients (e.g., removal of amino acids) stimulates autophagy activity, promoting lysosomal degradation and the recycling of cellular components for cell survival. Importantly, insulin and amino acids are two main inhibitors of autophagy. They both activate the mTOR complex 1 (mTORC1) signaling pathway to inhibit the autophagy upstream of the uncoordinated-51 like kinase 1/2 (ULK1/2) complex that triggers autophagosome formation. In particular, insulin activates mTORC1 via the PI3K class I-AKT pathway; while amino acids activate mTORC1 either through the PI3K class III (hVps34) pathway or through a variety of amino acid sensors located in the cytosol or lysosomal membrane. These amino acid sensors control the translocation of mTORC1 from the cytosol to the lysosomal surface where mTORC1 is activated by Rheb GTPase, therefore regulating autophagy and the lysosomal protein degradation.

Keywords

Amino acids · Autophagy · Mammalian target of rapamycin complex 1 · Calcium/calmodulin-dependent protein kinase kinase · Autophagosome · Lysosome · Rheb · Rag GTPase · Leucine · Arginine

Abbreviations

AMPK	AMP-activated protein kinase
Akt	Protein kinase B
BCAA	Branched-chain amino acid
ERK	Extracellular signal-regulated kinases
MAPK	Mitogen-activated protein kinase
mTOR	Mechanistic target of rapamycin
PI3K	Phosphatidylinositol-3-kinase
ULK1/2	Uncoordinated-51 like kinase 1/2

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1332,
https://doi.org/10.1007/978-3-030-74180-8_4

4.1 Introduction to Autophagy

Autophagy refers to a dynamic catabolic process in which the eukaryotic cells develop double-membrane vesicles called autophagosomes to sequester and deliver intracellular components to lysosomes for degradation. Under normal conditions (without metabolic stress signals), the eukaryotic cells maintain a basal level of autophagy for the clearance of misfolded proteins, protein aggregates, and damaged/obsolete organelles. These processes are necessary for cellular metabolic homeostasis (Mizushima and Komatsu 2011; Rubinsztein et al. 2011; Jiang et al. 2015; Tan et al. 2017). Upon receiving stimulation from metabolic stress signals such as amino acid deprivation, the activity of autophagy is upregulated, which promotes lysosomal degradation and the recycling of intracellular components for utilization by cells. Thus, autophagy is widely recognized as an important pro-survival intracellular mechanism in eukaryotic cells. Nevertheless, once the cells are under an extremely stressful condition, autophagy will drive the cells to either apoptotic or autophagic cell death by excessive "self-digestion" and degradation of essential cellular components (Pattingre et al. 2005; Shore et al. 2011; Urra et al. 2013; Yonekawa and Thorburn 2013). The dual functions (pro-survival and pro-death) of autophagy facilitate the eukaryotes to adapt to different environments, whereas dysfunction of autophagy makes individuals susceptible to neurodegeneration, metabolic disorders, muscle atrophy, or cancer (Uversky et al. 2008; Mizushima and Komatsu 2011; Menzies et al. 2017).

There are three major types of autophagy in the eukaryotic cells: macroautophagy, microautophagy, and chaperone-mediated autophagy that are classified by the type of cargo delivery (Meijer and Codogno 2011; Wesselborg and Stork 2015; Galluzzi et al. 2017). In macroautophagy, the components to be degraded are wrapped by autophagosome and thereafter delivered to the lysosome for degradation. In microautophagy, the intracellular components directly invaginate into the lysosome and proceed with lysosomal degradation by enzymes such as proteases. In the chaperon-mediated autophagy, damaged and misfolded proteins are transported into the lumen of the lysosome via the processes that are facilitated by cytosolic and lysosomal chaperones. Among these three types of autophagy, macroautophagy (hereinafter referred to as autophagy) is the most characterized pathway. This process consists of several key steps: development of autophagy precursor (i.e., the omegasome), formation of autophagosomes, fusion between the autophagosome and the lysosome to form the autophogolysosome, lysosomal degradation inside the autophagolysosome, and the efflux of degraded products to the cytoplasm (see Fig. 4.1) (Hayat 2016).

In this chapter, we will discuss the regulation of autophagy under either removal or presence of amino acids. In particular, the upstream signaling pathways of serine/threonine kinase mammalian target of rapamycin complex 1 (mTORC1) controlled by amino acid sensors are summarized to highlight the role of the mTOR cell signaling pathway in autophagy under physiological and pathological conditions.

4.2 General Process of Autophagy

The initiation of autophagy is triggered by uncoordinated-51 like kinase-1/2 (ULK1/2), which can be activated through either AMPK-mediated phosphorylation at serine 555 (S555) or a decrease of mTORC1-mediated phosphorylation at serine 757 (S757) (Kim et al. 2011; Yu et al. 2018). Active ULK1/2 forms a complex by binding autophagy-related protein 13 (ATG13), ATG101, and FAK family interacting protein of 200 kDa (FIP200) (Ghislat et al. 2012; Noh et al. 2016; Nazio and Cecconi 2017). The ULK1/2 complex then translocates to a discrete region of the endoplasmic reticulum (ER) characterized by ATG9 (Orsi et al. 2012; Yu et al. 2018), followed by recruitment of the beclin1-class III phosphatidylinositol 3-kinase (PI3K C-III) complex to form the omegasome. Driven by PI3K C-III, the lipid composition of the omegasome membrane is

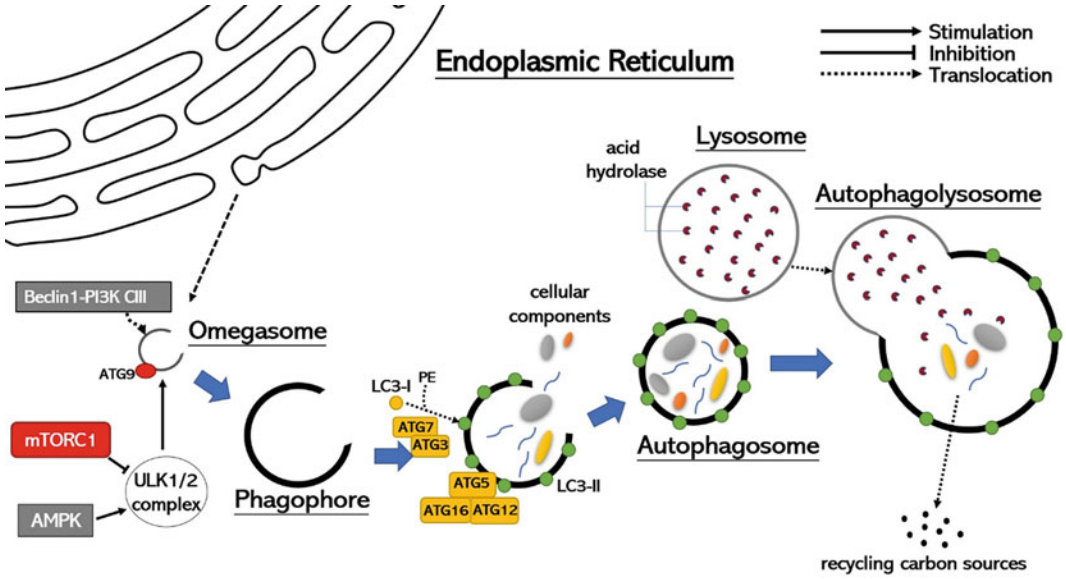

Fig. 4.1 General process of autophagy. Autophagy process is initiated by the ULK1/2 complex which can be activated by AMPK or suppressed by mTORC1. Activated ULK1/2 complex translocates to the endoplasmic reticulum marked by ATG9, followed by recruitment of the beclin1-PI3K C-III complex to form the omegasome. As the lipid composition is changed by PI3K, omegasome transforms into a phagophore. The phagophore starts to collect cellular components and turns into the mature autophagosome after conjugating with the LC3-II and ATG5-ATG12-ATG16 complex. The mature autophagosome fuses with the lysosome to generate the autophagolysosome in which cellular components are degraded and recycled

changed and becomes a phagophore. The phagophore undergoes elongation to internalize cellular components, resulting in the formation of the autophagosome. This process of elongation is regulated by two ubiquitin-like conjugation systems, namely the ATG5-ATG12-ATG16 complex and the lipidated LC3-I (i.e., LC3-II) driven by ATG7/ATG3 (Liu et al. 2009). Both systems are inserted into the autophagosomal membrane to bring it to maturity. Mature autophagosomes fuse with the lysosome to form autophagolysosomes in which lysosomal degradation occurs. The products generated from lysosomal degradation are recycled to cytosol for cell utilization (Fig. 4.1) (Ouimet et al. 2011; Yu et al. 2018).

4.3 Amino Acid Starvation-Induced Autophagy

The negative correlation between concentrations of amino acids and autophagy flux has been extensively reported over the past decades. Mortimore and his research group demonstrated that amino acids deprivation in perfused rat liver induced autophagy (Mortimore and Schworer 1977; Mortimore and Reeta 1986). Nevertheless, the exact mechanisms about the amino acid starvation-induced autophagy have yet been well-established, although several compelling models have been proposed. In general, amino acid starvation activates autophagy mainly through the mTORC1-dependent signaling pathway, although mTORC1-independent signaling pathways do exist (Bar-Peled and Sabatini 2014; Ghislat and Knecht 2015; Meijer et al. 2015). Upon removal of amino acids, cytosolic mTORC1 would not actively translocate to the surface of the lysosome in which mTORC1 turns into its active form. Without being suppressed by mTORC1, ULK1/2 proceeds to trigger its downstream pathways to stimulate autophagy. On the other hand, the amino acid starvation-induced Raf-MEK-ERK1/2 pathway is one of the mTORC1-independent pathways (Ogier-Denis et al. 2000; Pattingre et al. 2003; Corcelle et al.

2007; Cagnol and Chambard 2010). Recent studies reported that mTORC1 could also be one of the downstream targets of ERK1/2 (Wang et al. 2009, 2017; Yang and Klionsky 2010). More importantly, amino acid starvation is usually accompanied by upregulation of intracellular Ca^{2+} concentration (Fig. 4.2), which is important for the Ca^{2+}-CaMKKβ-dependent autophagy signaling (Ghislat and Knecht 2015). The details of Ca^{2+} and Raf-MEK-ERK1/2 pathways on autophagy activation will be discussed in this section, and the details of amino acid-induced mTORC1 translocation and activation will be elaborated in the next section.

Amino acid starvation that activates autophagy through the Ca^{2+}-CaMKK-β signaling pathway Ca^{2+} is an important intracellular second messenger that is involved in many cellular activities, such as cell–cell adhesion/communication, intracellular energy balance, glucose homeostasis, cytoskeleton remodeling, and even autophagy (Means et al. 2012; Racioppi and Means 2012). An increase in cytosolic Ca^{2+} concentration was observed in human or mouse embryonic fibroblasts (MEF cells), human embryonic kidney cells, and Hela cells in response to the removal of amino acids (Ghislat and Knecht 2012). Nevertheless, in other cell types, for example, in neurons, intracellular Ca^{2+} concentration is elevated by amino acid addition, rather than amino acid starvation. Indeed, the relationship between elevation of intracellular Ca^{2+} concentration and amino acid availability depends on the cell types and the intracellular signaling cascades (Ghislat and Knecht 2015). In fact, amino acid starvation-induced elevation of intracellular Ca^{2+} mainly occurs in non-excitable cells (e.g., hepatocytes and MEF cells), and Ca^{2+} is mainly exported from intracellular compartments (e.g., the endoplasmic reticulum) through inositol 1,4,5-trisphosphate receptors (IP_3Rs), a major Ca^{2+}-releasing channel localized in the ER (Gordon et al. 1993; Pattingre et al. 2005; Decuypere et al. 2013). In this case, the increase of intracellular Ca^{2+} concentration promotes the binding between Ca^{2+} and calcium/calmodulin-dependent protein kinase

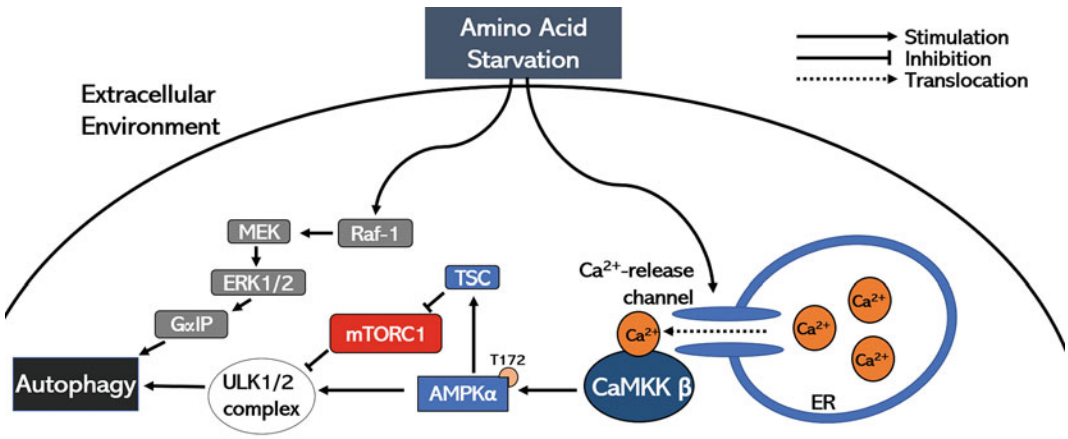

Fig. 4.2 Amino acid starvation induces autophagy in non-excitable cells. Upon amino acid starvation, Ca^{2+} is secreted from intracellular compartments (e.g., ER) through the Ca^{2+}-releasing channel to interact with CaMKK-β in the cytosol. Ca^{2+}-CaMKK-β promotes the phosphorylation of α subunit of AMPK at T172, leading to activation of the ULK1/2 complex either in an mTORC1-independent manner (direct phosphorylation at S555 of ULK (not shown in the figure)) or in an mTORC1-dependent manner (TSC-mTORC1 pathway). Alternatively, amino acid starvation can be sensed by Raf-1, which further triggers the MEK-ERK1/2-GαIP signaling cascade to activate autophagy

kinase β (CaMKK-β), leading to CaMKK-β activation, which further activates AMPK (Høyer-Hansen et al. 2007; Ghislat and Knecht 2012; Dalle Pezze et al. 2016). AMPK could activate autophagy either through directly phosphorylating ULK1 at S555 or through indirectly dephosphorylating ULK1 at S757 via inhibiting mTORC1 (Noh et al. 2016). In the AMPK-mTORC1-ULK1 pathway, AMPK activates tuberous sclerosis complex (TSC1/2), which is a key inhibitor of mTOR. The function of TSC1/2 will be elaborated in the next section (Woods et al. 2005; Høyer-Hansen et al. 2007; Xu et al. 2012; Dalle Pezze et al. 2016).

Apart from being a key element in the early stage of autophagy, Ca^{2+} released from the lysosome through the voltage-gated calcium channel (VGCC) is necessary for the fusion between the lysosome and the autophagosome. Mutation in the subunits of VGCC causes defective lysosomal calcium homeostasis, which slows down the turnover of long-lived proteins and accumulation of autophagosomes, leading to neurodegeneration (Coen et al. 2012; Tian et al. 2015).

4.3.1 Amino Acid Starvation Induces Autophagy Activation via the Raf-MEK-ERK1/2 Signaling Cascade

Apart from the Ca^{2+}-CaMKK-β pathway, an alternative pathway involved in the amino acid starvation-induced autophagy is the Raf-MEK-ERK1/2 pathway (Fig. 4.2). Ras is a family of related proteins that belongs to small GTPase and is known for its master regulatory role in cell growth and tissue development. Active Ras is GTP-bound, while inactive Ras is GDP-bound. Overexpression of the Ras gene causes uncontrolled cell growth, resulting in cancer development (McCormick 1999; Shaw and Cantley 2006). The absence of amino acids stimulates Ras activity, with the protein directly binding to Raf-1 serine/threonine kinase to form an active form (Pattingre et al. 2003; White 2013). Ras-induced Raf-1 activation further phosphorylates mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK), eventually leading to ERK1/2 phosphorylation and activation (Thiaville et al. 2008). Active ERK1/2 continues to phosphorylate Gα-interacting protein (GαIP) at S151 and upregulate GαIP activity. GαIP is a regulator of G protein signaling (RGS), which promotes the hydrolysis of GTP through the α-subunit of the trimeric Gi3 protein, subsequently promoting autophagy. Addition of amino acids demonstrated an inhibitory effect on ERK1/2 and GαIP phosphorylation in HT-29 cells (Pattingre et al. 2003; Cagnol and Chambard 2010; Schmukler et al. 2014).

It is worth noting that activation of ERK and autophagy is not in a straightforward causal relationship. Indeed, either carcinogens (e.g., lindane) or amino acid starvation has an ability to activate the Raf-MEK-ERK cascade but results in the opposite direction on autophagy regulation. Carcinogens impair autophagy maturation by disrupting the lysosome-autophagosome fusion through sustainably stimulating the ERK cascade, while amino acid starvation only transiently activates the ERK cascade to a certain level, thereby favoring autophagolysosome formation (Ogier-Denis et al. 2000; Corcelle et al. 2007). MAPK/p38, another member of the MAPK family, possesses a similar paradoxical effect on autophagy activity. In the lipopolysaccharide (LPS)-treated microglia, MAPK/p38 was reported to inhibit autophagy and promote inflammation via the direct phosphorylation of ULK1 at S757 (He et al. 2018). On the other hand, in oxidant (hydrogen peroxide)-treated C2Cl2 myotubes, MAPK/p38 upregulated the expression of autophagy-related genes and the autophagy-lysosome-dependent proteolytic mechanism (McClung et al. 2009). Furthermore, the activation of ERK and autophagy could be reciprocal interactions. It has been demonstrated that during the formation of the autophagosome, the autophagosome membrane provides docking sites for the Raf-MEK-ERK signaling, facilitating the phosphorylation of ERK (Martinez-Lopez et al. 2013). The involvement of Raf-MEK-ERK1/2 pathway in autophagy activation

is complicated and requires further investigation. Thus, the stimulation of the Raf-MEK-ERK1/2 pathway leads to activation of autophagy relies on the types of extracellular stimuli (e.g., amino acid starvation or carcinogens), pathological conditions, and cell types (Meijer and Codogno 2011).

4.4 Amino Acids Sensing and Regulation of Autophagy

Based on studies over several decades, an intracellular signaling network involving amino acids in sensing and controlling autophagy activity has been proposed, and nearly, all these pathways converge in the nutrient sensor mTOR. mTOR, which is a member of the phosphatidylinositol 3-kinase-related kinase (PIKKs) family of protein kinases, exists in two distinct complexes, mTORC1 and mTORC2. They are characterized by their unique components. mTORC1 contains the regulatory associated protein of mTOR (Raptor) and proline-rich AKT/PKB substrate 40 kDa (PRAS40), whereas mTORC2 contains the rapamycin-insensitive companion of mTOR (Rictor) and mammalian stress-activated MAP kinase-interacting protein 1 (mSin1) (Bar-Peled and Sabatini 2014; Meijer et al. 2015; Dalle Pezze et al. 2016). The regulation of mTORC2 remains an enigma and seems to be mainly responsible for growth factor signaling and the AKT-dependent pathway. A recent study revealed that autophagy-promoted lifespan extension relied on serum/glucocorticoid regulated kinase 1 (SGK1), which is a downstream of mTORC2 to control mitochondrial permeability (Zhou et al. 2019). On the other hand, mTORC1 has a critical role in sensing cell energy status and nutrient availability (e.g., amino acids). A nutrient-rich condition promotes mTORC1 activity, leading to anabolic pathways to synthesize intracellular components, whereas a nutrient-poor condition suppresses mTORC1 activity, resulting in catabolic pathways such as autophagy to recycle intracellular components (Meijer and Codogno 2011; Meijer et al. 2015).

4.4.1 Upstream Signaling of mTORC1

The activation of mTORC1 can be induced by two major signals: insulin and amino acids (Laplante and Sabatini 2012; Manjarín et al. 2020; Meijer et al. 2015). Insulin-induced mTORC1 activation mainly initiates through insulin receptor substrate1/2-class I phosphatidylinositol 3-kinase (IRS1/2-PI3K class I) and depends on the activation of protein kinase B (PKB/AKT) (Cathy and Shaodong 2017). Activation of PKB/AKT causes the suppression of TSC1/2—Tre2-Bub2-Cdc16 1 domain family member 7 (TBC1D7), namely the TSC1/2-TBC1D7 complex, which can convert Rheb, a Ras-like small GTPase, from an active form (GTP-bound state) to an inactive form (GDP-bound state) (Long et al. 2005; Bar-Peled and Sabatini 2014). Active Rheb (GTP-bound state) would activate mTORC1 directly or indirectly through phospholipase-D 1 (PLD1) which is recruited to the lysosomal membrane (Sun et al. 2008; Groenewoud and Zwartkruis 2013; Porta et al. 2014; Rabanal-Ruiz and Korolchuk 2018).

On the other hand, the amino acid-induced mTORC1 pathway is largely independent of the PI3K class I-AKT signaling. Takahiro and his colleagues reported that amino acids activated mTORC1 through PI3K class III (or hVps34). Knockdown of PI3K class III protein by PI3K class III siRNA abolished the amino acid-induced mTORC1 signaling. It is worth noting that the aforementioned PI3K class III is different from the PI3K class III involved in the early stage of autophagy (formation of the phagophore). These PI3K class III directly binds with mTORC1 and appears on the lysosome (Nobukuni et al. 2005; Um et al. 2006). Indeed, later studies revealed that the Rag family of GTPase, which recruits mTORC1 to the lysosomal surface, is essential for mTORC1 activation (Ben-

Sahra and Manning 2017; Rabanal-Ruiz and Korolchuk 2018). There are four different units in the Rag family, RagA, RagB, RagC, and RagD, among which RagA or RagB (RagA/B) can dimerize with RagC or RagD (RagC/D) to form a heterodimer complex before they proceed to regulate mTORC1 (Groenewoud and Zwartkruis 2013; Brady et al. 2016). Like Rheb, Rags turn into the active state if they are present in the GTP-bound form. It has been demonstrated that the Rag GTPase heterodimer complex reaches the maximum activity when Rag A/B is in the active form (GTP-bound state, $RagA/B^{GTP}$) while RagC/D is in the inactive form (GDP-bound state, $RagC/D^{GDP}$) (Bar-Peled et al. 2012; Petit et al. 2013; Hsu et al. 2018). Amino acid-induced Rag GTPase then recruits mTORC1 to the surface of the lysosome, followed by the Rheb-dependent mTORC1 activation (see Fig. 4.3) (Buerger et al. 2006; Ben-Sahra and Manning 2017; Deng et al. 2019).

Although the amino acids-induced Rheb-dependent mTORC1 activation has been extensively studied, the exact spatial regulation of the Rheb-dependent mTORC1 activation requires further investigation. Rheb can be observed throughout the endomembrane system (Buerger et al. 2006). One widely accepted model is that mTORC1 is activated by Rheb after Rheb is farnesylated in its C-terminal CaaX box and stably localized on the surface of the lysosome (Clark et al. 1997; Sancak et al. 2008; Laplante and Sabatini 2012; Amick et al. 2016; Ben-Sahra and Manning 2017). However, some other studies reported that a large proportion of Rheb was found in the Golgi apparatus, and the Golgi provides a region called the Golgi-lysosome contact site (GLCS) to facilitate the Rheb-mediated mTORC1 activation. Upon recruitment of mTORC1 to the lysosomal surface, the lysosome starts to accumulate in the perinuclear region where the Golgi can be found. Meanwhile, the number of GLCS increases, offering platforms for the Golgi-bound Rheb to interact with the mTORC1 on the lysosome. A transient membrane interaction is optimal for the Rheb-dependent mTORC1 activation (Fawal et al. 2015; Hao et al. 2018; Angarola and Ferguson 2019).

4.4.2 Amino Acid Sensing and mTOR Regulation

At an early age of study on the relationship between amino acids and autophagy, it has been reported that the generation of ammonia from amino acid catabolism accounts for the basic inhibitory effect of amino acids on autophagy since ammonia increases the pH inside the lysosome, thereby altering the optimal condition for lysosomal degradation (Seglen and Gordon 1984). However, this phenomenon cannot explain the inhibitory effect of amino acids on the formation of the autophagosome. Now, we know that amino acids can inhibit autophagy through either PI3K class III or amino acids sensors, both of which converge in the mTORC1 signaling. In particular, some of these amino acid sensors possess a specificity for certain types of amino acids. Leverve et al. (1987) reported that the combination of leucine and alanine was able to replace the entire amino acid mixture to provide a similar inhibitory effect on autophagy proteolysis in rat hepatocytes. Subsequent studies further demonstrated that leucine, but not other branched-chain amino acids (BCAAs) isoleucine and alanine, is a primary target of amino acid sensing. Recent research has also revealed a role of L-valine in activating mTOR to stimulate protein synthesis and inihibt protein degradation in mammalian cells (Zhang et al. 2019). BCAAs are abundant in both protein and animal proteins (Hou et al. 2019; Li and Wu 2020). Even small lipophilic modifications of the leucine side chain and α-hydrogen would not significantly change the stimulatory effect of leucine on mTOR signaling (Lynch et al. 2000). Shvets et al. (2008) utilized flow cytometry to monitor autophagy activity (i.e., intensity of GFP-LC3) in Chinese hamster ovary (CHO) and HeLa cell lines, under amino acid-rich, all amino acids-lacking (EBSS), or one amino acid-lacking conditions. They found that the absence of arginine, leucine, lysine, or methionine in medium could significantly increase intracellular GFP-LC3 intensity, indicating a strong autophagy induction; while the absence of glutamine, histidine, phenylalanine, threonine, tryptophan, tyrosine, or valine only exerted a mild potential on the elevation of intracellular GFP-LC3. The absence of alanine, asparagine, aspartic acid, cysteine, glutamic acid,

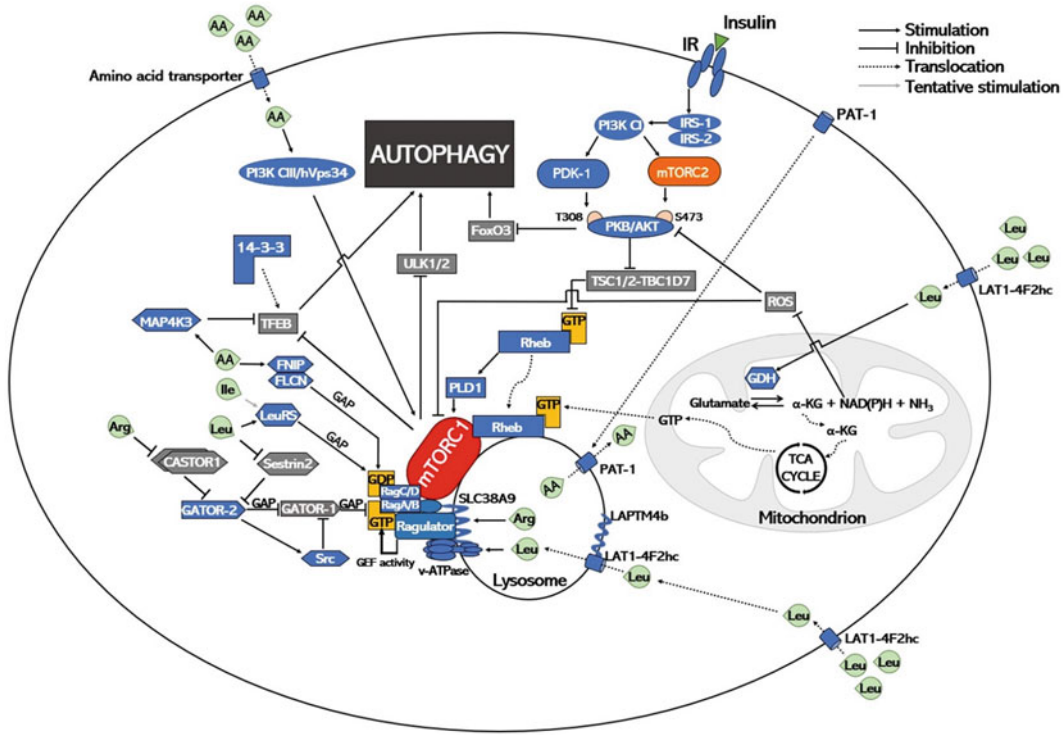

Fig. 4.3 Autophagy is inhibited by amino acids and insulin mainly through mTORC1-mediated pathways. The activation of mTORC1 requires interaction between mTORC1 and $Rheb^{GTP}$ (active form). Autophagy is mainly inhibited by mTORC1 which can be activated by either insulin or amino acids through different mechanisms. In one pathway, insulin stimulates the IRS1/2-PI3K ClassI-AKT signaling pathway to inhibit TSC1/2-TBC1D7, which prevents Rheb from activating mTORC1 by either direct interaction, or the phospholipase-D 1 (PLD1)-mediated pathway. In the second pathway, amino acids interact with a variety of amino acid sensors to activate Rag GTPase, which recruits mTORC1 onto the lysosomal surface to interact with Rheb. Rag GTPase is a heterodimer complex comprised of RagA/B and RagC/D. The most active form of this Rag heterodimer complex is $RagA/B^{GTP}$ (active form) and $RagC/D^{GDP}$ (inactive form). Inactivation of RagC/D relies on LeuRS and FLCN-FNIP, which act as GTPase-activating proteins (GAP) after receiving signals from leucine and arginine, respectively. On the other hand, activation of RagA/B requires sestrin2 and CASTOR1 that can be suppressed by either leucine or arginine, resulting in the activation of GATOR 2, which negatively regulates RagA/B suppressor GATOR 1. In addition, the lysosomal protein LAPTM4b recruits leucine transporter LAT1-4F2hc from the cell surface to the lysosome to facilitate the import of leucine into the lysosome. v-ATPase on the surface of lysosome sense intra-lysosomal leucine to activate guanine nucleotide exchange factor (GEF) activity of Ragulator, then activate RagA/B. Furthermore, leucine acts as an allosteric activator of glutamate dehydrogenase (GDH) and activates mTORC1 via two ways: (1) promoting NAD(P)H generation to reduce reactive oxygen species (an inhibitor of mTORC1); (2) accelerating α-ketoglutarate (α-KG) production to favor the TCA cycle to generate GTP for Rheb activation. Lastly, besides those amino acid sensors, amino acids can activate mTORC1 directly through the PI3K Class III pathway. Upon activation of mTORC1, mTORC1 not only suppresses the autophagosome formation by inhibiting the ULK1/2 complex but also inhibits the nuclear translocation of transcriptional factor FoxO3 and TFEB (which can also be inhibited by MAP4K3 and then be sequestered by 14-3-3 in cytosol), leading to the downregulation of gene expression of ATG or lysosomal proteins. Names with gray color background indicate that they have a stimulatory effect on autophagy.

glycine, isoleucine, proline, or serine, however, failed to induce autophagy (Shvets et al. 2008). All these studies, as well as other similar studies (Tan et al. 2008; Chantranupong et al. 2016; Verdon et al. 2017; Wyant et al. 2017; Wang et al. 2018; Wilden et al. 2018) indicate that leucine and arginine are two major amino acids involved in amino acid sensing and mTORC1 activation to inhibit autophagy.

Leucyl-tRNA synthetase

Leucyl-tRNA synthetase (LeuRS), in addition to its canonical role on charging its cognate tRNA with leucine, possesses an ability to sense leucine in the cytosol. Upon binding with leucine and translocating to the lysosome, LeuRS can serve as GTPase-activating protein (GAP) which can convert RagD from the GTP-bound state ($RagD^{GTP}$) to the GDP-bound state ($RagD^{GDP}$), favoring the activation of the $RagA/B^{GTP}$-$RagC/D^{GDP}$ heterodimer complex (Han et al. 2012). LeuRS has the highest affinity (very low Km value) for leucine, although LeuRS does demonstrate an occasional misacylation on isoleucine, methionine, or other leucine analogs, such as norvaline and α-amino butyrate, with a relatively high Km value (Wang et al. 2010). In fact, isoleucine can also activate mTORC1 through LeuRS, even though the efficiency is much lower than leucine (Han et al. 2012). These findings indicate that although leucine is one of the major contributors to mTORC1 activation, maximum activation of mTORC1 by amino acids cannot be reached until a complete profile of amino acids is introduced to cells (Shvets et al. 2008).

Glutamate dehydrogenase

Glutamate dehydrogenase (GDH), apart from its catalytic role in converting glutamate to α-ketoglutarate (α-KG) and ammonium (NH_4^+) in the mitochondria, serves as a leucine-specific amino acid sensor through two mechanisms (Wu 2013). The first mechanism relies on the production of NADPH. Leucine is a specific allosteric activator of GDH. Leucine facilitates the catalytic reaction controlled by GDH and favors NADPH generation. An increase in NADPH helps to remove reactive oxygen species (ROS). ROS not only suppresses mTORC1 activity (Alexander et al. 2010) but also promotes the nuclear translocation of a transcriptional factor called forkhead box protein O3 (FoxO3) to upregulate ATG gene expression (Zhou et al. 2012; Gómez-Puerto et al. 2016). The second mechanism involves the production of α-ketoglutarate in the tricarboxylic acid (TCA) cycle. Upon binding with leucine, the enzymatic activity of GDH is accelerated. Thus, more α-ketoglutarate is produced for biological oxidation via the TCA cycle, favoring GTP production during the conversion of succinyl-CoA to succinate. As a result, more GTP is available to charge Rheb and Rags, leading to mTOR activation (Lorin et al. 2013; Tan et al. 2017).

Sestrin2

Sestrin2 is another cytosolic amino acid sensor responsible for leucine sensing. It is known that the Rag GTPase-activating protein complex 1 and complex 2 (GATOR1 and GATOR2) are two members of the GAP family. GATOR1 has an ability to switch RagA/B from the GTP-bound form to the GDP-bound form, leading to the suppression of the $RagA/B^{GTP}$-$RagC/D^{GDP}$ heterodimer complex, while GATOR2 can negatively regulate GATOR1. Sestrin2 can directly interact and then inactivate GATOR2, stabilizing GATOR1 functionality, resulting in the suppression of the Rag heterodimer complex as well as mTORC1 activity (Parmigiani et al. 2014; Dong 2015). The presence of leucine in the cytosol can disrupt the sestrin2-GATOR2 complex in a dose-dependent manner, which favors mTORC1 activation. On top of that, similar to LeuRS, methionine and isoleucine can also be sensed by Sestrin2, promote Sestrin2 to convert into the inactive form. However, the sensitivity of Sestrin2 to methionine and isoleucine is much weaker than that of leucine, which requires methionine and isoleucine at a concentration tenfold and 25-fold greater than the concentration of leucine in the cytosol (Wolfson et al. 2016).

CASTOR1

CASTOR1, which usually exists either as a homodimer of CASTOR1 or a heterodimer of CASTOR1 and CASTOR2, is a key molecule for arginine sensing. As CASTOR1 does not possess a transmembrane domain, CASTOR1 mainly locates in the cytosol and senses free arginine. CASTOR1 and Sestrin2 share a same target, GATOR2. Arginine can directly bind to CASTOR1, but not CASTOR2, in the binding pocket

with I280. Upon binding with arginine, the CASTOR1-GATOR2 complex is disrupted, subsequently promoting mTORC1 activation (Chantranupong et al. 2016).

Folliculin

Folliculin (FLCN) is a tumor suppressor which localizes to the lysosome. Germline mutation in the *FLCN* gene results in Birt-Hogg-Dube (BHD) syndrome (Schmidt and Linehan 2018). It is reported that FLCN can respond to amino acids by regulating mTORC1. Indeed, FLCN binds to its interacting protein (FNIP) to form the FLCN-FNIP complex, which can further activate the RagA/B-RagC/D heterodimer complex on the surface of the lysosome. Basically, during amino acid starvation, the $RagB^{GDT}$ of Rag heterodimer serves as the specific docking site for the FLCN-FNIP complex. Upon amino acid stimulation, the FLCN-FNIP complex disperses rapidly from RagB and then stimulates the hydrolysis of GTP by RagC, which converts RagC and RagD into the inactive form (GDP-bound), thereby generating the active form of the Rags heterodimer complex for mTORC1 recruitment onto the lysosome (Tsun et al. 2013; Schmidt and Linehan 2018). Moreover, once the FLCN-FNIP complex activates mTORC1 by amino acids stimulation, mTORC1 would stimulate the phosphorylation of the transcriptional factor EB (TFEB) at serine 211. TFEB is the major transcriptional factor regulating the synthesis of lysosomal proteins as well as the proteins necessary for lysosome-autophagosome fusion. The phosphorylation at serine 211 of TFEB facilitates the binding TFEB and 14-3-3 (a chaperone that sequesters TFEB in the cytosol), preventing TFEB from translocating to the nucleus. As a result, the expression of genes related to lysosomal proteins is downregulated, therefore suppressing autophagic degradation (Roczniak-Ferguson et al. 2012; Settembre et al. 2012; Petit et al. 2013).

MAP4K3

MAP4K3 belongs to a family of germinal-center kinase-like kinase which can activate MAPK, thereby playing a critical role in the regulation of cell growth, apoptosis, or inflammation (Chen et al. 2012). It is recently reported that MAP4K3 can act as a cytosolic amino acid sensor that tackles TFEB nuclear translocation (Hsu et al. 2018). Upon sensing amino acids, MAP4K3 phosphorylates TFEB at Serine 3, then forms the MAP4K3-TFEB complex which is ready to be recruited to the surface of the lysosome by Rag GTPase. Like the FLCN-FNIP complex, after translocating onto the lysosomal surface, MAP4K3 renders TFEB susceptible to mTORC1-catalyzed phosphorylation at Serine 211, therefore sequestering TFEB in the cytosol by recruiting 14-3-3 and subsequently suppressing lysosomal protein degradation. In the absence of amino acids, MAP4K3 localizes on the surface of the lysosome so that MAP4K3 fails to interact with TFEB in the cytosol. Under these circumstances, TFEB proceeds with nuclear translocation and upregulates the expression of genes for lysosome-related proteins (Hsu et al. 2018; Zhang et al. 2020).

v-ATPase

It has been reported that essential amino acids can be transported into the lumen of the lysosome through amino acid transporters. For instance, a leucine transporter, LAT1-4F2hc, can be recruited by the lysosomal protein LAPTM4b from the cell membrane to the surface of the lysosome, facilitating the influx of leucine to the intra-lysosomal space (Milkereit et al. 2015). On the other hand, under induction through the Rheb pathway, the proton-assisted amino acid transporter (PAT-1) complex can translocate from the cell surface to the lysosomal membrane to drive intra-lysosomal amino acid export (Fig. 4.3) (Ögmundsdóttir et al. 2012; Ji et al. 2017). Previous studies have shown that several amino acid sensors localize on the surface of the lysosome and sense amino acid concentrations inside the lysosome. On the surface of the lysosome, a Ragulator complex serves as a guanine nucleotide exchange factor (GEF) specifically for RagA/B (Bar-Peled et al. 2012). At least five important components in the Ragulator complex (LAMTOR1/2/3/4/5) work together to mediate RagA/B activity through GDP disassociation and

GTP binding (Bar-Peled and Sabatini 2014). Particularly, the GEF activity of the Ragulator complex is tightly regulated by vacuolar adenosine triphosphatase (v-ATPase) which is one of amino acid sensors on the lysosomal surface. v-ATPase is firstly known for its ability on pumping H^+ ions to optimize the acidic environment inside the lysosomal lumen for hydrolases (Fuchs et al. 1989). It has been reported that v-ATPase can directly sense the concentration of amino acids (e.g., leucine) inside the lysosome and proceed with conformational changes, followed by interaction with Ragulator, leading to full activation of GEF in the Ragulator complex (Milkereit et al. 2015; Brady et al. 2016). Cells pretreated with v-ATPase inhibitors such as salicylihalamide A (SalA) (Xie et al. 2004; Poëa-Guyon et al. 2013) or concanamycin A (ConA) (Bowman et al. 2004; Newton et al. 2015) had a weak binding between Ragulator and Rag A/B, regardless the presence of amino acids, indicating that v-ATPase is necessary for amino acids-induced Ragulator-Rag interactions (Bar-Peled et al. 2012).

SLC38A9

Human number 9 of solute carrier family 38 (SLC38A9) is one of members in the SLC group that transports amino acids across the plasma membrane. The important role of SLC38A9 in lysosomal amino acid sensing was firstly discussed by Manuele Rebsamen and his colleagues (Rebsamen et al. 2015). SLC38A9 is a cross-membrane channel and also an integral part of the Ragulator-Rag GTPase machinery on the surface of the lysosome (Jung et al. 2015; Rebsamen et al. 2015). SLC38A9 can transport some amino acids, particularly leucine, out of the lysosome, thereby playing an essential role in regulating amino acid concentration in the lysosomal lumen and controlling the mTORC1 activation. Accumulation of essential amino acids inside the lysosome was observed in SLC38A9-null HEK293T cells. Moreover, the efficiency of SLC38A9 in amino acid transport is regulated by intra-lysosomal arginine concentration, indicating that SLC38A9 is an important lysosomal arginine sensor (Wyant et al. 2017).

Src

Pal et al. (2018) recently discovered that Src, which is an important factor in the regulation of cancer cell growth, could be activated through the sensing of amino acids, causing disconnection between GATOR1 and the Rag-GTPase complex. This favors the GTP-loading of Rag. Src could receive amino acid signals from GATOR2 and then could undergo conformational changes and gain the ability to separate GATOR1 from the Rag-GTPase complex. Upon removal of amino acids, Src occurs in the inactive form, which fails to prevent GATOR1 from inhibiting the Rag complex. In this case, mTORC1 is kept in the cytosol and fails to interact with the lysosomal membrane-bound Rheb for activation (Pal et al. 2018).

4.5 Summary and Perspectives

This chapter summarizes the important functions of amino acids in the regulation of autophagy, particularly through the mTORC1 cell signaling pathway. In addition to amino acids, other nutrients like glucose and fatty acids have their regulatory roles in autophagy, but the underlying mechanisms are still controversial. For instance, some studies demonstrated that autophagy could be induced by AMPK under depletion of glucose (Kim et al. 2011; Moruno et al. 2012); while some other studies indicated that the ER stress was triggered by high glucose concentration, resulting in autophagy activation (Dong et al. 2017). Moreover, autophagy was reported to be suppressed by long-term exposure to a mixture of fatty acids and glucose in pancreatic β-cells (Las et al. 2011). In short, we should consider the regulation of autophagy by both nitrogenous and non-nitrogenous nutrients as an integrative topic, although amino acids always play a central role. The fundamental mechanisms for the control of cell survival, growth, and development by individual nutrients or a combination of different nutrients remain to be further elucidated.

Acknowledgements This work was supported by National Institutes of Health grants (R01 DK095118 and R01 DK120968), an American Diabetes Association

Career Development Award (1-15-CD-09), faculty startup funds from Texas A&M University Health Science Center and AgriLife Research, a US Department of Agriculture National Institute of Food and Agriculture grant (Hatch 1010958) to S. G. (principal investigator), and a Hatch project of Texas A&M AgriLife Research #H-8200 (To G.W.). S.G. is the recipient of the 2015 American Diabetes Association Research Excellence Thomas R. Lee Career Development Award.

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5 Oxidation of Energy Substrates in Tissues of Fish: Metabolic Significance and Implications for Gene Expression and Carcinogenesis

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Abstract

Fish are useful animal models for studying effects of nutrients and environmental factors on gene expression (including epigenetics), toxicology, and carcinogenesis. To optimize the response of the animals to substances of interest (including toxins and carcinogens), water pollution, or climate changes, it is imperative to understand their fundamental biochemical processes. One of these processes concerns energy metabolism for growth, development, and survival. We have recently shown that tissues of hybrid striped bass (HSB), zebrafish, and largemouth bass (LMB) use amino acids (AAs; such as glutamate, glutamine, aspartate, alanine, and leucine) as major energy sources. AAs contribute to about 80% of ATP production in the liver, proximal intestine, kidney, and skeletal muscle tissue of the fish. Thus, as for mammals (including humans), AAs are the primary metabolic fuels in the proximal intestine of fish. In contrast, glucose and fatty acids are only minor metabolic fuels in the fish. Fish tissues have high activities of glutamate dehydrogenase, glutamate–oxaloacetate transaminase, and glutamate-pyruvate transaminase, as well as high rates of glutamate uptake. In contrast, the activities of hexokinase, pyruvate dehydrogenase, and carnitine palmitoyltransferase 1 in all the tissues are relatively low. Furthermore, unlike mammals, the skeletal muscle (the largest tissue) of HSB and LMB has a limited uptake of long-chain fatty acids and barely oxidizes fatty acids. Our findings explain differences in the metabolic patterns of AAs, glucose, and lipids among various tissues in fish. These new findings have important implications for understanding metabolic significance of the tissue-specific oxidation of AAs (particularly glutamate and glutamine) in gene expression (including epigenetics), nutrition, and health, as well as carcinogenesis in fish, mammals (including humans), and other animals.

Keywords

Amino acids · Fish · Glutamate · Tissues · Metabolism

Abbreviations

AAs	Amino acids
BCAT	Branched-chain AA transaminase
CPT-I	Carnitine palmitoyltransferase 1
GDH	Glutamate dehydrogenase
HSB	Hybrid striped bass
LMB	Largemouth bass
PDH	Pyruvate dehydrogenase

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1332,
https://doi.org/10.1007/978-3-030-74180-8_5

5.1 Introduction

Nutrition plays an important role in human health and the pathogenesis of many metabolic diseases (Fontana and Partridge 2015; Wu 2020). It is unethical to recruit human subjects for studies that are known to have potentially adverse effects, such as the exposure to chemical pollution, toxins, and carcinogens. Much evidence has shown that fish are useful animal models for studying the effects of nutrients and environmental factors on growth (Kaushik and Seiliez 2010; Li et al. 2009), gene expression (including epigenetics) (Cossins and Crawford 2005; Schartl 2014), environmental toxicology (Hawkins et al. 1985; Malins et al. 1985a, b; Van Veld et al. 1997), and carcinogenesis (Bailey et al. 1996; Black and Baumann 1991; Bunton 1996; Couch and Harshbarger 1985; Stoletov and Klemke 2008; Williams et al. 1998). Diverse ranges of toxic and potential carcinogens include aflatoxins, pharmaceuticals, herbicides, pesticides, polycyclic aromatic hydrocarbons, metabolites of brominated flame retardants, and metals. An advantage to use fish as animal models for studies is that it is easy and convenient to manage them in recirculating aquaculture systems (Ebeling and Timmons 2012). In addition, the costs of fish are relatively low.

To optimize the response of the animals to substances of interest, water pollution, or climate changes, it is imperative to understand their fundamental biochemical processes. One of these processes concerns energy metabolism necessary for the maintenance, nutrient transport, tissue integrity, intracellular protein turnover, apoptosis, and cell survival (Chen et al. 2020; Ishak Gabra et al. 2020; Wu 2018). We have recently reported that tissues of hybrid striped bass (HSB; Jia et al. 2017), zebrafish (Jia et al. 2017) and largemouth bass (LMB; Li et al. 2020b) use amino acids (AAs, such as glutamine, glutamate, aspartate, alanine, and leucine) as major energy sources in a tissue-specific manner. Of particular note, AAs are the primary metabolic fuels in the small intestine of fish, as previously reported for mammals (including swine and humans; Wu 1998, 2018). In this regard, it is noteworthy that malignant tumors of the small intestine are rare in humans (Pan and Morrison 2011) and fish (Roberts 1989; Schlumberger and Lucké 1948). Thus, it is tempting to postulate whether the unique metabolism of AAs in the small intestine may protect it from malignant tumors. The main objective of this article is to highlight the tissue-specific metabolism of nutrients in fish and its relevance to understand nutrition, gene expression, and carcinogenesis in mammals (including humans and other animals).

5.2 Amino Acids Are Major Energy Substrates in Fish

Depending on species, age, size, and feeding habits, the dietary AA requirements of fish range from 30–60% on the dry matter basis (Ballantyne 2001; Li et al. 2020a; Wilson 2002). These values are much greater than those for mammals and birds such as swine (12–20%), chickens (14–22%), and cattle (10–18%) (Cao et al. 2021; Gilbreath et al. 2021; He et al. 2021; Wu 2014; Zhang et al. 2021). However, fish, such as HSB and LMB (Li et al. 2020b, c, d, e) raised in recirculating aquaculture systems, do not grow as fast as pigs (Haynes et al. 2009) and chickens (He et al. 2021), and the composition of most AAs in the whole fish body is similar to that of terrestrial mammals (including humans) and other animals (Kaushik and Seiliez 2010; Li et al. 2020a, Wu 2013). The fact that only about 30% of dietary AAs are deposited into the body of juvenile LMB indicates that most of the absorbed AAs are oxidized to CO_2 in the fish (Li et al. 2020f, 2021c). It has been estimated that the oxidation of AAs contributes up to 70% of the biological energy needed by the marine fish embryos and yolk-sac larvae (Rønnestad et al. 1999; Rønnestad and Fyhn 1993). Similarly, up to 85% of the energy requirement of teleost fish is provided by AAs, depending on the fish's developmental stage (Jürss and Bastrop 1995; Van Waarde 1983). Thus, in contrast to mammals and birds (Wu 2018), carbohydrates and

fatty acids are only the minor sources of energy in fish (Li et al. 2021c). The use of AAs for ATP production in fish confers physiological advantages by generating both HCO_3^- (a component of the major buffering system in the blood) and NH_3. The latter takes up H^+ to form NH_4^+, which is eliminated directly through the gills and the kidneys as a mechanism for the regulation of acid–base balance in fish (Li et al. 2020a, b). This is analogous to the high rate of glutaminolysis in tumors to produce NH_3 to remove the intracellularly generated H^+. Neither glucose nor fatty acids can fulfill such a buffering function of AAs. Compared with mammals and birds, the removal of AA-derived ammonia as NH_4^+ in fish does not require energy and the use of AAs for ATP production is energetically more favorable (Tng et al. 2008; Wu 2018).

$$\text{Amino acids} + O_2 \rightarrow CO_2 + H_2O + NH_3$$
$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- \text{(retained in the blood)} + H^+$$
$$NH_3 + H^+ \rightarrow NH_4^+ \text{(to be eliminated through the gills and kidneys in fish)}$$

5.3 Tissue-Specific Utilization of AAs as Energy Substrates in Fish

5.3.1 HSB and Zebrafish

We have demonstrated the tissue-specific utilization of AAs as the major energy source in tissues of HSB and zebrafish (Jia et al. 2017). Specifically, in the proximal intestine, liver, kidney, and skeletal muscle of HSB (Fig. 5.1) and in all zebrafish tissues studied except for the proximal intestine, the rate of CO_2 production from glutamate oxidation is lower in the presence of a mixture of energy substrates, compared with the presence of 5 mM unlabeled glucose alone (Jia et al. 2017). In the proximal intestine, kidney, and skeletal muscle of both fish species, the rate of the oxidation of glutamate is the highest among the tested nutrients under all the experimental conditions. The rate of CO_2 production from glutamine oxidation is the highest in the liver, and the second highest (after glutamate) in the kidney of HSB and zebrafish in the presence of 5 mM glucose or a mixture of substrates or in the proximal intestine in the presence of a mixture of substrates. This is consistent with the reports that: (1) ammonia is produced mainly in the liver mitochondrial matrix of ammoniotelic fishes (Ip and Chew 2010) and (2) glutamine degradation via glutaminase can account for 85% of the total ammonia excreted from some fish (Campbell et al. 1983). By contrast, there is a very low rate of glutamine oxidation in the skeletal muscle of HSB and zebrafish in the presence of a mixture of energy substrates. This is in contrast to the report that mitochondria isolated from the lateral red muscle of teleost (*Salvelinus namaycush*) and nonteleost fish (*Amia calva*) fish actively oxidize glutamine (10 mM in the incubation medium) to CO_2 (Chamberlin et al. 1991). It is unknown whether this occurs in fish in the presence of physiological concentrations of glutamine in the plasma (e.g., 0.2–0.3 mM). Unlike glutamine, the rate of CO_2 production from leucine oxidation is the second highest in the skeletal muscle of both fish species but is the lowest in the proximal intestine and liver of HSB and in the proximal intestine and kidney of zebrafish, in the presence of a mixture of energy substrates. The rate of hepatic CO_2 production from leucine oxidation differs markedly between HSB and zebrafish, indicating a species difference in BCAA catabolism among fish species.

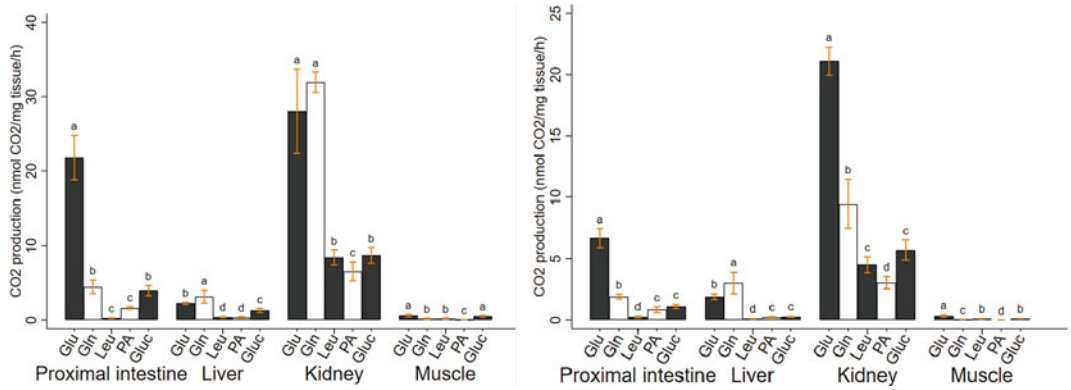

Fig. 5.1 Oxidation of a labeled nutrient by tissues of hybrid striped bass (HSB) in the presence of 5 mM glucose (Glu) or mixed substrates. Slices (15–40 mg) of the liver, proximal intestine, kidney, or skeletal muscle isolated from zebrafish and HSB were incubated at 26 °C for 2 h in oxygenated Krebs–Henseleit bicarbonate (KHB) buffer (95% O_2/5% CO_2; pH 7.4, with 5 mM D-glucose) containing 2 mM L-[U-^{14}C]glutamine, L-[U-^{14}C]glutamate, L-[U-^{14}C]leucine, or L-[U-^{14}C]palmitate, or a trace amount of D-[U-^{14}C]glucose. In parallel experiments, tissues were incubated with a tracer and a mixture of unlabeled substrates [glutamine, glutamate, leucine, and palmitate (2 mM each) plus 5 mM D-glucose]. $^{14}CO^2$ was collected in 0.2 ml Soluene to calculate the rates of substrate oxidation. Data, expressed as nmol CO_2/mg tissue/h, are mean ± SEM, n = 6. Adapted from Jia et al. (2017). **a–d** For a tissue, means not sharing the same superscript differ ($P < 0.05$), as analyzed by one-way ANOVA

In contrast to mammals (Hou et al. 2020; Wu 2021), the rate of CO_2 production from palmitate oxidation is the lowest among the tested nutrients in the skeletal muscle of HSB and in the liver and skeletal muscle of zebrafish in the presence of 5 mM glucose or a mixture of substrates (Jia et al. 2017). Of note, oxidation of palmitate is absent in HSB skeletal muscle in the presence of a mixture of energy substrates. Also in contrast to mammals, palmitate oxidation in the liver of both fish species is limited. The rate of glucose oxidation differs between HSB and zebrafish. Specifically, in HSB, in the presence of a mixture of energy substrates, the rate of CO_2 production from glucose oxidation is much lower than that from glutamate or glutamine oxidation in the proximal intestine, liver, and kidney and is similar to that from leucine oxidation in skeletal muscle. Under the same experimental conditions, the rates of CO_2 production from glucose oxidation in the proximal intestine and kidney are much higher in zebrafish than those in HSB, making glucose the second and third most oxidative substrate in the intestine and kidney of zebrafish, respectively. These results indicate a species difference in glucose catabolism among fish species.

Rates of ATP production from the oxidation of energy substrates can be estimated from the rates of CO_2 production by multiplying the coefficient (ATP/CO_2) according to the following equations (Jia et al. 2017, Li et al. 2020b).

$$\text{Glutamate}: C_5H_9NO_4 + 4.5O_2 \rightarrow NH_3 + 5CO_2 + 3H_2O \quad (ATP/CO_2 = 4.5;\ \text{mol/mol})$$

$$\text{Glutamine}: C_5H_{10}N_2O_3 + 4.5O_2 \rightarrow 2NH_3 + 5CO_2 + 2H_2O \quad (ATP/CO_2 = 4.5;\ \text{mol/mol})$$

$$\text{Alanine}: C_3H_7NO_2 + 3O_2 \rightarrow NH_3 + 3CO_2 + 2H_2O \quad (ATP/CO_2 = 5;\ \text{mol/mol})$$

$$\text{Aspartate}: C_4H_7NO_4 + 3O_2 \rightarrow NH_3 + 4CO_2 + 2H_2O \quad (ATP/CO_2 = 3.75;\ \text{mol/mol})$$

$$\text{Leucine}: C_6H_{13}NO_2 + 7.5O_2 \rightarrow NH_3 + 6CO_2 + 5H_2O \quad (ATP/CO_2 = 5.75;\ \text{mol/mol})$$

$$\text{Palmitate}: C_{16}H_{32}O_2 + 23O_2 \rightarrow 16CO_2 + 16H_2O \quad (ATP/CO_2 = 6.625;\ \text{mol/mol})$$

$$\text{Glucose}: C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad (ATP/CO_2 = 5;\ \text{mol/mol})$$

Results of the comparison of ATP production from nutrients among tissues of HSB and zebrafish are generally similar to those for the rates of nutrient oxidation noted previously. In the presence of a mixture of energy substrates, the percentage of ATP produced from the oxidation of AAs (glutamate plus glutamine plus leucine) is approximately 79%, 89%, 77%, and 80%, respectively, for the proximal intestine, liver, kidney, and skeletal muscle of HSB (Fig. 5.2), and is approximately 77%, 81%, 75%, and 78%, respectively, for the proximal intestine, liver, kidney, and skeletal muscle of zebrafish (Jia et al. 2017). Kidneys from both fish species have the highest rate of ATP production per g of tissue from glutamate, glutamine, glucose, palmitate, and leucine in the presence of 5 mM glucose or a mixture of energy substrates (Jia et al. 2017). The proximal intestine of HSB has the second highest rate of ATP production per g of tissue for all nutrients. Based on tissue weights of 20-g juvenile HSB and 0.5-g zebrafish, the rates of ATP production from nutrient oxidation per tissue in the presence of a mixture of substrates can be calculated (Jia et al. 2017). Our results indicate that glutamate produces most ATP in the proximal intestine, kidneys, and skeletal muscle of HSB and zebrafish, whereas glutamine is the most predominant metabolic fuel in the liver of both fish species. Both glutamate and glutamine are highly abundant in plant and animal proteins (Hou et al. 2019; Li and Wu 2020).

In the HSB, the proximal intestine has a low ability to oxidize physiological concentrations of glycine (e.g., about 0.9 nmol CO_2/30 min/mg tissue in the presence of 2 mM glycine), whereas there is no detectable oxidation of glycine (as indicated by $^{14}CO_2$ production from [U-^{14}C] glycine) in its skeletal muscle (our unpublished work). The liver and caudal kidney of HSB have relatively high rates of glycine oxidation (e.g., about 3 and 10 nmol CO_2/30 min/mg tissue, respectively, in the presence of 2 mM glycine; our unpublished work), as reported for mammals (Wu 2013). These results indicate that unlike glutamate, glutamine, and glycine, most of the dietary glycine enters the portal circulation and that glycine undergoes tissue-specific degradation in the fish.

5.3.2 LMB

In the liver, proximal intestine, kidney, and skeletal muscle of LMB, the rates of CO_2 production from glutamine oxidation are the highest among all the substrates studied, but the rates of CO_2 production from palmitate oxidation are the

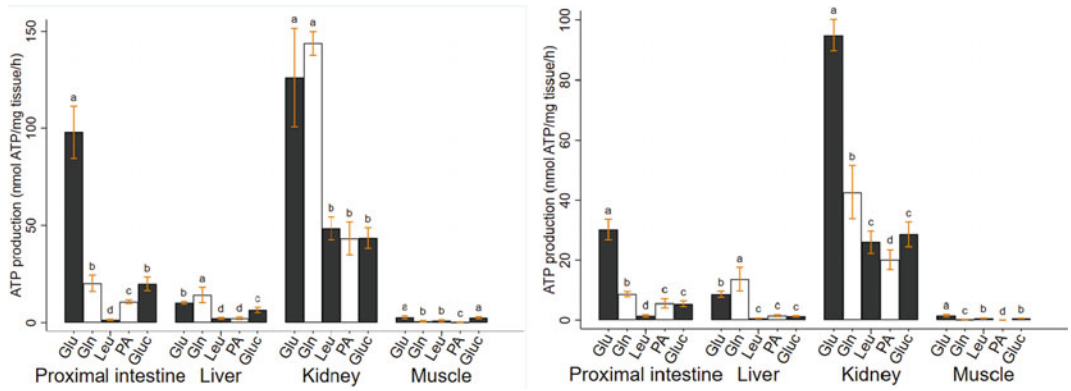

Fig. 5.2 Production of ATP from the oxidation of a nutrient by tissues of hybrid striped bass (HSB) in the presence of 5 mM glucose (Gluc) or a mixed substrates. See Fig. 5.1 for experimental details. Data, expressed as nmol ATP/mg tissue/h, are mean ± SEM, n = 6. Adapted from Jia et al. (2017). **a–d** For a tissue, means not sharing the same superscript differ ($P < 0.05$), as analyzed by one-way ANOVA

lowest among all the substrates studied (Table 5.1). In contrast to mammals, the rate of the oxidation of aspartate in the LMB liver is the second highest among all the substrates studied, followed by glutamate, leucine, and alanine in descending order. In the proximal intestine of LMB, the rates of CO_2 production from glutamate, aspartate, or alanine (similar rates) are the second highest among all the substrates studied, followed by glucose, leucine, and palmitate in descending order. In the kidneys of LMB, the rate of CO_2 production from glutamate is the second highest among all the substrates studied, followed by aspartate and alanine (similar rates), glucose, leucine, and palmitate in descending order. In skeletal muscle, the rates of CO_2 production from aspartate and glutamate (similar rates) are the second highest among all the substrates studied, followed by leucine, alanine, glucose, and palmitate in descending order. The rates of oxidation of glutamine do not differ between the liver and skeletal muscle of LMB. However, compared with skeletal muscle, the rates of oxidation of glucose, palmitate, alanine, and leucine in the liver are higher, but the rates of oxidation of glutamate and aspartate in the liver are lower, in the presence of 5 mM glucose. The liver and skeletal muscle of LMB have a limited ability to oxidize glucose and palmitate in the presence of either 5 mM glucose or a mixture of energy substrates.

Both the proximal intestine and the kidneys of LMB have higher rates of oxidizing all the substrates studied in the presence of 5 mM glucose, as compared with the liver and skeletal muscle of LMB. This is consistent with the rates of O_2 consumption by the tissues (Li et al. 2020b). In LMB, the rates of oxidation of glutamate, aspartate, alanine, leucine, and palmitate in the kidney are higher than those in the proximal intestine, whereas the rates of oxidation of glucose or glutamine to CO_2 do not differ between the proximal intestine and the kidney. Interestingly, the rates of oxidation of glutamate in the proximal intestine and kidney of LMB are approximately 280% and 580% higher than those in the skeletal muscle of LMB, respectively (Li et al. 2020b).

The rates of ATP production from individual substrates in LMB tissues are generally similar to those for the rates of substrate oxidation (Table 5.2). Of particular note, the rates of ATP production from the oxidation of glutamine are the highest among all the substrates studied in the liver, proximal intestine, kidney, and skeletal muscle of LMB. In this fish, the second most important substrates for ATP production vary among tissues: aspartate, leucine and glutamate (similar rates) in the liver, glutamate and alanine (similar rates) in the proximal intestine, glutamate in the kidney, and glutamate and aspartate (similar rates) in skeletal muscle. The third most important substrates for ATP production also vary among LMB tissues: alanine in the liver, aspartate and glucose (similar rates) in the proximal intestine, alanine in the kidney, and leucine in skeletal muscle. Based on the rates of ATP production by tissues in the presence of a mixture of energy substrates and the weights of individual tissues, we have estimated that skeletal muscle (45% of the body weight) produces most ATP from all substrates per whole tissue in LMB, followed by the proximal intestine, liver, and kidney in descending order.

5.4 Uptake of Amino Acids, Glucose, and Palmitate by Tissues of Fish

The uptake of nutrients by fish tissues can be measured with the use of ^{14}C-labeled substrates as described for the oxidation experiments (Jia et al. 2017; Li et al. 2020b), except that ^{3}H-inulin is included in the incubation medium as an extracellular marker (Lei et al. 2012; Li et al. 2002b). Briefly, a weighed tissue slice is incubated at 26 °C for 5 min in 1 ml medium. After the incubation period, the tissue is quickly transferred to a new petri dish with cold phosphate-buffered saline for washing. The washing step is quickly repeated three times to ensure that no radio-labeled substrate remains on the surface of tissue slices. Thereafter, the tissue is transferred into a 1.5-ml microtube containing 0.5 ml of 1 M NaOH for solubilization

Table 5.1 Oxidation of a labeled nutrient by tissues of largemouth bass (LMB) in the presence of 5 mM unlabeled glucose or a mixture of unlabeled substrates

	Glucose (5 mM)	Palmitate (2 mM)	Glutamate (2 mM)	Glutamine (2 mM)	Aspartate (2 mM)	Alanine (2 mM)	Leucine (2 mM)
In the presence of 5 mM glucose[1]							
Liver	$0.35 \pm 0.02^{e,B}$	$0.21 \pm 0.01^{f,C}$	$1.06 \pm 0.07^{c,D}$	$3.27 \pm 0.24^{a,B}$	$1.41 \pm 0.07^{b,D}$	$0.75 \pm 0.04^{d,C}$	$0.91 \pm 0.04^{c,C}$
Proximal intestine	$4.55 \pm 0.50^{c,A}$	$1.41 \pm 0.12^{d,B}$	$7.23 \pm 0.52^{b,B}$	$19.1 \pm 1.6^{a,A}$	$6.91 \pm 0.18^{b,B}$	$6.97 \pm 0.33^{b,B}$	$1.81 \pm 0.14^{d,B}$
Kidney	$4.82 \pm 0.56^{d,A}$	$2.27 \pm 0.21^{f,A}$	$13.0 \pm 0.62^{b,A}$	$18.8 \pm 0.70^{a,A}$	$9.94 \pm 0.73^{c,A}$	$9.69 \pm 0.73^{c,A}$	$3.62 \pm 0.09^{e,A}$
Skeletal muscle	$0.19 \pm 0.01^{e,C}$	$0.15 \pm 0.01^{f,D}$	$1.91 \pm 0.09^{b,C}$	$3.89 \pm 0.29^{a,B}$	$2.24 \pm 0.16^{b,C}$	$0.39 \pm 0.03^{d,D}$	$0.55 \pm 0.03^{c,D}$
In the presence of a mixture of energy substrates[2]							
Liver	$0.23 \pm 0.02^{e,B}$	$0.22 \pm 0.02^{e,C}$	$0.59 \pm 0.04^{c,B}$	$1.15 \pm 0.07^{a,B}$	$0.89 \pm 0.06^{b,B}$	$0.28 \pm 0.01^{d,B}$	$0.13 \pm 0.01^{f,C}$
Proximal intestine	$2.65 \pm 0.22^{c,A}$	$0.80 \pm 0.07^{e,B}$	$4.65 \pm 0.20^{a,A}$	$4.86 \pm 0.31^{a,A}$	$3.85 \pm 0.17^{b,A}$	$1.90 \pm 0.20^{d,A}$	$0.29 \pm 0.04^{f,B}$
Kidney	$2.29 \pm 0.15^{c,A}$	$1.91 \pm 0.14^{c,A}$	$5.05 \pm 0.24^{a,A}$	$5.34 \pm 0.16^{a,A}$	$3.39 \pm 0.21^{b,A}$	$1.93 \pm 0.10^{c,A}$	$0.72 \pm 0.04^{d,A}$
Skeletal muscle	$0.15 \pm 0.01^{c,C}$	$0.11 \pm 0.01^{d,D}$	$0.24 \pm 0.01^{b,C}$	$0.25 \pm 0.02^{b,C}$	$0.81 \pm 0.03^{a,B}$	$0.15 \pm 0.01^{c,C}$	$0.11 \pm 0.004^{d,C}$

Adaptd from Li et al. (2020b). Data are expressed as nmol CO_2/mg tissue/2 h. A tissue slices (20–30 mg for liver and intestine, 10–20 mg for kidney, and 40–50 mg for skeletal muscle) was incubated at 26 °C for 2 h in oxygenated Krebs–Henseleit bicarbonate (KHB) buffer (95% O_2/5% CO_2; pH 7.4, with 5 mM D-glucose). Adaptd from Li et al. (2020b)

[1]Values are means ± SEM, n = 8. The basal KHB medium contained 5 mM glucose. Either 2 mM palmitate + [U-^{14}C]palmitate, 2 mM glutamate + [U-^{14}C]glutamate, 2 mM glutamine + [U-^{14}C]glutamine, 2 mM aspartate + [U-^{14}C]aspartate, 2 mM alanine + [U-^{14}C]alanine, or 2 mM leucine + [U-^{14}C]leucine was added to the basal KHB medium

[2]Values are means ± SEM, n = 10. The basal KHB medium contained a mixture of energy substrates (i.e., 5 mM glucose + 2 mM palmitate + 2 mM glutamate + 2 mM glutamine + 2 mM aspartate + 2 mM alanine + 2 mM leucine)

[a–f]Means within a row that do not share the same superscript are different ($P < 0.05$)

[A–D]Means within a column that do not share the same superscript are different ($P < 0.05$)

Table 5.2 Production of ATP by tissues of largemouth bass (LMB) in the presence of 5 mM unlabeled glucose or a mixture of unlabeled substrates

	Glucose (5 mM)	Palmitate (2 mM)	Glutamate (2 mM)	Glutamine (2 mM)	Aspartate (2 mM)	Alanine (2 mM)	Leucine (2 mM)
In the presence of 5 mM glucose[1]							
Liver	$1.75 \pm 0.08^{d,B}$	$1.36 \pm 0.09^{d,C}$	$4.78 \pm 0.29^{b,D}$	$14.7 \pm 1.1^{a,B}$	$5.27 \pm 0.28^{b,D}$	$3.73 \pm 0.20^{c,C}$	$5.25 \pm 0.24^{b,C}$
Proximal intestine	$22.7 \pm 2.5^{c,A}$	$9.34 \pm 0.81^{d,B}$	$32.5 \pm 2.2^{b,B}$	$85.8 \pm 7.2^{a,A}$	$25.9 \pm 0.66^{c,B}$	$34.9 \pm 1.7^{b,B}$	$10.4 \pm 0.80^{d,B}$
Kidney	$24.1 \pm 2.8^{e,A}$	$15.1 \pm 1.40^{e,A}$	$58.4 \pm 2.8^{b,A}$	$84.5 \pm 3.2^{a,A}$	$37.3 \pm 2.8^{d,A}$	$48.4 \pm 3.7^{c,A}$	$20.8 \pm 0.53^{e,A}$
Skeletal muscle	$0.94 \pm 0.06^{e,C}$	$0.99 \pm 0.07^{e,D}$	$8.58 \pm 0.41^{b,C}$	$17.5 \pm 1.3^{a,B}$	$8.40 \pm 0.61^{b,C}$	$1.93 \pm 0.13^{d,D}$	$3.16 \pm 0.18^{c,D}$
In the presence of a mixture of energy substrates[2]							
Liver	$1.13 \pm 0.08^{d,B}$	$1.47 \pm 0.16^{d,C}$	$2.64 \pm 0.16^{c,B}$	$5.15 \pm 0.30^{a,B}$	$3.35 \pm 0.21^{b,B}$	$1.38 \pm 0.06^{d,B}$	$0.77 \pm 0.07^{e,C}$
Proximal intestine	$13.2 \pm 1.1^{b,A}$	$5.31 \pm 0.49^{d,B}$	$20.9 \pm 0.87^{a,A}$	$21.8 \pm 1.4^{a,A}$	$14.4 \pm 0.64^{b,A}$	$9.52 \pm 0.95^{c,A}$	$1.72 \pm 0.22^{e,B}$
Kidney	$11.5 \pm 0.76^{b,A}$	$12.7 \pm 1.0^{b,A}$	$22.7 \pm 1.1^{a,A}$	$24.1 \pm 0.71^{a,A}$	$12.7 \pm 0.81^{b,A}$	$9.63 \pm 0.47^{c,A}$	$4.13 \pm 0.22^{d,A}$
Skeletal muscle	$0.73 \pm 0.06^{c,C}$	$0.72 \pm 0.09^{c,D}$	$1.10 \pm 0.04^{b,C}$	$1.13 \pm 0.09^{b,C}$	$3.03 \pm 0.12^{a,B}$	$0.75 \pm 0.04^{c,C}$	$0.65 \pm 0.02^{c,C}$

Adaptd from Li et al. (2020b). Data are expressed as nmol ATP/mg tissue/2 h

[1]Values are means ± SEM, $n = 8$. The basal KHB medium contained 5 mM glucose. Either palmitate, glutamate, glutamine, aspartate, alanine, or leucine (2 mM each) was added to the basal KHB medium

[2]Values are means ± SEM, $n = 10$. Values in the parentheses indicate the percentage contribution of all the amino acids to ATP production in the tissue. The basal KHB medium contained 5 mM glucose. Either palmitate, glutamate, glutamine, aspartate, alanine, or leucine (2 mM each) was added to the basal KHB medium

[a–d]Means within a row that do not share the same superscript are different ($P < 0.05$)

[A–D]Means within a column that do not share the same superscript are different ($P < 0.05$)

overnight. An aliquot (0.3 ml) of the solubilized tissue solution is mixed with the scintillation cocktail (Hionic-Fluor, PerkinElmer), and ^{14}C and ^{3}H radioactivities are measured using the dual counting program in a Packard Scintillation Counter (Zhang et al. 2019).

5.4.1 HSB

Data on the uptake of nutrients by HSB tissues are summarized in Table 5.3. In the proximal intestine, kidneys, and skeletal muscle of HSB, the rate of uptake of glutamate is the greatest among all the tested substrates (including glutamine, leucine, palmitate, and glucose), followed by leucine. In the liver of HSB, the rate of uptake of glucose is the greatest among all the tested substrates, followed by glutamate, glutamine, and leucine (with a similar rate among the AAs). The proximal intestine has the highest rate of palmitate uptake among the liver, kidney, and skeletal muscle, but skeletal muscle barely takes up palmitate. The rates of uptake of glutamine, leucine, palmitate, and glucose by the proximal intestine, liver, kidney, and skeletal muscle are not affected by the presence of a mixture of energy substrates compared with glucose alone. This is also true for glutamate, except that its uptake by skeletal muscle is markedly reduced by the presence of a mixture of energy substrates compared with glucose alone. These results provide a biochemical basis for the extensive catabolism of glutamate by all HSB tissues, as well as the high rates of AA oxidation by the HSB intestine, kidneys, and liver.

5.4.2 LMB

We have measured the transport of nutrient by LMB tissues incubated in the presence of mixed substrates (Li et al. 2020b), and the results are summarized in Table 5.4. In the liver and kidney

Table 5.3 Uptake of nutrients by HSB tissues in the presence of 5 mM unlabeled glucose or a mixture of unlabeled substrates

Substrate	[U-^{14}C] Glutamate (2 mM)	[U-^{14}C] Glutamine (2 mM)	[U-^{14}C]Leucine (2 mM)	[U-^{14}C]Palmitate (2 mM)	[U-^{14}C]Glucose (5 mM)
Proximate Intestine					
Glucose[1]	108 ± 21.8[a]	48.1 ± 6.1[b]	82.6 ± 7.2[a,b]	50.2 ± 9.2[b]	59.9 ± 9.2[b]
Mixture[2]	99.5 ± 8.8[a]	44.0 ± 6.7[b]	79.0 ± 16.1[a,b]	49.1 ± 5.2[b]	56.6 ± 7.2[b]
Liver					
Glucose[1]	22.6 ± 3.4[a,b]	19.4 ± 2.6[b]	23.3 ± 2.4[a,b]	6.50 ± 0.53[c]	31.2 ± 3.1[a]
Mixture[2]	19.1 ± 2.2[b]	21.2 ± 3.1[b]	24.9 ± 4.6[a,b]	6.66 ± 0.66[c]	33.5 ± 4.3[a]
Kidney					
Glucose[1]	92.7 ± 16.7[a]	46.5 ± 6.0[b]	48.2 ± 4.7[b]	17.0 ± 2.4[c]	42.6 ± 7.4[b]
Mixture[2]	82.2 ± 8.5[a]	41.6 ± 8.8[b]	43.7 ± 5.6[b]	15.4 ± 4.4[c]	42.0 ± 7.0[b]
Skeletal muscle					
Glucose[1]	33.2 ± 6.7[a*]	5.94 ± 1.4[c]	22.8 ± 4.3[a,b]	0.22 ± 0.08[c]	19.0 ± 3.5[b]
Mixture[2]	13.4 ± 1.9[a]	5.73 ± 1.7[b]	18.8 ± 3.1[a]	0.23 ± 0.08[b]	19.5 ± 3.9[a]

Taken from Jia (2019). Data, expressed as pmol/mg tissue per min, are mean ± SEM, n = 6. Slices of a tissue from juvenile hybrid striped bass (HSB) were incubated 26 °C for 5 min in 1 ml of the KHB medium

[1]Uptake rate of the indicated labeled substrate in the presence of 5 mM unlabeled glucose

[2]Uptake rate of the indicated labeled substrate in the presence of a mixture of unlabeled substrates (2 mM glutamate, 2 mM glutamine, 2 mM leucine, 2 mM palmitate, and 5 mM glucose)

[a–d]Within a row, means not sharing the same superscript differ ($P < 0.05$), as analyzed by one-way ANOVA

[*]$P < 0.05$ vs the value for the oxidation of substrate alone

Table 5.4 Uptake of individual nutrients in LMB tissues incubated in the presence of a mixture of energy substrates[1]

	Glucose (5 mM)	Palmitate (2 mM)	Glutamate (2 mM)	Glutamine (2 mM)	Aspartate (2 mM)	Alanine (2 mM)	Leucine (2 mM)
Liver	$250 \pm 15^{c,C}$	$92 \pm 10^{e,B}$	$373 \pm 32^{b,C}$	$607 \pm 41^{a,B}$	$146 \pm 8^{d,C}$	$390 \pm 21^{b,B}$	$218 \pm 21^{c,B}$
Proximal intestine	$883 \pm 74^{a,A}$	$197 \pm 13^{c,A}$	$856 \pm 40^{a,A}$	$853 \pm 56^{a,A}$	$398 \pm 26^{b,A}$	$870 \pm 64^{a,A}$	$431 \pm 45^{b,A}$
Kidney	$202 \pm 26^{d,C}$	$192 \pm 34^{d,A}$	$497 \pm 37^{b,B}$	$787 \pm 53^{a,A}$	$231 \pm 28^{d,B}$	$373 \pm 21^{c,B}$	$214 \pm 24^{d,B}$
Skeletal muscle	$668 \pm 51^{b,B}$	$103 \pm 11^{d,B}$	$890 \pm 91^{a,A}$	$846 \pm 57^{a,A}$	$439 \pm 66^{c,A}$	$459 \pm 35^{c,B}$	$424 \pm 51^{c,A}$

[1]Taken from Li et al. (2020b). Data, expressed as pmol/mg tissue/5 min, are means ± SEM, n = 6. Slices of a tissue from juvenile largemouth bass (LMB) were incubated 26 °C for 5 min in 1 ml of the KHB medium containing a mixture of energy substrates (i.e., 5 mM glucose + 2 mM palmitate + 2 mM glutamate + 2 mM glutamine + 2 mM aspartate + 2 mM alanine + 2 mM leucine)

[a–f]Means within a row that do not share the same superscript are different ($P < 0.05$)

[A–D]Means within a column that do not share the same superscript are different ($P < 0.05$)

of LMB, the rates of glutamine transport are the highest among all the substrates studied, followed by either glutamate and aspartate (similar rates) in the LMB liver or glutamate and aspartate in the LMB kidney in descending order. In the proximal intestine of LMB, the rates of the transport of glucose, glutamate, glutamine, and alanine (similar rates) are the highest among all the substrates studied, followed by aspartate and leucine (similar rates). In the skeletal muscle of LMB, the rates of the transport of glutamate and glutamine (similar rates) are the highest among all the substrates studied, followed by glucose, with the rates of the transport of aspartate, alanine and leucine (similar rates) being lower than those for glucose. In all of these LMB tissues, the rates of the transport of palmitate are the lowest among the substrates studied, and the rates of the transport of glucose, palmitate, and leucine are similar in the kidneys. The proximal intestine of LMB has the highest rates of transporting all the nutrients among the tissues studied, and the rates of the transport of glutamate, glutamine, aspartate, and leucine are similar between the proximal intestine and skeletal muscle of LMB. The LMB liver has the lowest rates of transporting all the substrates studied, and the rates of the transport of glucose, alanine, and leucine do not differ between the liver and skeletal muscle of the fish. Consistent with the metabolic rates, the rates of transport of glucose in the LMB liver, kidney, and skeletal muscle are lower than those for glutamate and glutamine, but the rates of transport of glucose in the LMB liver, proximal intestine, and skeletal muscle are higher than those for aspartate. The data on nutrient uptakes by LMB tissues help to explain why these fish have the high rates of degrading AAs.

5.5 Determination of Enzyme Activities in Fish Tissues

Many tissues (including the liver, intestine, kidney, and muscle) of teleost and nonteleost fish possess a series of enzymes (including malic enzyme) to convert α-ketoglutarate into pyruvate (Chamberlin et al. 1991; Weber and Haman 1996), which is subsequently oxidized to CO_2 via pyruvate dehydrogenase (PDH) and the Krebs cycle (Wu 2018). To further provide a biochemical basis for the different patterns of nutrient catabolism in the liver, proximate intestine, kidney, and muscle of fish, we have determined the activities of key enzymes in these tissues using spectrophotometric, chromatographic, and radiochemical methods (Jia 2019; Li et al. 2020b). The enzymes of interest include glutamate dehydrogenase (GDH), glutamate-pyruvate transaminase (GPT), glutamate–oxaloacetate transaminase

(GOT), kidney-type glutaminase, liver-type glutaminase, glutamine synthetase (GS), branched-chain AA transaminase (BCAT), PDH, pyruvate kinase, hexokinase (HK), lactate dehydrogenase (LDH), phosphofructokinase-1 (PFK-1), and carnitine palmitoyltransferase 1 (CPT-1).

5.5.1 HSB

Data on the activities of enzymes in HSB tissues are summarized in Table 5.5. In the proximal intestine, kidneys, and skeletal muscle, the GDH activity is the greatest among all the measured enzymes, followed by GOT. The GS activity is the lowest among all the measured enzymes, followed by CPT-I. Thus, the fish can synthesize glutamine, but the amount of endogenous synthesis of glutamine may not be sufficient for their maximum growth or optimum health. In HSB liver, the activities of GOT and GPT are the greatest among all the measured enzymes, followed by GDH. High activities of both GDH and glutamate transaminases have been reported in the livers of rainbow trout (French et al. 1981) and the sea bass (Enes et al. 2006). Interestingly, the GS activity is nearly absent from the proximal intestine and skeletal muscle and is very low in the liver and kidney. The activity of K- or L-type phosphate-activated glutaminase in the skeletal muscle is much lower than that in the liver, kidney, and proximal intestine. CPT-I activity is appreciable in the liver, proximal intestine, and kidney, but is barely detected in the skeletal muscle. The activities of key enzymes of glycolysis are relatively high in all the tissues studied. Thus, HSB tissues have high activities of GDH, GOT and GPT to support the extensive catabolism of glutamate and other AAs in the body, while having a limited ability to oxidize both glucose and fatty acids. These results help to explain why the HSB cannot tolerate a high percentage of starch in diets and can rapidly store a large amount of lipids in the body when fed a high-fat diet (Li et al. 2021c).

5.5.2 LMB

Data on the activities of key enzymes involved in the oxidation of AAs, glucose, and fatty acids in LMB tissues are summarized in Table 5.6. The

Table 5.5 Activities of enzymes in tissues of juvenile hybrid striped bass (HSB)[1]

Enzyme	Liver	Proximal Intestine	Kidney	Skeletal muscle
Glutamate dehydrogenase	446 ± 57.0^{b}	603 ± 43.0^{a}	201 ± 7.10^{c}	17.8 ± 0.47^{d}
Glutamate-pyruvate transaminase	502 ± 30.0^{a}	68.7 ± 4.20^{b}	41.4 ± 3.50^{c}	13.2 ± 1.20^{d}
Glutamate–oxaloacetate transaminase	518 ± 45.0^{a}	352 ± 49.0^{b}	101 ± 13.0^{c}	64.1 ± 5.60^{c}
Branched-chain amino acid transaminase	25.6 ± 0.99^{b}	36.4 ± 5.60^{a}	4.92 ± 1.00^{c}	2.28 ± 0.44^{c}
K-type phosphate-activated glutaminase	3.27 ± 0.33^{a}	3.42 ± 0.18^{a}	1.79 ± 0.27^{b}	0.18 ± 0.03^{c}
L-type phosphate-activated glutaminase	1.23 ± 0.18^{b}	2.67 ± 0.35^{a}	0.89 ± 0.09^{b}	0.23 ± 0.07^{c}
Glutamine synthetase	0.23 ± 0.05^{a}	0.17 ± 0.02^{ab}	0.29 ± 0.05^{a}	0.014 ± 0.001^{b}
Pyruvate kinase	16.9 ± 0.97^{d}	126 ± 9.90^{b}	41.5 ± 4.30^{c}	281 ± 5.10^{a}
Hexokinase	7.41 ± 0.67^{a}	6.47 ± 1.00^{a}	2.04 ± 0.19^{b}	3.41 ± 0.40^{b}
Phosphofructokinase-1	33.2 ± 3.80^{c}	69.6 ± 4.50^{b}	26.2 ± 3.30^{c}	89 ± 5.60^{a}
Carnitine palmitoyltransferase-I	1.70 ± 0.10^{a}	1.78 ± 0.05^{a}	2.03 ± 0.22^{a}	0.17 ± 0.02^{b}

[1]Taken from Jia (2019). Values, expressed as nmol/mg protein per min, are means and pooled SEM, $n = 6$
$^{a–d}$Within a row, means not sharing the same superscript letter differ ($P < 0.05$)

Table 5.6 Activities of enzymes in tissues of juvenile largemouth bass[1]

	Liver	Proximal intestine	Kidney	Skeletal muscle
Carnitine palmitoyltransferase-I (n = 8)	$1.67 \pm 0.15^{b,I}$	$1.59 \pm 0.11^{b,J}$	$1.45 \pm 0.22^{b,K}$	$2.17 \pm 0.17^{a,G}$
BCAA transaminase (n = 8)	$9.81 \pm 0.54^{b,G}$	$9.41 \pm 0.71^{b,G}$	$24.6 \pm 1.2^{a,G}$	$7.46 \pm 0.39^{c,E}$
Liver-type glutaminase (n = 8)	15.1 ± 0.94^{F}	–	–	–
Kidney-type glutaminase (n = 8)	–	$15.9 \pm 1.1^{b,F}$	$62.0 \pm 4.5^{a,C}$	$5.21 \pm 0.41^{c,F}$
Glutamate dehydrogenase (n = 16)	$52.3 \pm 2.4^{a,C}$	$51.2 \pm 2.9^{a,C}$	$46.6 \pm 1.2^{a,D}$	$5.25 \pm 0.37^{b,F}$
Glutamate-pyruvate transaminase (n = 20)	$95 \pm 6.0^{b,A}$	$106 \pm 2.9^{b,A}$	$229 \pm 9^{a,A}$	$227 \pm 15^{a,C}$
Glutamate-OAA transaminase (n = 20)	$45.7 \pm 2.0^{b,D}$	$32.2 \pm 2.6^{c,D}$	$82.8 \pm 3.7^{a,B}$	$47.6 \pm 2.1^{b,D}$
Hexokinase (n = 20)	$5.45 \pm 0.15^{a,H}$	$3.81 \pm 0.16^{c,H}$	$5.52 \pm 0.24^{a,I}$	$4.49 \pm 0.16^{b,F}$
Pyruvate kinase (n = 20)	$38.6 \pm 1.8^{b,E}$	$26.7 \pm 0.92^{c,E}$	$37.9 \pm 2.0^{b,E}$	$225 \pm 12^{a,C}$
Phosphofructose kinase-1 (n = 20)	$38.7 \pm 2.0^{b,E}$	$15.0 \pm 0.48^{d,F}$	$29.3 \pm 1.2^{c,F}$	$577 \pm 38^{a,B}$
Lactate dehydrogenase (n = 20)	$81.1 \pm 3.5^{b,B}$	$61.2 \pm 2.6^{c,B}$	$16.8 \pm 1.0^{d,H}$	$8507 \pm 428^{a,A}$
Pyruvate dehydrogenase (n = 8)	$0.91 \pm 0.05^{c,J}$	$2.66 \pm 0.13^{b,I}$	$2.57 \pm 0.09^{b,J}$	$4.86 \pm 0.34^{a,F}$

[1]Taken from Li et al. (2020b). Data, express as nmol/mg protein/min, are means ± SEM, with the number of fish indicated in the parentheses

[a–d]Values within in a row that do not share the same superscript are different ($P < 0.05$)

[A–K]Values within in a column that do not share the same superscript are different ($P < 0.05$)

BCAA branched-chain amino acid; *OAA* oxaloacetate

activity of GPT is the highest ($P < 0.05$) among all the measured enzymes in the LMB liver, proximal intestine, and kidney, whereas the activity of LDH is the highest in skeletal muscle. In both the liver and proximal intestine of LMB, LDH, GDH, and GOT have the second, third, and fourth highest activities, respectively. In the LMB kidney, GOT, kidney-type glutaminase, and GDH have the second, third, and fourth highest activities, respectively. In the LMB skeletal muscle, PFK-1, pyruvate kinase, and GPT have the second, third, and fourth highest activities, respectively. In all of the studied LMB tissues, CPT-I, hexokinase, and PDH have the lowest activities. Just like the rates of nutrient oxidation, the activities of enzymes vary among LMB tissues. The LMB kidney has the highest activities of BCAT, glutaminase, and GOT among the tissues studied. The activities of GPT in the kidney and skeletal muscle of LMB are similar and are about 116–141% higher than those in the liver and proximal intestine. Among all the studied LMB tissues, skeletal muscle has relatively high activities of CPT-I, LDH, pyruvate kinase, PFK-1, and PDH, but relatively low activities of BCAT, glutaminase, and GDH. Collectively, these data help to explain why LMB tissues have high capacities for degrading glutamate, glutamine, and other AAs, while having a limited ability to oxidize both glucose and fatty acids. These biochemical data aid in understanding the previous observations that: (1) excessive glycogen is rapidly accumulated in the liver of LMB to cause glycogenosis (a metabolic disease) and the associated abnormal hepatic structure, when the fish are fed a diet containing $\geq 10\%$ starch (the dry matter basis); (2) an adequate provision of dietary AAs (e.g.,

45% protein; dry matter basis) and a low intake of dietary starch are crucial to prevent this metabolic disease in LMB; and (3) a large amount of lipids is rapidly accumulated in LMB when fed a high-fat diet (Li et al. 2020c, d, e).

5.6 Implications for Gene Expression (Including Epigenetics) and Carcinogenesis in Mammals

The expression of genes to produce mRNAs and the translation of mRNAs into proteins require a large amount of energy. For example, the incorporation of an AA molecule into a protein needs five molecules of ATP (Wu 2021). The intracellular trafficking and processing (e.g., post-translational modifications) of newly synthesized proteins are also dependent on energy. In addition, intracellular protein continuously undergoes not only synthesis but also degradation, and, on average, 2 molecules of ATP are hydrolyzed to provide energy necessary for the breakdown of a peptide bond via ATP-dependent proteases such as proteasomes (Wu 2013). Thus, adequate provision of energy from AA catabolism in cells play an important role in regulating gene expression. Knowledge from our studies on fish can be applied to enhance our understanding of the crucial roles of glutamate, glutamine, and aspartate in modulating gene transcription and translation in mammalian tissues such as the small intestine and lymphoid organs that also use AAs as major metabolic fuels (Jobgen et al. 2006). For example, microarray analysis has shown that dietary supplementation with glutamine stimulates the expression of anti-oxidative genes and inhibits the expression of pro-inflammatory genes in the small intestine of weanling piglets (an excellent model for studying human nutrition and metabolism) (Wang et al. 2008). Similar results have been obtained for fish (Li et al. 2020f). This new knowledge has important implications for our understanding of fish nutrition and health (Andersen et al. 2016), as well as the identification of alternative protein sources or crystalline AA (e.g., glutamate and glutamine) to replace fishmeal (Gatlin et al. 2007; Gaylord and Rawles 2007; Trushenski and Gause 2013; Li et al. 2021a, b; Yoshida et al. 2016).

Epigenetics refers to stable and heritable alterations of gene expression through covalent modifications of DNA and core histones without alterations in the DNA sequence. This process is affected by *S*-adenosylmethionine-dependent DNA and histone methylation, histone acetylation by acetyl-CoA, histone phosphorylation, and histone ubiquitination (Wang et al. 2012). Of note, all of these biochemical reactions require ATP. In addition, glutaminolysis is closely linked with serinogenesis and one-carbon metabolism in cells, including tumors (Bazer et al. 2021; Seo et al. 2021; Wong et al. 2017), which then contribute to changes in their epigenetics (Bazer et al. 2018; Johnson et al. 2018). Thus, the metabolism of AAs (including ATP production) can influence epigenetics in mammals (including pigs and humans; Ji et al. 2017; Wu et al. 2006), fish (Li et al. 2020f), and crustaceans (Li et al. 2021d). In support of this notion, there is evidence that glutamine can modify metabolically sensitive histone marks and alter cancer cell phenotype (Simpson et al. 2012). In addition, dietary supplementation with glutamine during gestation alleviates intrauterine growth restriction-induced intestinal dysfunction in postnatal piglets (Zhu et al. 2018). Furthermore, Ishak Gabra et al. (2020) recently reported that dietary glutamine supplementation suppressed epigenetically activated oncogenic pathways to inhibit melanoma tumor growth. This may be a potential dietary intervention to treat tumors via epigenetic reprogramming.

Metabolism is vital to the maintenance and health of all organisms, including fish and humans. Abnormal metabolism of AAs contributes to the development and inhibition of tumorigenesis (Lieu et al. 2020; Vettore et al. 2020). Malignant tumors may occur in any tissues but are very rare in the small intestine of humans (Pan and Morrison 2011) and fish (Roberts 1989; Schlumberger and Lucké 1948). This may be attributed to the rapid turnover of the epithelial cells of the small intestine (Arike et al. 2020) and the apoptosis of these cells (Baxt

and Xavier 2015; Patankar and Becker 2020; Yang et al. 2021). These normal physiological processes are critically dependent on energy to maintain cell homeostasis (Hou et al. 2015). In addition, glutamine can enhance the function of cells of the small-intestinal and whole-body immune systems (Li et al. 2007), which in turn suppresses tumor growth and migration along the gut. Furthermore, high rates of glutamate, glutamine, and aspartate oxidation in the small intestine result in relatively low intracellular concentrations of glutamine (Hou and Wu 2018; Zhang et al. 2021). This naturally limits the availability of glutamine to any tumor cells that preferentially use glutamine as the primary metabolic fuel (Kovacevic and McGivan 1983). Such a process is analogous to the use of glutamine-blocking drugs to effectively slow tumor growth and strengthen anti-tumor response (Leone et al. 2019). Thus, it is tempting to speculate that AA oxidation in the small intestine, and other tissues play an important role in inhibiting gut tumorigenesis. Future studies with fish are warranted to test this hypothesis.

5.7 Conclusion and Perspectives

Radioactive or stable carbon-labeled substrates are useful for investigating the oxidation of amino acids and other nutrients (e.g., glucose and fatty acids) in tissues and animals (Wu 2018). This method has been well applied to our recent studies of HSB, LMB, and zebrafish (Jia et al. 2017; Li et al. 2020b). Moreover, the rates of CO_2 production from potential energy substrates, along with data on oxygen consumption by tissues, provide a useful tool to assess their metabolic activities and the role of nutrients in generating ATP. Furthermore, measurements of the activities of key enzymes in metabolic pathways and nutrient transport by tissues can provide biochemical bases to explain differences in the metabolic patterns of nutrients among animal tissues. Using these vertically integrated approaches, we have identified that the liver, proximal intestine, kidney, and skeletal muscle of HSB, LMB, and zebrafish have a limited ability to oxidize glucose and palmitate for ATP production, compared with AAs, because fish tissues have low activities of CPT-I, hexokinase, and PDH but high activities of enzymes for AA degradation. AAs (e.g., glutamate, glutamine, aspartate, alanine, and leucine) contribute to about 80% of ATP production in the liver, proximal intestine, kidney, and skeletal muscle of the fish. These results may explain, in part, why LMB fed commercial diets containing high starch levels develop hepatic disorders (e.g., glycogenosis and abnormal structure) and rapidly gain fats in the body. Results of this work have important implications for understanding metabolic significance of tissue-specific oxidation of AAs (particularly glutamate and glutamine) in gene expression (including epigenetics), nutrition, and health, as well as carcinogenesis in fish, mammals (including humans), and other animals.

Acknowledgements This work was supported by Texas A&M AgriLife Research (H-8200). We thank graduate students and research assistants in our laboratory for helpful discussions.

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6 Arginine, Agmatine, and Polyamines: Key Regulators of Conceptus Development in Mammals

Katherine M. Halloran, Claire Stenhouse, Guoyao Wu, and Fuller W. Bazer

Abstract

Arginine is a key amino acid in pregnant females as it is the precursor for nitric oxide (NO) via nitric oxide synthase and for polyamines (putrescine, spermidine, and spermine) by either arginase II and ornithine decarboxylase to putrescine or via arginine decarboxylase to agmatine and agmatine to putrescine via agmatinase. Polyamines are critical for placental growth and vascularization. Polyamines stabilize DNA and mRNA for gene transcription and mRNA translation, stimulate proliferation of trophectoderm, and formation of multinucleated trophectoderm cells that give rise to giant cells in the placentae of species such as mice. Polyamines activate MTOR cell signaling to stimulate protein synthesis and they are important for motility through modification of beta-catenin phosphorylation, integrin signaling via focal adhesion kinases, cytoskeletal organization, and invasiveness or superficial implantation of blastocysts. Physiological levels of arginine, agmatine, and polyamines are critical to the secretion of interferon tau for pregnancy recognition in ruminants. Arginine, polyamines, and agmatine are very abundant in fetal fluids, fetal blood, and tissues of the conceptus during gestation. The polyamines are thus available to influence a multitude of events including activation of development of blastocysts, implantation, placentation, fetal growth, and development required for the successful establishment and maintenance of pregnancy in mammals.

Keywords

Pregnancy · Arginine · Agmatine · Polyamines · Uterus

Abbreviations

ADC	Arginine decarboxylase
AGMAT	Agmatinase
AMD1	*S*-Adenosylmethionine decarboxylase
Azin1	Antizyme inhibitor 1
AZI	Antienzymes
DFMO	Difluoromethylornithine
eIF5A	Eukaryotic translation initiation factor 5A-1
IFNT	Interferon tau
LE	Uterine luminal epithelia
MAO	Morpholino antisense oligonucleotide
MTOR	Mechanistic target of rapamycin
MAPK	Mitogen-activated protein kinases
NFKB	Nuclear factor kappa B
NO	Nitric oxide

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1332,
https://doi.org/10.1007/978-3-030-74180-8_6

NOS	Nitric oxide synthases
OAZ1	Ornithine decarboxylase antizyme 1
ODC	Ornithine decarboxylase-1
oTr1	Ovine trophectoderm
Paox	Peroxisomal N^1-acetyl-spermine/spermidine oxidase
SAM	*S*-Adenosylmethionine
SAT1	Spermidine/spermine N^1-acetyltransferase 1
sGE	Superficial glandular epithelia
Smox	Spermine oxidase
SMS	Spermine synthase
SRM	Spermidine synthase

6.1 Introduction

6.1.1 Establishment of Pregnancy in Mammals

Following the fertilization of ova released from ovarian follicles, the early stages of embryonic development occur in the oviduct, but further development to the blastocyst stage and beyond occurs in the uterus to allow for implantation and placentation, regardless of species (see Bazer et al. 2009; Aplin and Ruane 2017). Implantation may be non-invasive (central) as in most domestic animals or invasive (interstitial or eccentric) as in rodents and primates, depending on whether or not the trophectoderm invades through uterine luminal (LE) and superficial glandular (sGE) epithelia into the stroma. The key events in implantation of blastocysts include (1) hatching of the blastocyst from the zona pellucida; (2) precontact of trophectoderm with uterine LE/sGE and orientation of blastocyst/conceptus (embryo and extraembryonic membranes); (3) apposition between trophectoderm and uterine LE/sGE; (4) adhesion of trophectoderm to uterine LE/sGE; and 5) depending on species, limited or extensive, invasion of the blastocyst through the uterine LE/sGE into the uterine stroma (see Fig. 6.1). These periimplantation events are prerequisites for placentation required for fetal–placental growth and development throughout the remainder of pregnancy. A complex mixture of molecules, either secreted or transported into the uterine lumen by maternal uterine epithelia, form nutrient-rich histotroph to support growth and development of the conceptus. This is followed by placentation and development of uterine and placental vasculares of the conceptus for hematotrophic transfer of essential gases and nutrients. During the periimplantation period of pregnancy, there is coordinated signaling between conceptus trophectoderm and uterine epithelia critical to conceptus growth and development, pregnancy recognition signaling, implantation, and placentation.

6.1.2 Pathways for Metabolism of Arginine for the Synthesis of Nitric Oxide (NO), Ornithine, Agmatine, and Polyamines

Both plant and animal proteins contain arginine (Hou et al. 2019; Li and Wu 2020). In all animal tissues, arginine is metabolized to NO and polyamines (Fig. 6.2). However, the decarboxylation of arginine to agmatine is cell- and species-specific. For example, this pathway is present in the ovine conceptus (Wang et al. 2014) but is absent from the porcine conceptus (Wu et al. 2005, 2010). In mammals, the three polyamines of interest are spermine, spermidine, and putrescine, but there is a fourth named cadaverine. The latter is produced from the decarboxylation of lysine by ornithine decarboxylase-1 (ODC1) in animal cells at a very low rate but by bacterial lysine decarboxylase at a much greater rate (Wu 2013). Metabolic pathways leading to the synthesis of arginine and methionine are key, as the classical pathway for synthesis involves arginine conversion to ornithine by arginases I and II, and ornithine decarboxylation by ODC1 to putrescine. Putrescine is the substrate for spermidine synthase to generate spermidine, which can then be catabolized to spermine by spermine synthase (Wang et al. 2014). This 'classical pathway' was believed to be the only one present in mammals for the synthesis of polyamines. However, there is an alternate pathway in the ovine conceptus and some other tissues whereby arginine is decarboxylated

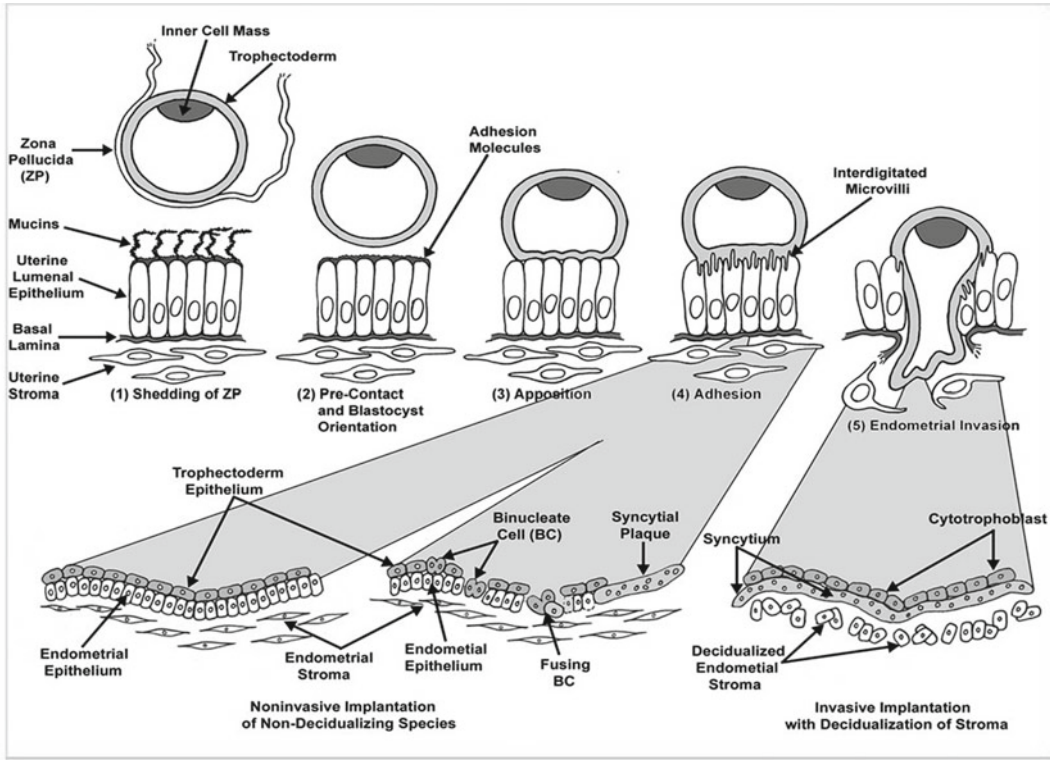

Fig. 6.1 Apposition and adhesion phases of blastocyst implantation. (1) The preattachment phase involves shedding of the zona pellucida (Phase 1) and then pre-contact of the blastocyst as it undergoes orientation (phase 2) for the next phase of implantation. Phase 3 is the period of contact of the trophectoderm with adhesive receptors such as integrins on uterine luminal epithelium (LE) during apposition and transient attachment of blastocyst/conceptus to uterine luminal epithelium. Phase 4 is the adhesion phase when mucin-1 declines on the uterine LE to expose constitutively expressed integrins on the uterine LE and trophectoderm. Species, such as rodents and humans, next enter Phase 5 that is a period of invasion of the uterine stroma by the blastocyst/conceptus to gain close proximity to maternal blood for exchange of nutrients. Species in which implantation ends at Phase 4 generally have a large surface area of trophectoderm for transport of nutrients and gases from the maternal vasculature to the fetal-placental vasculature

by arginine decarboxylase (ADC) to produce agmatine which is then converted to putrescine by agmatinase (Wang et al. 2014). Spermidine and spermine can be also be synthesized from *S*-adenosylmethionine (SAM) after it is decarboxylated by SAM decarboxylase (AMD1) to form decarboxylated *S*-adenosylmethionine. The latter is converted to spermidine by spermidine synthase (SRM) or to spermine by spermine synthase (SMS). Thus, ODC1 and AMD1 are rate-limiting enzymes in polyamine biosynthesis. Knockout of genes for either *Odc1* (Pendeville et al. 2001) or *Amd1* (Nishimura et al. 2002) in mice is embryonic lethal. Three different enzymes are involved in the reverse reactions that convert spermine to spermidine, and spermidine to putrescine. Spermine oxidase converts spermine to spermidine, and spermidine/spermine N^1-acetyltransferase and acetylpolyamine oxidase converts spermine to spermidine and spermidine to putrescine.

Ornithine decarboxylase activity and, thus, polyamine biosynthesis is controlled by a family of antienzymes (AZI) that inactivate ODC1 and maintain homeostasis for polyamines within cells. The abundance of polyamines in cells is maintained within a narrow range as they have adverse effects if present either in insufficient amounts or in excessive amounts. Insufficient amounts of

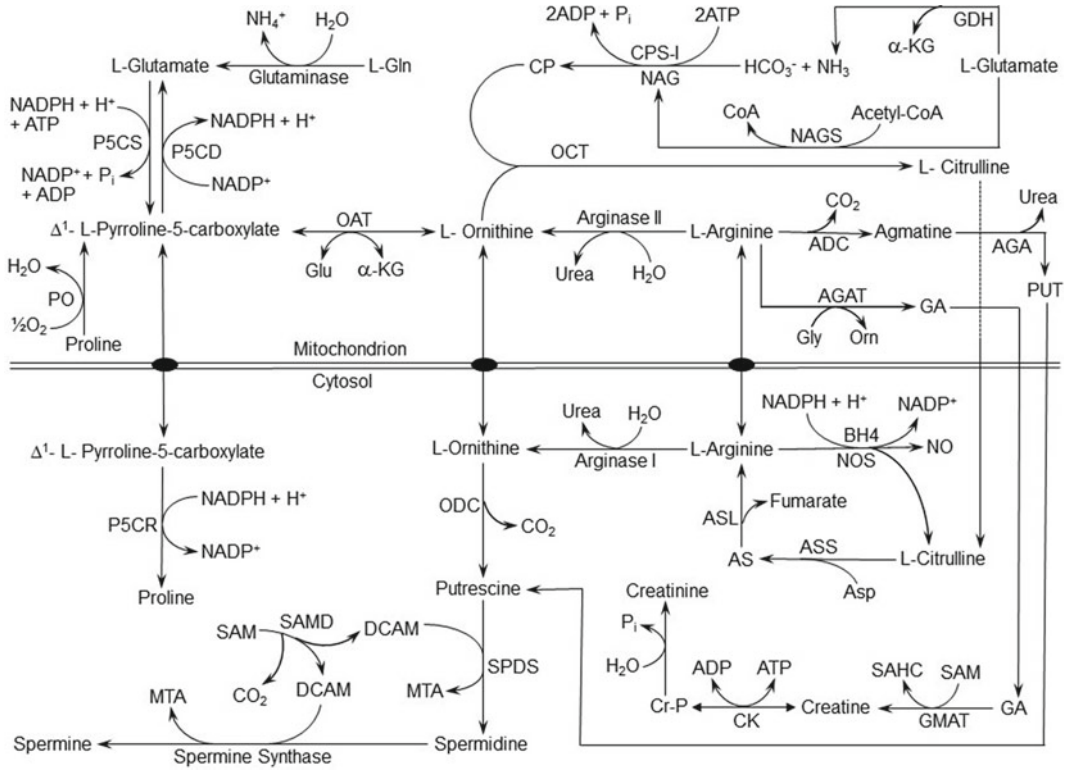

Fig. 6.2 Arginine metabolism in mammals. ADC arginine decarboxylase, AGA agmatinase, AGAT arginine:glycine amidinotransferase, ASL argininosuccinate lyase, ASS argininosuccinatesynthase, AS argininosuccinate, Asp aspartate, BH4 (6R)-5,6,7,8-tetrahydro-L-biopterin, CP carbamoylphosphate, CPS-I carbamoylphosphate synthetase-I (ammonia), DCAM decarboxylated S-adenosylmethionine, Glu glutamate, Gln glutamine, GDH glutamate dehydrogenase, GA guanidinoacetate, GMAT guanidinoacetate N-methyltransferase, CK creatine kinase, Cr–P creatine-phosphate, α-KG α-ketoglutarate, MTA methylthioadenosine, NAG N-acetylglutamate, NAGS N-acetylglutamate synthase, NO nitric oxide, NOS nitric oxide synthase, OAT ornithine aminotransferase, OCT ornithine carbamoyltransferase, ODC ornithine decarboxylase, PO proline oxidase, P5CD pyrroline-5-carboxylate dehydrogenase, P5CR pyrroline-5-carboxylate reductase, P5CS pyrroline-5-carboxylate synthase, PUT putrescine, SAM S-adenosylmethionine, SAMD S-adenosylmethionine decarboxylase, SAHC S-adenosylhomocysteine, SPDS spermidine synthase.

polyamines inhibit proliferation and migration of cells, whereas an over-abundance of polyamines results in apoptosis and cell transformation due to their catabolism to toxic reactive aldehydes and reactive oxygen species that enhance oxidative stress on cells (see Martin et al. 2003). At normal physiological levels, polyamines have antioxidant activity. To ensure that concentrations of polyamines in cells are not excessive, there are antizymes (AZIs), specifically AZI1, AZI2, and AZI3, that regulate ODC1 activity. Ornithine decarboxylase antizyme 1 (OAZ1) inhibits ODC1 activity by directing it to the antizyme–26S proteasome complex for proteasomal degradation and OAZ1 can inhibit the import of polyamines into cells. In contrast, there is an antizyme inhibitor 1 (AZIN1) that is structurally similar to ODC1. AZIN1 binds OAZ1 causing it to dissociate from ODC1 and that prevents ODC1 from being degraded (see Lefèvre et al. 2011).

6.1.3 Roles of Polyamines in Eukaryotic Cells

The functions of polyamines are discussed in detail by Pegg (2009), Igarashia and Kashiwagic (2010), Lenis et al. (2017) and Bae et al. (2018). Those reviews discuss the important roles of polyamines in cells to stimulate: (1) proliferation, migration, and apoptosis; (2) autophagy; (3) DNA transcription and mRNA translation; (4) ion channel activity; (5) cell signaling; (6) immunological functions; (7) wound healing; (8) metabolism; (9) aging; and (10) cancer. Both polyamines and iron influence proliferation of cells by affecting the ribonucleotide reductase catalyzed rate-limiting step in the synthesis of DNA. Cellular levels of iron, like those of polyamines, influence the cell cycle, proliferation of cells, and suppression of metastasis of cells suggesting that iron and polyamine metabolism is interlinked.

6.2 Pregnancy

6.2.1 Arginine, a Key Nutrient in Pregnancy (See Fig. 6.3)

Nitric oxide and polyamines (putrescine, spermidine, and spermine) are products of arginine catabolism and are essential for growth and development of the embryo to the blastocyst stage and then for further development of blastocyst to a conceptus (embryo/fetus and associated placental membranes) (Bazer et al. 2015; Gao 2020; Wu et al. 2009). In mice, outgrowth of trophectoderm of expanding blastocysts in preparation for implantation in the uterus requires leucine and arginine (Gwatkin 1969; Martin et al. 2003). Both leucine and arginine initiate cell signaling via serine-threonine kinase and mechanistic target of rapamycin (MTOR) to regulate protein synthesis and catabolism, and induce expression of genes such as insulin-like growth factor 2, nitric oxide synthases (NOS), and ODC1 (Murakami et al. 2004) required for growth and development of the conceptus. Polyamines associate with DNA and nuclear proteins to produce normal chromatin required for gene transcription, proliferation of trophectoderm, and formation of multinucleated trophectoderm cells or giant cells in placentae of mice (Kwon et al. 2004a). Polyamine-induced cell signaling pathways include tyrosine and mitogen-activated protein kinases (MAPK) and proto-oncogenes, c-myc, c-jun, and c-fos (Kwon et al. 2004b). Polyamines also activate MTOR cell signaling to stimulate protein synthesis in porcine trophectoderm cells (Kong et al. 2012). ODC1 is required for conversion of ornithine to polyamines that enhance motility, integrin signaling via focal adhesion kinases, cytoskeletal organization, and invasiveness of mouse blastocysts during implantation. Polyamines stimulate motility of trophectoderm cells of mice through modification of beta-catenin phosphorylation and changes in uterine epithelial cells that allow blastocysts to adhere to uterine LE/sGE and undergo superficial implantation (see Martin et al. 2003).

6.2.2 Polyamines and Reproduction

Polyamines are important in the reproductive system of both male and female mammals. Like taurine (Wu 2020), physiological concentrations of polyamines have antioxidative effects in animal cells (Agostinelli 2020). In males, Sertoli and Leydig cells synthesize and store polyamines which regulate differentiation of spermatogonia during spermatogenesis. Spermine is considered a vital polyamine for fertility in mice, as mice incapable of producing spermine are sterile due to meiotic arrest during spermatogenesis at the primary spermatocyte stage (see Lefèvre et al. 2011). Concentrations of spermine and spermidine are greater in seminal plasma than in any other body tissue or fluid suggesting that they are required for sperm motility and capacitation leading to fertilization of ova. Spermine and spermidine increase the utilization of glucose by sperm cells that increases the production of ATP required for sperm motility (Lefèvre et al. 2011).

In females, polyamines influence meiosis and oocyte maturation in ovarian follicles, ovulation, and luteinization of theca and granulosa cells, as

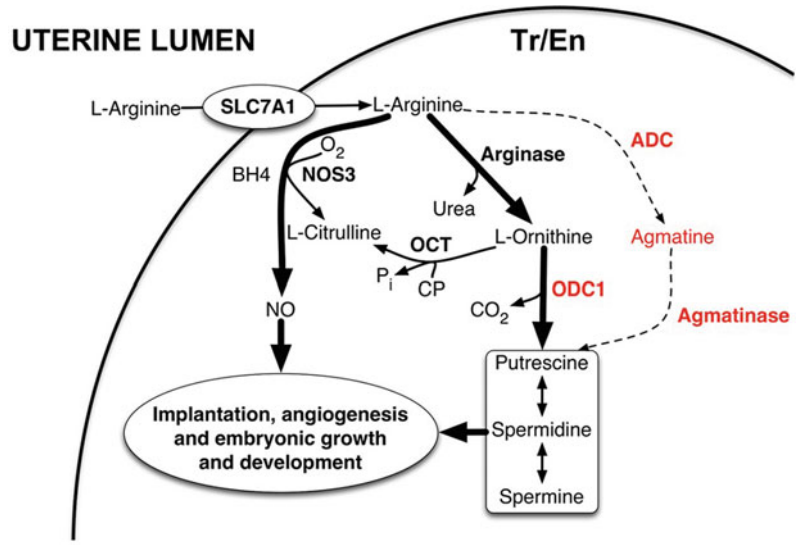

Fig. 6.3 Schematic pathway whereby arginine decarboxylase (ADC) and agmatinase (AGMAT), as well as arginase and ornithine decarboxylase (ODC1) provide for de novo biosynthesis of polyamines to support growth and development of the ovine conceptus. In the absence of translation of ODC1 mRNA to ODC1 protein, the expression of ADC and AGMAT increases to provide adequate amounts of polyamines for support of conceptus development in sheep (see Wang et al. 2014)

well as stimulating angiogenesis to increase blood flow to reproductive tissues, including the placenta (Kwon et al. 2003; Lefèvre et al. 2011). During the peri-implantation period of pregnancy in ewes, polyamines, agmatine, ornithine, and arginine are abundant in uterine flushings and conceptus tissues as the conceptuses transition from tubular to fully elongated forms and secrete interferon tau (IFNT) to signal pregnancy recognition and to act in concert with progesterone to increase transport of nutrients into the uterine lumen (Wang et al. 2014). Total amounts of polyamines (nmol) in uterine flushings decrease between Days 12 and 16 of pregnancy, whereas concentrations (nmol/g conceptus) of polyamines, arginine, ornithine, and agmatine increase in conceptus tissue between Days 14 and 16 of pregnancy. In uterine flushings, total amounts of agmatine also increase between Days 12 and 16 of gestation. For pregnant ewes, concentrations of polyamines in reproductive tissues vary due to the stage of gestation and are generally greater during the first half of gestation in placentomes, intercotyledonary placenta, and endometrial caruncles (Kwon et al. 2003). Polyamines are associated with proliferation, migration, and adhesion of trophectoderm cells, processes that are vital for establishment and maintenance of pregnancy, as well as growth and development of the conceptus (Wang et al. 2005). These positive effects of polyamines are critical for embryonic survival during the peri-implantation period of pregnancy in mammals (Wang et al. 2004; Kim et al. 2011). Embryonic death loss in mammals is estimated to be about 20–40% during the peri-implantation period of pregnancy. As these losses may be associated with insufficient amounts of polyamines in the reproductive tract, understanding the roles of polyamines and how their abundances can be enhanced aids in developing new strategies to reduce embryonic death loss.

6.2.3 Key Enzymes for Synthesis of Polyamines

Knockout of the Odc1 gene in mice is embryonic lethal, but not until the gastrulation stage of mouse embryogenesis when the lack of

polyamines leads to death at the early blastocyst stages due to apoptosis of cells in the embryonic disc; however, loss of cells in the embryonic disc can be prevented by providing putrescine (a precursor of spermidine and spermine) in drinking water of the dam up to the early stages of implantation, but not later in pregnancy (Pendeville et al. 2001). Furthermore, the inner cell mass is unable to proliferate in vitro in the absence of Odc1 (Pendeville et al. 2001). Methionine can also be used for synthesis of polyamines and this is dependent on AMD1. *Amd1*-null mouse embryos appear normal at Day 4, but die by Day 7 of pregnancy; however, *Amd1*-null embryos undergo trophoblast outgrowth in vitro when the medium is supplemented with spermidine (Nishimura et al. 2002). Thus, spermidine and spermine are required for cell cleavage in embryos, but putrescine, spermidine, and spermine all contribute to development of the blastocyst and implantation. The expression of Odc1 is low in uterine epithelia of mice before increasing between Days 4 and 5 specifically at sites of implantation (Zhao et al. 2008) and is greatest on Day 8 and then declines to Day 9 (Lopez-Garcia et al. 2008). Spermidine is detectable as early as Day 2 in uteri of pregnant mice and increases on Day 6, but the abundance of spermine is static during that time (Fozard et al. 1980a; b). In addition to Amd1, the expression of SRM, spermine oxidase (Smox), spermidine/spermine N^1-acetyltransferase 1 (Sat1), Azin1, ornithine decarboxylase antizyme 2 (Oaz2), spermidine/spermine N^1-acetyltransferase (Sat1), and peroxisomal N^1-acetylspermine/spermidine oxidase (Paox) and Odc1 increase only at sites of implantation in mice (Zhao et al. 2008).

6.2.4 Polyamines and Embryogenesis

Polyamines are likely essential for embryogenesis. Spermine is abundant in the cleavage stages of sea urchin embryos (Kusunoki and Yasumasu 1978), chick embryos in which ODC1 activity and polyamine levels increase during cleavage and gastrulation stages (Lowkvist et al. 1980), and frogs (*Xenopus laevis*), in which ODC1 activity increases between the 2-cell to the early blastula stage to increase abundances of putrescine and spermidine (Russell 1971). The development of parthenote pig embryos from the 2-cell stage to blastocyst stage is reduced when ODC1 activity is inhibited and the blastocysts that do form have fewer numbers of cells and greater expression of apoptotic genes (Cui and Kim 2005). Similarly, for hamsters (Galliani et al. 1983), rats (Saunderson and Heald 1974) and sheep (Kwon et al. 2003) expression of ODC1 increases in uterine epithelia and trophectoderm cells during the peri-implantation period of pregnancy. In addition, the abundance of ODC1 protein is regulated by progesterone in uteri of hamsters (Luzzani et al. 1982). Inhibition of ODC1 activity during the peri-implantation period of pregnancy using difluoromethylornithine (DFMO, an ODC inhibitor) prevents implantation in mice, rats, and hamsters (Mehrotra et al. 1998; Zhao et al. 2008).

6.2.5 Polyamines and Reproduction in Mink

Mink have been studied extensively regarding the roles of polyamines in the termination of diapause (delayed implantation of blastocysts) with emphasis on expression of ODC1, SAT1, and AZIN1 (see Lefevre et al. 2011a, b; Fenelon et al. 2014; Fenelon and Murphy 2019). Uterine expression of ODC1, SAT1 and AZIN1 in mink is markedly low during embryonic diapause, but expression of all three genes increases coordinately with reactivation of blastocysts upon termination of diapause. Treatment of mink with DFMO to inhibit ODC1 during the reactivation period for blastocysts, results in reactivated blastocysts reentering diapause. Treatment of pregnant mink with DFMO decreases the abundances of putrescine and spermidine, as well as ODC1, SAT1, and AZIN1 in uteri to levels similar to those in uteri with blastocysts in diapause. Polyamines appear to directly effect reactivation of mink blastocysts, likely by increasing proliferation of cells of the

trophectoderm and embryonic disc. In vivo treatment of mink embryos with DFMO decreases proliferation of cells in blastocysts, while culture of blastocysts in diapause with putrescine reactivates them and increases their rates of survival. Similarly, during diapause in mice, uterine expression of *Odc1*, *Srm*, *Sms* and *Smox* genes for synthesis of polyamines and *Oaz1* and *Azin1* mRNAs for enzymes that control *Odc1* activity is significantly lower compared to levels of expression in uteri with activated blastocysts (Fenelon and Murphy 2017).

6.2.6 Polyamines and Pregnancy in Rodents

From Day 4 to Day 8 of pregnancy in mice, expression of most genes for synthesis of polyamines increases, except for Srm, Paox, Oaz1 and Azin2 for which expression does not change, and Smox, for which expression decreases (Zhao et al. 2008; Fenelon and Murphy 2017). Consistent with those results, concentrations of polyamines in uteri of mice and mink were measured to reveal that DFMO treatment decreased concentrations of putrescine and spermidine, but not spermine, probably due to compensatory increases in AMD1 (Mamont et al. 1978; Fozard et al. 1980a, b; Pegg 1984; Lefevre et al. 2011b; Silva et al. 2015). In contrast, during embryonic diapause in mice, uterine expression of *Odc1*, *Sms*, *Srm* and *Smox* is less than that on Day 4 of pregnancy (Fenelon and Murphy 2017). Thus, coordinated changes in expression of enzymes that decrease synthesis of putrescine and spermidine result in blastocysts entering into diapause that compensatory increases in *Amd1* and spermine cannot overcome.

6.2.7 Polyamines and Pregnancy in Sheep

The abundances of arginine, citrulline, and methionine increase in uterine flushings of sheep between Days 10 and 15 of pregnancy, but not between Days 10 and 15 of the estrous cycle (Gao et al. 2009a). Similarly, members of the arginine family of amino acids are highly abundant in ovine and porcine allantoic fluid during the periods of rapid placental growth; therefore, rates of synthesis of NO and polyamines in both porcine and ovine placentae are greatest during early gestation when placental growth is most rapid (Kwon et al. 2003, 2004a, b; Wu et al. 2005; Wu et al. 2013). The enzymes argininosuccinate synthase and argininosuccinate lyase allow for citrulline to be converted to arginine, which can then be utilized to synthesize polyamines. The ovine placenta expresses arginase, therefore, citrulline is abundant in allantoic fluid, whereas the pig placenta does not express arginase, which allows for the accumulation of a large amount of arginine in allantoic fluid. Synthesis of polyamines is greatest in ovine placentomes and endometria between Days 30 and 60 of gestation when their growth and morphological changes are most rapid, and high levels of polyamines in ovine placental and endometrial tissues in the second half of pregnancy likely contribute to continued development of the placental vascular bed for increased uterine blood flow to support fetal growth (Kwon et al. 2004b). Similar results have been reported for porcine conceptuses (Wu et al. 2005). These observations suggest important biological roles for arginine and citrulline in growth and development of mammalian conceptuses.

The pathways for synthesis of polyamines and their importance in development of ovine conceptuses were first investigated by Wang et al. (2014), Yasser et al. (2017, 2018) (see Fig. 6.4). Intra-uterine delivery of morpholino antisense oligonucleotides to inhibit translation of ODC1 mRNA resulted in failure of 50% of the conceptuses to develop morphologically and functionally, but 50% of the conceptuses were apparently normal. The normal conceptuses increased expression of *ADC* and *agmatinase* (*AGMAT*) mRNAs to produce putrescine, spermidine, and spermine, and the abundance of AGMAT protein was significantly more abundant in the normal conceptuses. These results were the first to indicate that: (1) sheep conceptuses produce polyamines via the classical

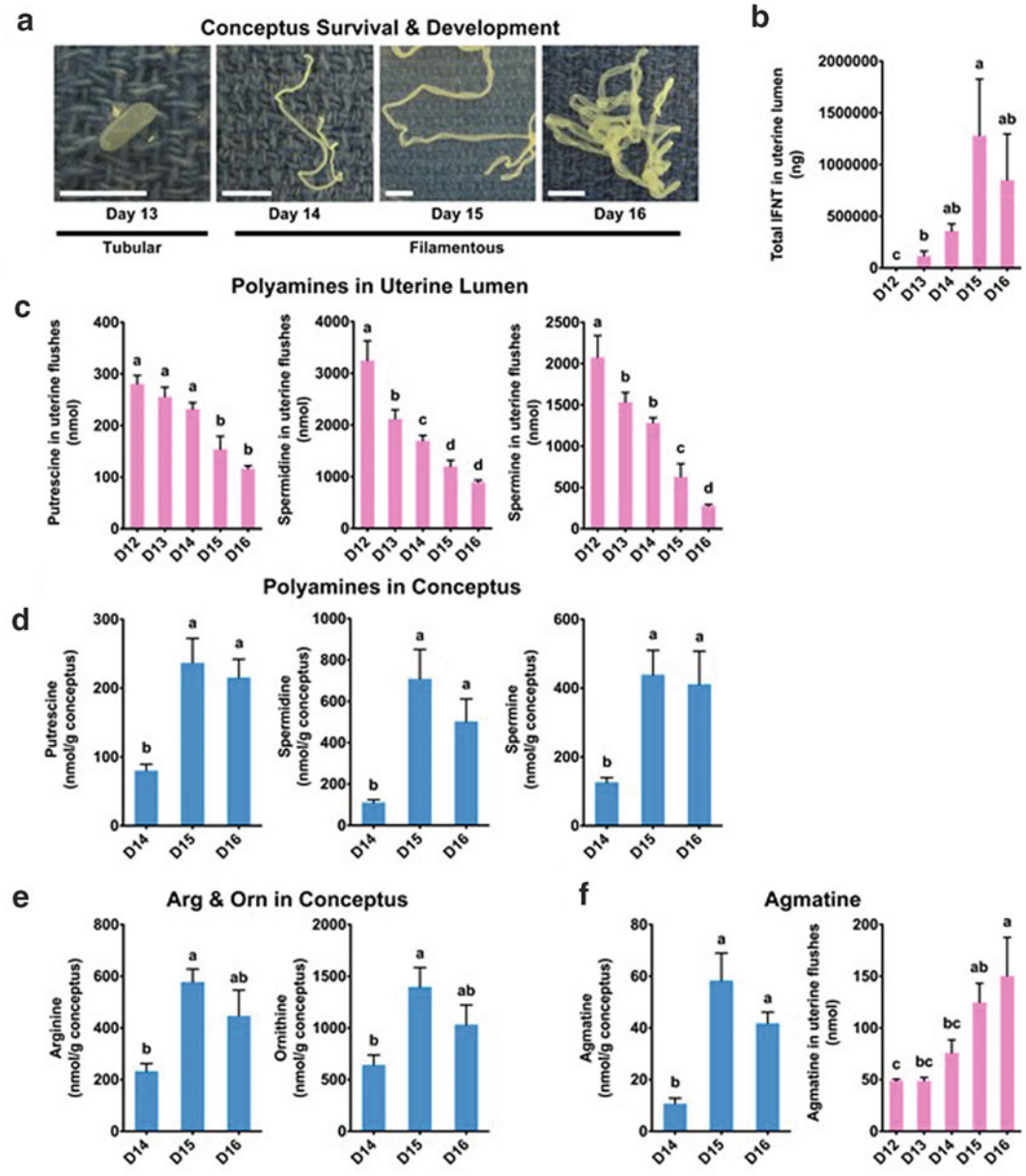

Fig. 6.4 Changes in the abundance of polyamines, agmatine, ornithine, and arginine in uterine flushings are associated with development of the conceptus as it transitions from a tubular to a fully elongated form that secretes IFNT to signal pregnancy recognition and increase transport of those nutrients into the uterine lumen (Panel **a**). Ovine conceptuses undergo a morphological transition from the tubular (Day 13) to elongated filamentous forms between Days 14 and 16 of pregnancy (Panel **a**). There is a corresponding increase in total amount (ng) of IFNT secreted by conceptuses and recovered in uterine flushings between Days 12 and 16 of pregnancy signals pregnancy recognition (Panel **b**). Total amounts of polyamines (nmol) in uterine flushings decrease from Day 12 to Day 16 of pregnancy (Panel **c**). However, concentrations of polyamines (nmol/g conceptus; Panel **d**), arginine and ornithine (nmol/g conceptus; Panel **e**), as well as agmatine (nmol/g) and total amount of agmatine (nmol; Panel **f**) increase in uterine flushes during the peri-implantation period of pregnancy (see Wang et al. 2014). Means with different superscript letters are different ($P < 0.05$)

ODC1-dependent pathway (arginine to ornithine to putrescine) and via an alternative pathway via ADC-AGMAT-dependent pathway; (2) polyamines are essential for development of the ovine conceptuses; and (3) knockdown of *ODC1* mRNA translation result in an abnormal conceptus phenotype and a significant decrease in their secretion of IFNT.

The next study used MAO to inhibit translational initiation of mRNAs for *ODC1* and *ADC*, in ovine conceptuses (Yasser et al. 2018). The morphologies of MAO control, MAO-ODC1, and MAO-ADC conceptuses were normal; however, double knockdown of ODC1 and ADC (MAO-ODC1:ADC) resulted in two phenotypes of conceptuses; 33% of conceptuses appeared morphologically and functionally normal (phenotype a) and 67% of the conceptuses had an abnormal morphology and functionality (phenotype b) (see Fig. 6.4). Further, the MAO-ODC1:ADC (a) conceptuses had greater concentrations of agmatine, putrescine, and spermidine than MAO control conceptuses, while MAO-ODC1:ADC (b) conceptuses only had greater tissue concentrations of agmatine, but lower amounts of putrescine, spermidine, spermine, alanine, aspartate, glutamine, tyrosine, phenylalanine, isoleucine, leucine, and lysine than for MAO-ODC1:ADC (a) conceptuses. From a functional standpoint, uterine flushings from the MAO-ODC1:ADC (a) conceptuses had 13.5 μg IFNT compared to 0.4 μg IFNT for MAO-ODC1:ADC (b) conceptuses.

Morpholino antisense oligonucleotides (MAOs) were again used to inhibit translation of mRNAs for ODC1 alone, AGMAT alone, and their combination (Yasser et al. 2017). This was because results from previous experiments revealed that agmatine is abundant in uterine flushings of control ewes during the estrous cycle and pregnancy (Wang et al. 2014). Inhibition of translation of both ODC1 and AGMAT mRNAs resulted in 22% of ewes having morphologically and functionally normal (elongated and healthy) conceptuses designated MAO-ODC1:MAO-AGMAT (A), whereas 78% of the MAO-ODC1:MAO-AGMAT ewes had morphologically and functionally abnormal (not elongated and fragmented) conceptuses designated MAO-ODC1:MAO-AGMAT (B) (see Fig. 6.5). The pregnancy rate was also less (22%) for MAO-ODC1:MAO-AGMAT ewes than for MAO-control (80%), MAO-ODC1 (75%), MAO-ADC (84%), and MAO-ODC1:MAO-ADC (44%) ewes. Moreover, inhibition of translational of both ODC1 and AGMAT mRNAs increased expression of ADC mRNA, as well as mRNAs for transporters of polyamines (SLC22A1, SLC22A2, and SLC22A3), and abundances of agmatine, putrescine, spermidine, and spermine in conceptus tissue. However, MAO-ODC1:MAO-AGMAT (B) ewes had greater abundances of agmatine, putrescine, and spermidine, but lesser amounts of spermine in uterine flushings. Thus, in vivo knockdown of translation of ODC1 and AGMAT mRNAs increased expression of genes for the synthesis and transport of polyamines in ovine conceptuses during the peri-implantation period of pregnancy.

6.2.8 Arginine, Agmatine, and Polyamines in Fetal-Placental Tissues, Fetal Fluids, and Plasma of Sheep

Agmatine, spermine, spermidine, and putrescine are present in maternal and fetal plasma on multiple gestational days, but agmatine is most abundant. Interestingly, the abundance of spermine in maternal plasma increases between Days 9 and 12 of gestation, which may highlight a potential role in early embryonic proliferation and elongation. The developing conceptus is entirely reliant on histotroph as a source of nutrients until placentation and the development of the vascular networks that comprise a functional placenta. When examining the abundance of agmatine and polyamines in uterine flushings from early pregnancy, agmatine is the least abundant and spermine is most abundant. Agmatine can be converted to spermine by a sequential series of enzymatic reactions involving agmatinase, spermidine synthase, and

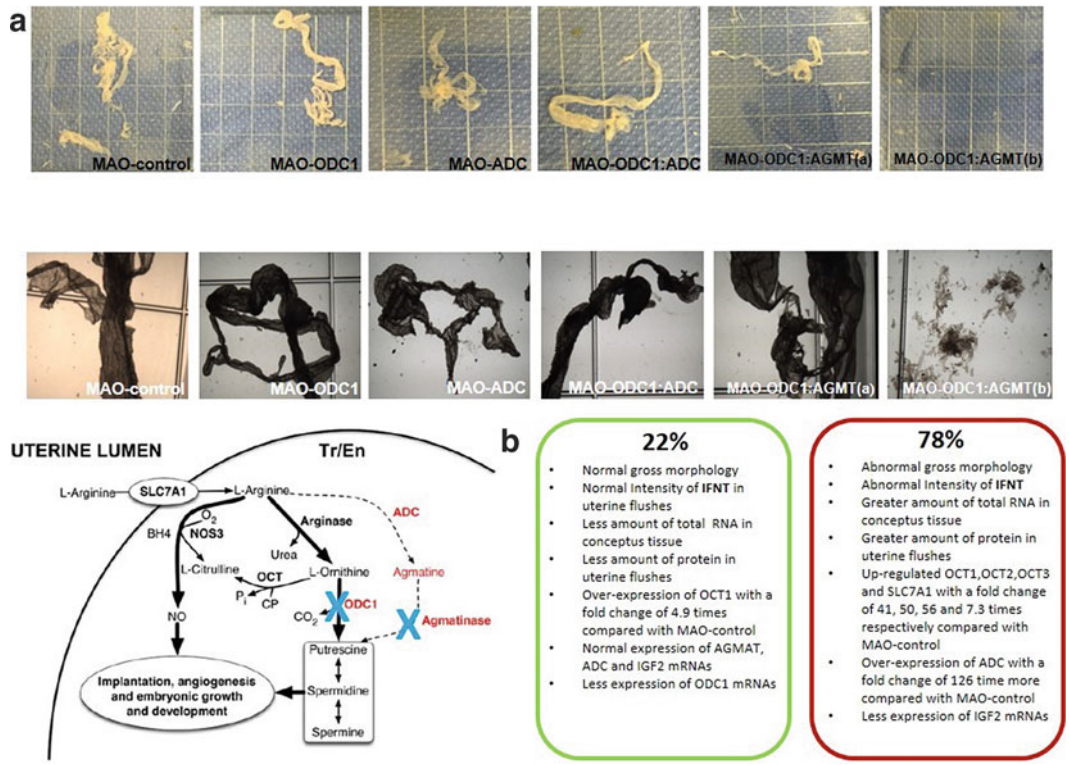

Fig. 6.5 This figure shows the gross morphology of ovine conceptus on Day 16 of pregnancy following inhibition of translation of mRNAs for ornithine decarboxylase (ODC1), arginine decarboxylase (ADC), both ODC1and ADC, and both ODC1 and agmatinase (AGMAT). In vivo knockdown of ODC1, ADC, and ODC1: ADC resulted in normal and healthy phenotypes of ovine conceptus, while the combination knockdown (ODC1: AGMAT) resulted in two phenotypes based on their morphological and functional development. The MAO-DC1:AGMAT (**a**) conceptuses were normal, healthy, and elongated while MAO-ODC1: AGMAT (**b**) conceptuses were abnormal, fragmented, and not elongated (Panel **a**). The differences in morphologies of the conceptuses, recoverable interferon tau (IFNT) in uterine flushings, and expression of other genes are summarized in Panel **b**. Legend: Organic cation transporters (CAT1, CAT2, CAT3, also known as Solute Carrier Family 22, Members 1, 2, 3), insulin-like growth factor 2, Solute Carrier Family 7 family member A1 (high-affinity cationic amino acid transporter 1), morpholino antisense oligonucleotide (MAO), nitric oxide synthase 3 (NOS_3; tetrahydrobiopterine (Lenis et al. 2018)

spermine synthase. This suggests that agmatine is converted into spermine, which can then be utilized by the conceptus for cellular proliferation and differentiation.

Polyamines are present in both allantoic and amniotic fluid across gestation (Kwon 2003). Total content of polyamines in these fluids are greatest during the latter half of gestation when there is significant fetal-placental growth and remodeling (Kwon 2003). Data from Day 125 of gestation revealed that agmatine is the most abundant polyamine in maternal and fetal plasma, and in allantoic fluid. Further, comparison of the abundances of agmatine and polyamines in fetal and maternal plasma demonstrated that agmatine, spermine, and putrescine are more abundant in fetal plasma than maternal plasma, indicating that fetal and placental tissues may have an increased requirement for polyamines compared to maternal tissues. These results suggest that these fetal fluids are reservoirs of polyamines in the conceptus during pregnancy. Additionally, like arginine, agmatine is more abundant in allantoic fluid compared to

amniotic fluid. In uterine flushings in early pregnancy, the concentration of agmatine is relatively low compared with arginine.

Further evidence for a role of polyamines in fetal growth and development has been demonstrated by comparison of singleton and twin pregnancies in late pregnancy. Interestingly, while most amino acids are significantly more abundant in fetal plasma compared to maternal plasma, arginine and citrulline (the immediate precursor of arginine) are present in equal abundances in ovine fetal and maternal plasma at Day 125 of gestation, suggesting that both are utilized extensively by the fetal-placental tissues. Spermidine, citrulline, and arginine are more abundant in maternal plasma from ewes carrying a single fetus compared with those carrying a twin pregnancy at Day 125 of gestation, indicating increased metabolism of these nutrients from maternal stores when two fetuses are present. Single fetuses have more spermine than twin fetuses, and greater placental expression of mRNAs encoding cationic amino acid transporters solute carrier family 7, members 1 and 2 (*SLC7A1* and *SLC7A2*). This suggests that citrulline and arginine are utilized extensively in support of fetal growth and production of polyamines. In contrast, there is greater expression of *ADC mRNA* in endometria of ewes with twin pregnancies compared with single pregnancies at Day 125 of gestation, indicating an increased demand for polyamines in ewes carrying two fetuses.

To further understand the role of polyamines in conceptus development, it is necessary to explore the localization of the molecules involved in their synthesis (ODC1, ADC, and AGMAT) and transporter (solute carrier family 22, members 2 and 3; SLC22A2 and SLC22A3). *ODC1* mRNA increases in the ovine conceptus between Days 13 and 18 of gestation and ODC1 protein are localized to both the trophectoderm and the endoderm during this period (Gao et al. 2009c). ADC protein is localized to the uterine LE, sGE, myometrium, and stromal cells on Days 9 and 12 of gestation (Hoskins et al., unpublished results). AGMAT protein is localized in a similar manner on Days 9 and 12 of gestation, but is also localized to epithelia in the deep uterine glands (Hoskins et al., unpublished results). ODC1 protein is localized to the uterine LE, sGE, myometrium, and stromal cells between Days 9 and 16 of gestation, with decreased expression on Day 20 (Hoskins et al., unpublished results; Gao et al. 2009c). In the peri-implantation period of pregnancy, SLC22A2 protein localizes to the apical surface of the uterine LE, sGE, and GE, as well as endothelial cells within the ovine endometrium, while SLC22A3 protein localizes throughout the uterine LE, sGE, and GE (Gao et al. 2020b).

In late gestation (Day 125), ODC1 protein localizes to uterine LE and sGE and stratum compactum stromal cells (Halloran et al., unpublished results). In contrast, ADC protein localizes to the uterine LE, stromal cells, and endothelial cells, with week staining in the sGE. For placentomes from Day 125 of gestation, ADC protein localizes to the caruncle and syncytium of the cotyledons, while AGMAT localizes exclusively to the syncytium of the cotyledons (Halloran et al., unpublished results). The differential localization of ADC and AGMAT in the cotyledons and caruncle of placentomes indicate that specific cell types cooperate synergistically to ensure the synthesis of polyamines across the maternal–fetal interface.

6.2.9 Hormonal Regulation of Synthesis and Transport of Polyamines by the Ovine Uterus

In mammals, the corpus luteum is a temporary endocrine organ that produces progesterone, the hormone of pregnancy (Spencer and Bazer 2004). In sheep, the conceptus enters the uterus as a morula on Day 4, develops into a blastocyst by Day 6, and hatches from the zona pellucida on Day 8. At this stage, the conceptus is spherical, and by Days 11 to 12, it elongates into a tubular form. By Day 14, the conceptus is filamentous in shape (Wintenberger-Torres 1974). Importantly, the initiation of conceptus elongation is

coincident with the production and secretion of IFNT by the mononuclear cells of the trophectoderm (Bazer et al. 1997). IFNT secretion begins on Day 11 of gestation, reaching maximal secretion on Day 16 before production ends on Day 21 (Bazer et al. 1997). In ruminants, IFNT is the signal for maternal recognition of pregnancy, and its production by the conceptus is required to prevent regression of the corpus luteum that is required to produce progesterone, the hormone of pregnancy (Bazer et al. 2018). Paradoxically, progesterone downregulates its own receptor in uterine LE and sGE, which is required for implantation (Spencer et al. 1995), and the loss of the progesterone receptor in these cells results in upregulation of specific amino acid transporters, such as SLC7A2 (Gao et al. 2009b; Satterfield et al. 2009).

There are differences in total recoverable amino acids from uterine flushings of ewes due to both pregnancy status and day of pregnancy, highlighting the roles of hormones and other signaling molecules (i.e., progesterone and IFNT) on the expression of mRNAs for nutrient transporters in the ovine endometrium (Gao et al. 2009b). In previous studies, administration of progesterone to ewes during the first 9 to 12 days of pregnancy accelerated blastocyst development by Day 12 of pregnancy when progesterone-treated ewes had filamentous conceptuses while blastocysts in control ewes remained spherical (Satterfield et al. 2010). In those studies, exogenous progesterone up-regulated expression of key genes in uterine epithelia responsible for transport and/or secretion of nutrients into the uterine lumen to form histotroph. Notably, total recoverable amounts of arginine and citrulline were greater in uterine flushings of progesterone-treated ewes than CO-treated ewes on Day 9 of pregnancy, and while arginine remained greater in uterine flushings from progesterone-treated ewes on Day 12 of pregnancy, citrulline was reduced (Satterfield et al. 2010). As arginine is a precursor for the production of agmatine and polyamines, it is evident that progesterone and/or IFNT, by inducing expression of transporters that transport arginine into the uterine lumen, clearly play a role in the synthesis and secretion of polyamines in the ovine uterus.

Progesterone administered to ewes from Day 1.5 through Day 8 of pregnancy accelerates conceptus development, therefore, effects of progesterone on synthesis and transports of polyamines were determined. Ewes treated with progesterone had more citrulline, spermidine, and spermine in maternal plasma when compared with CO-treated ewes at Days 9 and 12 of gestation. This suggests that progesterone may increase the reservoir of polyamines available in maternal circulation for transport into the pregnant uterus to support development of the conceptus. Less agmatine and spermidine were present in uterine flushings of ewes treated with progesterone compared to CO treated ewes, indicating their increased utilization by conceptuses in progesterone-treated ewes. Immunohistochemical analysis of endometria from Day 12 of gestation revealed increased abundance of AGMAT in uterine LE/sGE of progesterone-treated compared with CO-treated ewes. In contrast, expression of ODC1 was less abundant in uterine LE/sGE of progesterone-treated compared with CO-treated ewes on both Days 9 and 12 of gestation. Overall, these findings suggest that aspects of polyamine metabolism are progesterone-dependent during the peri-implantation period of pregnancy.

Furthermore, treatment of ewes with exogenous progesterone in early gestation significantly altered the synthesis and secretion of polyamines at Day 125 of gestation, even though progesterone-supplementation occurred 117 days earlier. As amniotic and allantoic fluid are important nutrient reservoirs for the fetus, abundances of amino acids and polyamines were quantified in those fluids collected on Day 125 of gestation. In amniotic fluid, both arginine and spermidine were more abundant in fluids from progesterone-treated compared to CO-treated ewes, suggesting increased transport of arginine by the endometrium and subsequent enzymatic conversion at the maternal-conceptus interface. Additionally, expression of *AGMAT*, *ODC1*, *SLC7A1*, and *SLC7A2* mRNAs were determined

in endometria from progesterone-treated compared with CO-treated ewes at Day 125 of gestation. The expression of *ODC1* mRNA was greater in placentae from progesterone-treated compared to CO-treated ewes. Overall, these findings indicate that treatment of ewes with exogenous progesterone in early gestation can influence the synthesis, transport, and secretion of polyamines in reproductive tissues during late gestation, suggesting a potential programming effect.

To further understand the roles of hormones in the regulation of polyamine synthesis and secretion during pregnancy, ewes were fit with catheters into each uterine horn and treated with either IFNT or control proteins, and either progesterone or a progesterone receptor antagonist (RU486, also known as mifepristone). Concentrations of polyamines in maternal plasma was not affected by either progesterone or IFNT. Interestingly, uterine flushings from progesterone-treated ewes had less arginine, citrulline, and agmatine when compared with the RU486 treated ewes (Halloran et al., unpublished results). Further, uterine flushings from ewes treated with RU486 and IFNT had more putrescine than ewes not treated with RU486. These results indicate a role for progesterone in regulating the abundance of putrescine in the ovine uterus. In the same study, progesterone alone increased endometrial expression of mRNAs for the polyamine transporters *SLC22A2* and *SLC22A3*, indicating an effect of progesterone to increase the transport of polyamines to the conceptus during the peri-implantation period of pregnancy. Further, IFNT increased endometrial expression of *AGMAT* mRNA, suggesting that IFNT increases the availability of putrescine, spermidine, and spermine for the conceptus during the peri-implantation period. The decrease in precursors for the synthesis of polyamines in uterine flushings (i.e., arginine, citrulline, and agmatine) in progesterone-treated ewes suggests that progesterone and IFNT work synergistically to affect the synthesis and secretion of polyamines in the pregnant uterus of ewes.

6.2.10 Factors Affecting Synthesis of Polyamines by Ovine Trophectoderm Cells

Dopamine and norepinephrine are abundant in uterine flushings from pigs (Young et al. 1980), and ovine trophectoderm (oTr1) cells secrete dopamine, norepinephrine, and epinephrine (Yasser et al. 2016). Therefore, oTr1 cells were used to assess effects of dopamine, norepinephrine, and epinephrine on their proliferation, migration and adhesion, secretion of IFNT, and expression of genes for synthesis of polyamines and apoptosis (Elmetwally et al. 2018). Expression of mRNAs for AGMAT, ADC, ODC1, and SLC7A1 increased ($P < 0.05$) in oTr1 cells in response to epinephrine and dopamine, and migration of oTr1 cells increased in response to epinephrine, dopamine, and norepinephrine, but proliferation of oTr1 cells was inhibited by 300 pg/ml epinephrine after 96 h and dopamine at 20 and 100 ng/ml. Further, epinephrine increased adhesion of oTr1 cells and their secretion of IFNT increased in response to epinephrine, norepinephrine, and dopamine. Thus, catecholamines may affect conceptus development during the peri-implantation period of pregnancy through effects on synthesis of polyamines and secretion of IFNT.

Arginine, but not agmatine, increases proliferation and migration of oTr1 cells, but neither arginine nor agmatine affected cell adhesion (Yasser et al. 2016). Arginine increased the total amount of IFNT in culture medium of oTr1 cells whereas agmatine increased the amount of IFNT production per cell. Arginine and agmatine plus arginine decreased secretion of dopamine and norepinephrine by oTr1 cells, while agmatine alone increased expression of mRNAs for *SLC7A1*, *AGMAT*, and *OAZ2*. Additionally, the combination of arginine and agmatine decreased expression of mRNAs for *ODC1*, *SLC7A1*, *OAZ1* and *OAZ3* by oTr1 cells. Although agmatine did not stimulate proliferation, migration, or adhesion of oTr1 cells or their secretion of catecholamines, it increased the expression of

mRNAs for *SLC7A1*, *AGMAT*, and *OAZ2* to increase the capacity of oTr1 cells to produce polyamines. Thus, arginine and agmatine may regulate the transport of arginine into oTr1 cells to influence the synthesis of polyamines and catecholamines.

6.3 Roles of Polyamines in Eukaryotic Cells

Polyamines have a plethora of physiological functions in eukaryotic cells (Fig. 6.6). These substances regulate the expression of genes at both transcriptional and translational levels, as well as nutrient transport and metabolism, antioxidative responses, and cell signaling. Therefore, polyamines mediate, in part, the beneficial effects of arginine and related amino acids on embryonic/fetal survival, growth, and development (Wu et al. 2018; Gilbreath et al. 2021; Zhang et al. 2021).

6.3.1 Polyamines and Ion Channels

Snyder-Robinson syndrome is due to an X-linked mutation in the SMS gene (Cason et al. 2003). This mutation is responsible for mild-to-moderate mental retardation and skeletal abnormalities in males with Snyder-Robinson syndrome as a result of a deficiency of calcium phosphate mineralization and a depletion of osteoblasts due to failure of osteogenic differentiation (Lee et al. 2013; Yeon et al. 2014). Similarly, X-irradiation resulted in a mutant mouse strain identified as Gyro that produced male offspring exhibiting hypophosphatemia with rickets (osteomalacia) and neurological deficits associated with circling behavior (Lyon et al. 1986). The Gyro mice have a deletion of part of the X chromosome that includes the spermine synthase gene and the phosphate-regulating gene. The phenotype of these mice includes reduced size, sterility, deafness, neurological abnormalities, and a propensity to sudden death and a short

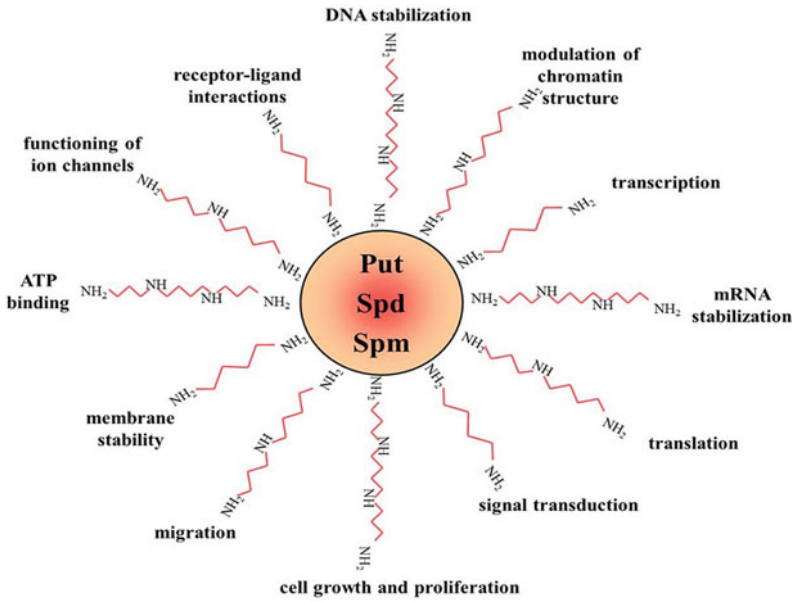

Fig. 6.6 Polyamines are required for many cellular processes including modulation of chromatin structure, gene transcription, and translation, DNA stabilization, signal transduction, cell growth and proliferation, migration, membrane stability, functioning of ion channels, and receptor-ligand interactions. All of those functions are essential for growth and development of mammalian conceptuses as inhibition of synthesis of polamines is embryonic lethal [see Pendeville et al. (2001) and Nishimura et al. (2002)]

life span due to an inability to produce spermine (Ikeguchi et al. 2004). The spermine:spermidine ratio has been reported to be critical for normal growth and development of male mice (Wang et al. 2004).

6.3.2 Cell Proliferation

Eukaryotic translation initiation factor 5A-1 (eIF5A) is the only known protein to contain the unusual amino acid hypusine [N(ε)-(4-amino-2-hydroxybutyl)-lysine], which is synthesized on eIF5A at a specific lysine residue from the polyamine spermidine by two catalytic steps. EIF5A is a translation factor that plays a role in both initiation and elongation of peptide bond formation. EIF5A has roles in transcription, mRNA turnover, and nucleocytoplasmic transport as it is constitutively abundant in most cells and essential for cell proliferation and apoptosis (Mathews and Hershey 2015). The genes for eIF5A, deoxyhypusine synthase, and deoxyhypusine hydroxylase, are essential for viability in mice (Nishimura et al. 2012; Sievert et al. 2014; Pällmann et al. 2015) as eIF5A is required for translation of mRNAs encoding proteins containing polyproline tracts or triplets of PPX (where X may be glycine, tryptophan, aspartate, or asparagine) (Dever et al. 2014). Ribosomes arrest on these nascent polyproline stretches and ribosome-bound hypusinylated eIF5A at the peptidyltransferase center of the ribosome stabilizes and orients the CCA end of the end of the peptidyl-tRNA to allow synthesis through these regions (Schmidt et al. 2016).

6.3.3 Gene Expression

Polyamines play a key role in gene transcription and translation (see Coffino 2000). Polyamines induce a ribosomal frameshift in the mRNA reading frame as required for the second sequence to be properly translated. Translation beginning from either the second ATG codon or the first ATG codon will cease soon after an in-frame termination codon, encoding for open reading frame 1. Open reading frame 2 involves the transfer of ribosomes to the reading frame to produce a mature functional OAZ (Kahana 2009). Physiological concentrations of Mg^{2+} are required for polyamine and RNA interactions leading to protein synthesis, including protein expression in cellular systems and promotion of assembly of the 30S ribosomal subunit (Yoshida et al. 2001). The abundances of spermidine and spermine in mammalian cells influence protein and nucleic acid synthesis and structure, protection from oxidative damage, activity of ion channels, cell proliferation, differentiation, and apoptosis. Spermidine is essential for viability of cells as it is the precursor of hypusine, a post-translational addition to eIF5A allowing for translation of mRNAs encoding proteins containing polyproline tracts. Spermidine acts as the aminobutyl group donor for posttranslational modification of a specific lysine residue of translation factor eIF5A by deoxyhypusine synthase. Subsequent hydroxylation by deoxyhypusine hydroxylase results in formation of N-(4-amino-2-hydroxybutyl)lysine (hypusine) essential for eIF5A activity (Caraglia et al. 2013).

6.3.4 Cell Signaling

Polyamines initiate intracellular signaling after binding membrane phospholipids regulating membrane-linked enzymes (Maruzzi et al. 1993). Polyamines stabilize cell membranes and this alters activities of ligands such as growth factors and activates protein kinases (tyrosine kinase and MAPK) that induce expression of proto-oncogenes like fos, jun, and myc (Johnson and McCormack 1999). They can also induce guanosine triphosphate phosphatase activity by binding with G proteins (Pegg 2013). Polyamines can also induce nuclear factor kappa B (NFKB), an inflammation-associated transcription factor that binds its unique response elements in DNA (Casero and Pegg 2009). The activity of NFKB increases as cells are depleted of polyamines (Pfeffer et al. 2001) and excessive levels of polyamines cause oxidative stress which stimulates NFKB (Moinard et al. 2005).

Further, Raf can be inhibited by spermine or stimulated by spermine plus spermidine or putrescine as those conditions alter the state of phosphorylation of Raf by casein kinase 2 (Minois 2014). Casein kinase 2 is likely responsive to variations in abundance of polyamines that influence activation of the MAPK pathway (Minois 2014). The phosphorylation status of MTOR and AMPK and their substrates are not affected by spermidine interactions (Moriselli et al. 2011), but the phosphorylation status of protein kinase B/Akt in lipopolysaccharide-activated BV2 microglial cells was reported to be decreased by spermidine (Choi and Park 2012).

6.3.5 Metabolism

The SAT1 gene encodes for diamine acetyltransferase 1 involved in regulating the amount of polyamines inside cells by regulating their transport in and out of the cell and it is involved in the first step for synthesis of N-acetylputrescine from putrescine (see Pegg and Michael 2010). Polyamine modulated factor 1 and nuclear factor erythroid 2–related factor 2 and SAT1 are part of a complex of transcription factors required for normal chromosome alignment and segregation and kinetochore formation during mitosis. Polyamine catabolism can also influence adipogenesis as over-expression of SAT1 in mice increases acetyl-CoA that depletes cellular energy, reduces white adipose tissue, increases glucose tolerance and insulin sensitivity, and increases basal metabolic rate (see Pegg and Michael 2010). These effects may be due to effects of SAT1 to modulate metabolic flux of polyamines due to an increase in ODC as SAT1 inhibits synthesis of polyamines and, therefore, AZ1.

6.3.6 Angiogenesis

Angiogenesis, the physiological process whereby new blood vessels are generated from existing blood vessels in tissues, requires migration, proliferation, and growth of endothelial cells (Billington 1991; Jasnis et al. 1994). Angiogenesis is critical in the female reproductive tract during pregnancy to allow increases in uterine and placental blood flows for the exchange of nutrients and gases required to support growth and development of the conceptus. DFMO significantly inhibits angiogenesis in tumors of mice, whereas exogenous putrescine reversed the effects of DFMO (Jasnis et al. 1994), and low physiological concentrations of polyamines are associated with poor angiogenic responses in gastric tumors (Takahashi et al. 2000). In the chicken yolk-sac model, spermine and spermidine increase angiogenesis with spermine being most stimulatory to formation of new blood vessels (Takigawa et al. 1990). There is clear evidence that polyamines and enzymes related to their synthesis in tissues stimulate angiogenesis (Takigawa et al. 1990; Takahashi et al. 2000), but the molecular pathway by which polyamines stimulate angiogenesis is poorly understood. Available evidence indicates that polyamines increase expression of vascular endothelial growth factor that induces migration and proliferation of endothelial cells during neovascularization (Neufeld et al. 1999) through transactivation of MEK1–ERK1/2 cell signaling that then activates expression of other pro-angiogenic molecules such an angiogenin and angiopoietins (Venuti et al. 2011).

6.4 Summary and Perspectives

Successful reproduction in animals depends on multiple systems and factors, but nutrition is of vital importance for proper synthesis and metabolism of polyamines. Wu et al. (1998) reported a decrease in ODC1 activity and a lower abundance of polyamines in endometria and placentae of primiparous sows subjected to a dietary protein restriction. That condition reduced the abundance of polyamines in the female reproductive tract and decreased reproductive performance of the sows (Wu et al. 1998). Current research is defining multiple pathways for synthesis of polyamines, hormonal regulation of

expression of gene encoding enzymes for those pathways, and the roles of polyamines in influencing the physiology of reproduction under normal and pathological conditions. Physiological levels of arginine, agmatine, and polyamines play a critical role in the functionality of cells regarding cell division and proliferation, gene expression, DNA and protein synthesis, apoptosis, oxidative stress, angiogenesis, and cell–cell communication. In ruminants, polyamines are critical to the secretion of IFNT by trophectoderm cells for pregnancy recognition signaling and, in rodents, they are required for activation of development of blastocysts and implantation. These key events are essential to the successful establishment and maintenance of pregnancy in mammals.

Acknowledgements Work in our laboratories was supported by Agriculture and Food Research Initiative Competitive Grants (Nos. 2016-67015-24958 and 2018-67015-28093) from the USDA National Institute of Food and Agriculture.

Conflict of Interest The authors declare no conflict of interest.

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7 Interorgan Metabolism, Nutritional Impacts, and Safety of Dietary L-Glutamate and L-Glutamine in Poultry

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Abstract

L-glutamine (Gln) is the most abundant amino acid (AA) in the plasma and skeletal muscle of poultry, and L-glutamate (Glu) is among the most abundant AAs in the whole bodies of all avian tissues. During the first-pass through the small intestine into the portal circulation, dietary Glu is extensively oxidized to CO_2, but dietary Gln undergoes limited catabolism in birds. Their extra-intestinal tissues (e.g., skeletal muscle, kidneys, and lymphoid organs) have a high capacity to degrade Gln. To maintain Glu and Gln homeostasis in the body, they are actively synthesized from branched-chain AAs (abundant AAs in both plant and animal proteins) and glucose via interorgan metabolism involving primarily the skeletal muscle, heart, adipose tissue, and brain. In addition, ammonia (produced from the general catabolism of AAs) and α-ketoglutarate (α-KG, derived primarily from glucose) serve as substrates for the synthesis of Glu and Gln in avian tissues, particularly the liver. Over the past 20 years, there has been growing interest in Glu and Gln metabolism in the chicken, which is an agriculturally important species and also a useful model for studying some aspects of human physiology and diseases. Increasing evidence shows that the adequate supply of dietary Glu and Gln is crucial for the optimum growth, anti-oxidative responses, productivity, and health of chickens, ducklings, turkeys, and laying fowl, particularly under stress conditions. Like mammals, poultry have dietary requirements for both Glu and Gln. Based on feed intake, tissue integrity, growth performance, and health status, birds can tolerate up to 12% Glu and 3.5% Gln in diets (on the dry matter basis). Glu and Gln are quantitatively major nutrients for chickens and other avian species to support their maximum growth, production, and feed efficiency, as well as their optimum health and well-being.

Keywords

Amino acids · Feed efficiency · Growth · Health · Productivity · And poultry

Abbreviations

α-KG	α-Ketoglutarate
AA	Amino acid
BCAA	Branched-chain amino acid

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1332,
https://doi.org/10.1007/978-3-030-74180-8_7

BW	Body weight
CP	Crude protein
EDL	Extensor digitorum longus
GABA	γ-Aminobutyrate
MSG	Monosodium glutamate
MTOR	Mechanistic target of rapamycin
NRC	National Research Council
PEPCK	Phosphoenolpyruvate carboxykinase

7.1 Introduction

Compared with mammals, birds have different digestive, urinary, and immune systems, as well as different metabolic patterns for the utilization of dietary amino acids (AAs) [including L-glutamate (Glu) and L-glutamine (Gln)] and the excretion of uric acid as the major nitrogenous metabolite (Wu 2018a, b). Among these uricotelic animals, poultry are agriculturally significant species to provide humans with high-quality animal proteins (e.g., meat and eggs), and also serve as useful models to study human physiology (e.g., angiogenesis and renal gluconeogenesis) and some human diseases, such as hyperglycemia, gout, ovarian cancers, Hashimoto's thyroiditis, and sex-linked dwarfism (Dodgson and Romanov 2004). Thus, research in avian AA metabolism is important for both economic developments and biomedical advancements worldwide. Because chickens grow fast and respond sensitively to dietary AA intake, they are most studied among avian species in nutrition, biochemistry, physiology, and immunology (Austic 1973; Austic and Nesheim 1971; Bailey 2020; Baker 2009; Brown 1966).

Glu and Gln are among the most abundant AAs in the diets and tissue proteins of poultry (He et al. 2021), as well as humans (Hou et al. 2019; Li and Wu 2020), ruminants (Gilbreath et al. 2021), and other animals such as swine (Zhang et al. 2021). These two AAs are interconverted in the tissues and cells of animals, including poultry (Fig. 7.1). Much is known about the interorgan metabolism of Glu and Gln, as well as their nutritional and physiological significance in mammals (Brosnan 2000, 2003; Brosnan and Brosnan 2004, 2006, 2013; Hou and Wu 2018; Pelicia et al. 2015; Watford 2000, 2008; Welbourne 1987). However, only a few studies investigated AA metabolism in birds in vivo (Tinker et al. 1986) and in isolated avian tissues (He et al. 2018; Wu and Thompson 1987, 1988, 1990; Wu et al. 1989, 1991, 1995). Although a previous study reported that adult roosters did not need dietary Glu or Gln to maintain their body at a zero or positive nitrogen balance when fed a purified diet during only a 3-day experimental period (Leveille and Fisher 1959), dietary Glu and Gln are crucial for the maximum growth and long-term survival of chickens (Maruyama et al. 1976). Thus, like mammals, birds have dietary requirements for these two AAs (Wu 2014). There is growing evidence that dietary supplementation with Glu and Gln can improve the growth, feed efficiency, and anti-oxidative responses in chickens particularly under stress conditions (e.g., Ayazi 2014; Porto et al. 2015; Shakeri et al. 2016), as reported for post-weaning, gestating, and lactating swine (Hou and Wu 2018; Rezaei et al. 2013; Wu et al. 1996, 2011; Zhang et al. 2021). The main objective of this article is to highlight recent advances in our understanding of the interorgan metabolism and the safety of dietary Glu and Gln in poultry.

7.2 Glu and Gln Catabolism in Poultry

7.2.1 An Overall View of Glu and Gln Catabolism in Poultry

The major sites of Glu catabolism in poultry appear to be the small intestine, liver, kidneys, skeletal muscle, and brain (He et al. 2018; Smith and Campbell 1983; Tinker et al. 1986; Watford et al. 1981; Watford and Wu 2005). This is also true for Gln catabolism in poultry except that their small intestine has a limited ability to degrade Gln (Wu et al. 1995). As the precursor of γ-aminobutyrate (GABA, an excitatory neurotransmitter), Glu and Gln (a precursor of Glu) play an important role in the behavior of all birds (He and Wu 2020). As the immediate precursor of α-KG, Glu is a major energy source in avian tissues,

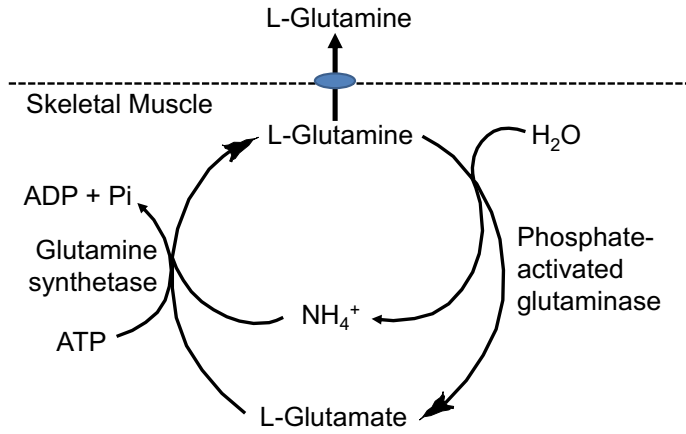

Fig. 7.1 Intracellular glutamine-glutamate cycle in chicken skeletal muscle. Skeletal muscle possesses mitochondrial phosphate-activated glutaminase to degrade glutamine to glutamate and ammonia, and also has cytosolic glutamine synthetase to generate glutamine from glutamate and ammonia. This constitutes the intracellular glutamine-glutamate cycle. The rates of glutamine synthesis and catabolism are the major determinant of glutamine release from skeletal muscle

whereas Gln is a major energy source in extra-intestinal tissues and cells (including bone marrow and lymphocytes) in birds. In the whole body of birds, Glu and Gln are interconverted through Gln synthetase, glutaminase, and Gln:fructose-6-phosphate transaminase (Wu 2013). Through its conversion into Gln, Glu further participates in many metabolic pathways, including: (1) the syntheses of uric acid in the liver, aminosugars, and nucleic acids in all tissues; (2) the removal of ammonia from the brain, liver, and muscle; (3) ammoniagenesis in the kidneys to regulate acid–base balance. This illustrates the metabolic versatility of Glu and Gln in poultry.

As in mammals (Hou and Wu 2018) and aquatic animals (Li et al. 2020, 2021a, b), the production of ATP from energy substrates is cell- and tissue-specific in birds (Wu 2018a, b). For example, Glu and Asp are major metabolic fuels for avian enterocytes, whereas these two AAs plus long-chain fatty acids provide most ATP in avian skeletal muscles. Long-chain fatty acids are the primary energy substrates for the avian heart, liver, and kidneys, but short-chain fatty acids fulfill this metabolic function in avian colonocytes. Glucose is nearly the sole source of ATP in the avian brain and red blood cells in the fed and post-absorptive states when the concentrations of ketone bodies in the blood are low. In birds, excessive Glu and Gln are either oxidized to CO_2, used by the kidneys for gluconeogenesis, or converted into lipids. Note that there is no synthesis of glucose from Glu, Gln and other AAs in avian livers (Wu 2018a).

7.2.2 Glu and Gln Catabolism in the Skeletal Muscle of Poultry

Glu and Gln are highly abundant AAs in both the free and protein pools of avian skeletal muscle (Wu et al. 1991). As the body weight (BW) increases with growth, the total amount of intramuscular Glu and Gln also increases to expand its pool size. All chicken muscles can oxidize extracellular and intramitochondrially generated Glu and Gln to CO_2 and water, but the rates of Glu and Gln catabolism differ markedly among their anatomical locations and fiber types. For example, the rates of Glu and Gln oxidation are greater in the breast muscle of chickens (mainly glycolytic fibers) than in their leg muscle (mainly oxidative fibers) (Wu et al. 1991, 1998). Thus, Glu and Gln may be extensively oxidized to produce ATP in chicken extensor digitorum

longus (EDL) and breast muscles (Wu and Thompson 1987; Wu et al. 1991). In avian skeletal muscles (e.g., the chicken EDL muscle; Wu and Thompson 1987; Wu et al. 1998), Glu is converted into Gln by the cytosolic Gln synthetase. This reaction helps to scavenge free ammonia in the blood and other tissues, and, therefore, the survival of birds. Interestingly, in chicken skeletal muscles, Gln is hydrolyzed into Glu by the mitochondrial phosphate-activated glutaminase. This reaction helps to remove excessive Gln from plasma as a defense against an adverse effect of high Gln levels on inhibiting the synthesis of nitric oxide (NO) by vascular endothelial cells and other cell types (Wu and Meininger 2002). The catabolism of Gln and its synthesis constitutes an intracellular Gln-Glu cycle in skeletal muscle (Fig. 7.1). We found that, in the chicken EDL muscle, both Glu and Gln are degraded to CO_2, with the net release of a large amount of Gln from this tissue (Wu and Thompson 1987). Intramuscular Glu may be channeled into oxidation, Gln synthesis, and protein synthesis pathways, whereas intramuscular Gln may be used for oxidation, Glu production, and protein synthesis, depending on nutritional and physiological conditions.

7.2.3 Glu and Gln Catabolism in the Small Intestine of Poultry

7.2.3.1 Glu Catabolism in the Small Intestine of Poultry

Porteous (1980) reported that the rate of the consumption of Glu by chicken enterocytes was only 20% of the rate of the consumption of glucose and that the nitrogenous products of Glu metabolism in these cells were aspartate and Gln, which together accounted for 74% of the utilized Glu. However, we recently found that, at equal substrate concentrations (i.e., 5 mM), the rate of CO_2 production from Glu oxidation in the enterocytes of 1- to 42-day-old broilers was about 10 times the rate of CO_2 production from glucose oxidation (He et al. 2018). In addition, the extensive degradation of Glu in the enterocytes of growing chickens generated not only aspartate and Gln, but also alanine and asparagine (He 2019). Among all the substrates studied (i.e., D-glucose, Glu, Gln, aspartate, alanine, palmitate, propionate, and butyrate), Glu is the major metabolic fuel for the broiler enterocytes (He et al. 2018), indicating a crucial role of this AA in gut maintenance and function. The metabolic pathways for Glu degradation are outlined in Fig. 7.2. In addition to its catabolism, Glu is utilized along with glycine and cysteine for glutathione synthesis in the avian intestine (Porteous 1980). Thus, although Glu is the most abundant AA in the common feedstuffs used for poultry diets (Table 7.1), the concentration of this AA is particularly low (<100 μM) in the plasma of chickens (Watford and Wu 2005). This is important for the survival of animals, because a high level of Glu in the circulation is neurotoxic (Wu 2013). At present, the rates of the first-pass utilization of dietary Glu by the avian small intestine in vivo are unknown. Due to the limited uptake of arterial Glu by enterocytes as assessed by the limited entry of ^{14}C-glutamate from the serosal side of the Ussing chamber into the mounted jejunal mucosa (our unpublished work), dietary (enteral) Glu is essential to sustain the integrity, function, and health of the chicken small intestine.

7.2.3.2 Gln Catabolism in the Small Intestine of Poultry

Porteous (1980) reported that the rate of consumption of Gln by chicken enterocytes was the highest among all substrates tested at 2.5 mM for each AA and 5 mM for glucose (Gln > proline > serine > Glu > aspartate > asparagine > glucose). The author also found that the nitrogenous products of Gln metabolism in these cells were ammonia, Glu, alanine, aspartate, and ornithine, which together accounted for >95% of the utilized Gln. However, we recently found that, at equal substrate concentrations (i.e., 5 mM), the rate of CO_2 production from Gln oxidation in the enterocytes of 1- to 42-day-old broilers was much lesser than that of Glu, aspartate and glucose (He et al. 2018). In addition, the low rate of Gln degradation in the enterocytes of growing chickens did not generate ornithine, citrulline, or urea

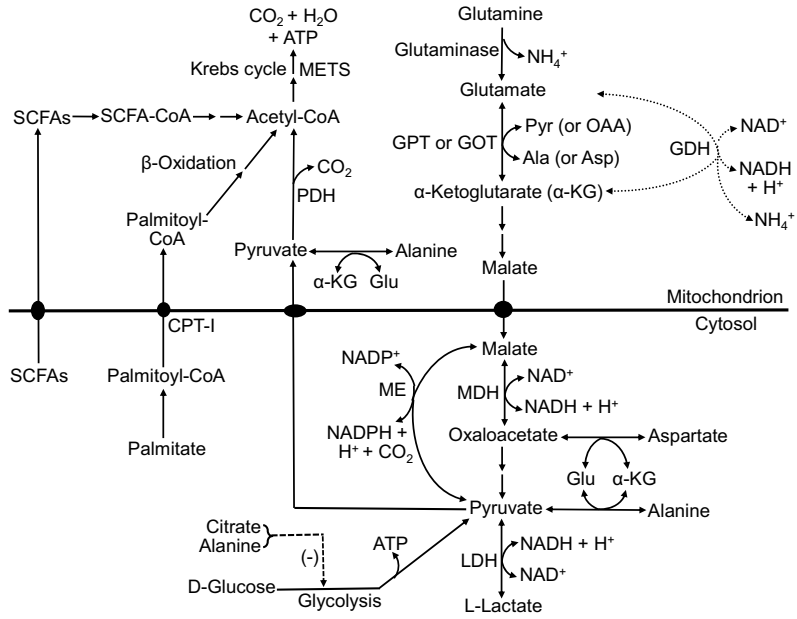

Fig. 7.2 Oxidation of amino acids, glucose, and fatty acids in birds. In many avian cells and tissues, glutamate, glutamine, aspartate, and alanine are metabolized to pyruvate through a series of pathways that involve both the cytosol and mitochondria. By contrast, glucose is converted into pyruvate via the cytosolic glycolysis pathway, and the glucose-derived pyruvate is either reduced to lactate in the cytosol or oxidized to acetyl-CoA in the mitochondria. Alanine and citrate (an intermediate of the Krebs cycle) inhibit glycolysis by inhibiting pyruvate kinase and phosphofructokinase-1, respectively. Palmitate, a long-chain fatty acid, is activated to palmitoyl-CoA in the cytosol by long-chain acyl-CoA synthase, and palmitoyl-CoA is transported into the mitochondrial matrix via carnitine palmitoyltransferase-I for oxidation to acetyl-CoA. Short-chain fatty acids (propionate and butyrate) are transported from the cytosol into the mitochondria for activation by short-chain acyl-CoA synthase into acyl-CoAs. The latter are metabolized to acetyl-CoA. Thus, glutamate, glutamine, aspartate, alanine, glucose, short-chain fatty acids, and palmitate are ultimately converted into acetyl-CoA, which is oxidized to CO_2 and H_2O via the Krebs cycle and the electron transport system in the mitochondria. Glutamate and aspartate are major metabolic fuels in avian enterocytes, whereas these two amino acids plus long-chain fatty acids provide most ATP in avian skeletal muscles. Long-chain fatty acids are the primary energy substrates for the avian heart, liver, and kidneys, but short-chain fatty acids fulfill this metabolic function in avian colonocytes. Glucose is nearly the sole source of ATP in the avian brain and red blood cells in the fed and post-absorptive states when the concentrations of ketone bodies in the blood are low. Ala, alanine; GDH, glutamate dehydrogenase (absent from chicken and pig enterocytes); Glu, glutamate; GOT, glutamate–oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; LDH, lactate dehydrogenase; MDH, NAD^+-linked malate dehydrogenase; ME, malic enzyme; METS, electron transport system; OAA, oxaloacetate; PDH, pyruvate dehydrogenase; PFK-1, phosphofructokinase-1; Pyr, pyruvate; SCFAs, short-chain fatty acids

(Wu et al. 1995). Among all the substrates studied (i.e., D-glucose, Glu, Gln, aspartate, alanine, palmitate, propionate, and butyrate), Gln is quantitatively only a minor metabolic fuel for the broiler enterocytes (He et al. 2018), but Glu and aspartate are the primary sources of ATP for gut maintenance and growth. In addition to its catabolism, Gln is utilized along with fructose-6-phosphate for the synthesis of glucosamine and thus glycoproteins (including mucins and membrane receptors) in the avian intestine (Wu 2013). Thus, because dietary Gln is not subject to extensive catabolism in the small intestine due to low glutaminase activity and because Gln is an abundant AA in the common feedstuffs used for poultry diets (Table 7.1), the concentration of this AA is particularly high (1 mM) in the plasma of chickens (Watford and Wu 2005), which doubles Gln concentration in the plasma of mammals (Wu 2018a, b). This is important for the survival and

Table 7.1 Composition of amino acids in the whole-body proteins of chicks and pigs and in common feedstuffs

AA	Chicks[a]		Pigs[b]		Corn grain[c]	Soybean meal[d]	Sorghum grain[e]	Meat and bone meal[f]
	mg AA/g protein	% of lysine	mg AA/g protein	% of lysine	g AA/100 g foodstuff			
Alanine	66.3	108	65.7	109	0.71	1.95	0.96	4.78
Arginine	68.5	111	67.7	112	0.38	3.18	0.41	3.67
Asparagine	36.5	59.3	36.0	59.7	0.35	2.10	0.31	2.21
Aspartate	43.1	70.1	42.8	71.0	0.43	3.14	0.36	3.08
Cysteine	15.0	24.4	13.2	21.9	0.20	0.70	0.19	0.49
Glutamine	50.5	82.1	51.2	84.9	1.02	3.80	0.85	2.81
Glutamate	82.9	135	84.6	140	0.64	4.17	1.18	4.05
Glycine	115	187	117	19.4	0.40	2.30	0.39	8.67
Histidine	21.1	34.3	20.8	34.5	0.23	1.13	0.23	1.19
Isoleucine	35.9	58.4	35.3	58.5	0.34	2.03	0.38	1.92
Leucine	69.2	113	68.3	113	1.13	3.44	1.21	3.56
Lysine	61.5	100	60.3	100	0.25	2.80	0.21	3.13
Methionine	18.9	30.7	18.7	31.0	0.21	0.60	0.20	1.10
Phenylalanine	34.8	56.6	34.3	56.9	0.46	2.21	0.51	1.85
Proline	85.3	139	86.1	143	1.06	2.40	0.96	5.86
OH-Proline	34.8	56.6	37.9	62.9	0.00	0.09	0.00	2.88
Serine	45.0	73.2	44.3	73.5	0.45	2.12	0.46	2.08
Threonine	36.3	59.0	35.1	58.2	0.31	1.76	0.32	2.42
Tryptophan	11.6	18.9	11.1	18.4	0.07	0.62	0.10	0.39
Tyrosine	26.6	43.3	27.2	45.1	0.43	1.66	0.45	1.45
Valine	41.8	68.0	42.2	70.0	0.44	2.09	0.50	2.23

Adapted from Wu (2014). Except for glycine, all amino acids are L-isomers. Calculations were based on the molecular weights of intact amino acids. OH-Pro = 4-hydroxyproline

[a]Chickens (10-day-old). The content of protein in the body is 14.3 g/100 g wet tissue

[b]Pigs (30-day-old). The content of protein in the body is 14.1 g/100 g wet tissue

[c]As-fed basis (89.0% dry matter). Corn grain contains 9.3% crude protein (as-fed basis)

[d]As-fed basis (89.0% dry matter). Soybean meal contains 43.6% crude protein (as-fed basis)

[e]As-fed basis (89.1% dry matter). Sorghum grain contains 10.1% crude protein (as-fed basis)

[f]As-fed basis (96.1% dry matter). Meat and bone meal contains 52.0% crude protein (as-fed basis)

growth of poultry, because a sufficiently high level of Gln in the circulation is necessary to activate the mechanistic target of rapamycin (MTOR) cell signaling, sustain a high rate of tissue protein synthesis, and support the very rapid growth of the animals (Xi et al. 2011, 2012). At present, the rates of the first-pass utilization of dietary Gln by the avian small intestine in vivo are unknown but are likely to be low based on the results of our in vitro studies with enterocytes (He et al. 2018; Wu et al. 1995). Because arterial Gln is actively taken up by enterocytes, dietary (enteral) and blood Gln are two major sources of Gln that are essential for the integrity, function, and health of the chicken small intestine.

7.2.4 Glu and Gln Catabolism in the Liver and Kidneys of Poultry

7.2.4.1 Glu Catabolism in the Liver and Kidneys of Poultry

In the fed state, but not the long-term (6-day) fasting state, the liver of chickens takes up Glu from the arterial blood, and the hepatic uptake of Glu is the highest among all AAs (Tinker et al. 1986). The avian liver can degrade Glu through the Glu dehydrogenase (oxidative deamination) and/or Glu transaminases (e.g., Glu-pyruvate transaminase and Glu-oxaloacetate transaminase), with the carbon skeletons of Glu being mainly converted into CO_2. This is because, in avian hepatocytes, phosphoenolpyruvate carboxykinase (PEPCK) is localized exclusively in mitochondria, and therefore, Glu is not converted into glucose in fed or fasted states (Watford et al. 1981). Instead, in the mitochondria of avian hepatocytes, Glu is either degraded by Glu dehydrogenase to ammonia plus α-KG or utilized for Gln synthesis by Gln synthetase (Vorhaben and Campbell 1977).

In contrast to the liver, the chick kidneys do not take up Glu from the arterial blood in either the fed or long-term (6-day) fasting state (Tinker et al. 1986). Also, in chicken kidneys, PEPCK is present in both the cytosol and mitochondria, which allows for the production of glucose from Glu under fed and fasting conditions (Watford et al. 1981). This is significant for the regulation of glucose homeostasis in birds (Wu 2018a). When renal Glu dehydrogenase activity is enhanced under acidotic conditions, the Glu-derived ammonia contributes to the maintenance of acid–base balance in the whole body.

7.2.4.2 Gln Catabolism in the Liver and Kidneys of Poultry

Whether the avian liver extracts Gln from the blood depends on the nutritional state. For example, in the fed state, the liver of chickens does not take up Gln from the arterial blood (Tinker et al. 1986). Thus, the high concentration of Gln in the liver of fed poultry results primarily from its endogenous synthesis of Gln from Glu and ammonia (both are products of AA catabolism), and Gln can be considered as the sink of both the nitrogen and carbon atoms of AAs. In the long-term (6-day) fasting state, the chicken liver actively takes up Gln from the arterial blood, and the extracted amount is the highest (the same order of ranking as alanine) among all AAs (Tinker et al. 1986). The physiological significance of this process is largely unknown, but it may help to regulate hepatic nutrient metabolism, such as inhibiting proteolysis, AA degradation, and glycogenesis to spare protein, AAs, and glucose. Unlike mammals, the avian liver lacks glutaminase for the hydrolysis of Gln into Glu. By contrast, in the avian liver, the predominant pathways for Gln metabolism other than protein synthesis are the syntheses of purine and pyrimidine nucleotides, with Glu being formed at the steps of Gln 5-phosphoribosyl-pyrophosphate amidotransferase and carbamoylphosphate synthase II, respectively. Purines are further converted into uric acid. Another pathway for hepatic Gln utilization, despite its low rate, is Gln-fructose-6-P transaminase. The avian liver can degrade the Gln-derived Glu through Glu dehydrogenase and/or Glu transaminases (e.g., glutamate-pyruvate transaminase and glutamate–oxaloacetate transaminase), with the carbon skeletons of Glu being mainly converted into CO_2 and water. As noted above, in avian hepatocytes, Gln-derived Glu is not converted into glucose in fed or fasted states because PEPCK is localized exclusively in mitochondria (Watford et al. 1981).

In contrast to the liver, the chicken kidneys take up Gln from the arterial blood in the fed state (Tinker et al. 1986). Interestingly, unlike mammals, there is no significant renal uptake of Gln by chickens in the long-term (6-day) fasting state (Tinker et al. 1986). As noted above, PEPCK is present in both the cytosol and mitochondria of avian kidneys to allow for the conversion of Gln-derived Glu into glucose under both fed and fasting conditions (Watford et al. 1981). This is significant for the regulation of glucose homeostasis in birds (Wu 2018a). When renal glutaminase and Glu dehydrogenase activities are enhanced under acidotic conditions, the

Gln-derived ammonia contributes to the maintenance of acid–base balance in the whole body.

7.3 Glu and Gln Synthesis in Poultry

Little is known about in vivo Glu and Gln synthesis in poultry. However, the endogenous synthesis of Glu and Gln must occur to account for its high abundance in the whole body of birds, as reported for pigs (Table 7.1). Furthermore, Glu and Gln syntheses are likely cell- and tissue-specific in poultry, as reported for mammals (Wu 2013). For example, a low activity of glutaminase in avian enterocytes limits the formation of Glu from Gln by these cells (Wu et al. 1995). Because phosphate-activated glutaminase is virtually absent from the liver of chickens (Watford and Wu 2005), this organ does not play a role in hydrolyzing Gln into Glu. However, glutaminase is present in many other tissues (including skeletal muscle and kidneys) of birds, as noted previously. Interestingly, a relatively high activity of Gln synthetase in chicken enterocytes (e.g., about 40 nmol/mg protein/min in the enterocytes of 21-day-old broiler chickens) can play a quantitatively significant role in the formation of Gln from Glu and ammonia by these cells (He 2019). For comparison, the activity of Gln synthetase in porcine enterocytes is very low (e.g., only about 0.5 nmol/mg protein/min in the enterocytes of 21-day-old pigs reared by sows; He 2019). In contrast to the chicken intestine, the chicken kidneys do not appear to be the site of net Glu or Gln synthesis (Tinker et al. 1986). Thus, the inter-organ metabolism of some AAs (e.g., arginine, BCAAs, histidine, and proline) involving the kidneys, skeletal muscle, intestine, and liver provides both the amino group and carbon skeletons to generate Glu and Gln in skeletal muscle (Wu 2013). In skeletal muscle (Wu and Thompson 1989), branched-chain AA (BCAAs; leucine, isoleucine and valine) donate the amino group for Glu formation, with its carbon skeleton being derived primarily from glucose and, to a much lesser extent, valine, isoleucine, and aspartate (Wu and Thompson 1989). Glu is subsequently amidated by the ATP-dependent Gln synthetase to produce Gln. Endogenous synthesis of Glu and Gln may contribute a substantial amount of these two AAs to poultry [including chicken embryos (Hu et al. 2017)], but in vivo quantitative data are not available.

Based on Glu content in the body, we can estimate that a growing broiler chicken must synthesize at least 83 mg Glu/day to gain 1 g protein in the body or 5 g BW per day. For broilers that grow from 50 g at day 0 to 2500 g at day 42, they would need to synthesize endogenously 0.97 g Glu/day on average [i.e., (2500 g − 50 g)/5 g × 0.083 g/42 days], which is equivalent to approximately 11.7 g of body protein per day (i.e., 0.97/0.083) or 25.2 g of dietary protein per day (11.7 g/46.5%).

As reported for mammals, the skeletal muscle of birds releases a large amount of Gln in both the fed and fasting states (Tinker et al. 1986). This occurs in the absence of a significant uptake of Glu by the muscle. Like Glu, in vivo synthesis of Gln in birds depends on the interorgan metabolism of AAs. Based on Gln content in the body, we can estimate that growing chickens must synthesize at least 51 mg Gln/day to gain 1 g protein in the body or 5 g BW per day. For broilers that grow from 50 g at day 0 to 2500 g at day 42, they would need to synthesize endogenously 0.60 g Gln/day. Thus, broilers within this age range need to synthesize de novo 1.57 g Glu plus Gln/day.

7.4 Glu and Gln Nutrition in Poultry

7.4.1 The Needs to Consider Dietary Glu and Gln in Poultry Nutrition

Chickens grow very rapidly and respond sensitively to dietary AA intake (Baker 2009). Of note, the commercial birds today are very different from those before 1991 because of differences in genetic selection, environment, and dietary composition (Bailey 2020). For example, the national average BW of a male turkey at 18 weeks of age may be over 18.2 kg today

versus only 10.9 kg in 1986 (Applegate and Angel 2014). Thus, although the basic principles of nutrition in poultry remain the same, the quantitative aspects of nutrient requirements, particularly AAs, differ among different breeds of birds (Wecke and Liebert 2013; Wecke et al. 2018). At present, NRC (1994) has not recommended dietary requirements of poultry for Glu or Gln. However, Glu is an excellent source of both nitrogen and carbon for the synthesis of AAs in chicks via amination and amidation. Importantly, although historically Glu was not considered as a nutritionally essential AA for birds, Glu had often been used to balance dietary nitrogen content in studies involving poultry (Baker and Han 1994). There is evidence that Glu and Gln must be provided in the diets of poultry for their maximum growth, as well as optimum development and health (Hou et al. 2015; Hou and Wu 2017; Kidd 2004; Maruyama et al. 1976). These findings refute the historical view that Glu and Gln were not nutritionally essential AAs for birds. These two nutrients must be taken into consideration when optimum AA patterns are formulated to feed poultry (He et al. 2021). The lack of Glu and Gln in rations compromises the growth performance, feed efficiency, and immune response in birds. Based on recent advances in AA biochemistry and nutrition, we have concluded that the term "nutritionally nonessential AA" is a misnomer in nutritional sciences and should no longer be used (Hou and Wu 2017).

Based on a short-term (3-day) study, Leveille and Fisher (1959) found that adult roosters did not need dietary Glu or Gln for maintaining a zero or positive nitrogen balance when fed a purified diet. However, this result should not be interpreted to indicate that dietary Glu and Gln are not necessary for maximum efficiency in the utilization of dietary protein AAs for poultry growth or for the optimal health of all poultry. Of note, these authors also reported that during the 3-day experimental period, adult roosters did not require dietary L-histidine for maintaining a zero or positive nitrogen balance (Leveille and Fisher 1959), but L-histidine is not synthesized de novo by any animal cells (Wu 2013, 2020). Thus, as recognized previously (Wu 2014), nitrogen balance, particularly within a short period of time, is not highly sensitive to assess dietary requirements for AAs (including Glu and Gln) in animals (e.g., chickens and other avian species). Because of a limited activity of arginase (Klain and Johnson 1962) and proline oxidase (Furukawa et al. 2018) in tissues of chickens for Glu and Gln syntheses from arginine and proline, dietary requirements for Glu and Gln are likely greater in birds than in mammals (He et al. 2020; Wu 2018a, b). Results of longer-term studies (> a 14-day period) involving purified diets indicate that the absence of dietary Glu intake markedly reduced BW gain in 1-to 14-day-old chicks and that the addition of 10% Glu to the Glu-free basal diet increased daily weight gain of the young chicks by fourfold (Maruyama et al. 1976).

Likewise, poultry do need dietary Gln for optimum health and maximum growth (Bortoluzzi et al. 2018; Fernandes et al. 2018; Wu 2018a, b; Xue et al. 2018). Collectively, these findings indicate that growing chickens and other avian species do not synthesize sufficient Glu or Gln de novo for maximum lean tissue gain or maximum feed efficiency.

7.4.2 Effects of Dietary Supplementation with Glu in Poultry

Extensive studies have shown that dietary supplementation with Glu can improve the growth performance and health of poultry. These results provide additional lines of evidence to support the view of insufficient Glu synthesis in birds. First, dietary supplementation with 0.4, 0.6 or 0.8% Glu to 1-day-old broiler chickens for 35 days enhanced their feed intake, BW gain, and feed conversion ratio without negatively affecting the percentage of carcass (Maslami et al. 2019). Second, dietary supplementation with 0.5–1% Glu augmented small-intestinal villus height, weight gain, and feed efficiency, while reducing post-hatching mortality in 1- to 42-day-old broilers under hot and humid conditions

(Olubodun et al. 2015a). Third, supplementing a mixture of biosynthesizable AA (1.21% Glu, 0.8% glycine, 0.43% proline, 0.33% alanine, and 0.99% aspartate) to a low protein [16.2% crude protein (CP)] diet improved weight gain by 14% and feed efficiency by 10% (Gain:Feed, 0.754 vs. 0.686) in 1- to 21-day-old broilers (Awad et al. 2014). Fourth, under heat stress conditions (room temperature being 5 °C higher than the thermoneutral temperature), dietary supplementation with 1% Glu between days 1 and 21 of age increased duodenal villus height and body-weight gain by 7% and 28%, respectively (Porto et al. 2015). Fifth, adding 0.34% Glu to a low protein (15.2% CP) diet for laying hens increased egg production during a 140-day period by 7% without affecting feed intake or egg mass, compared with the control group fed a low protein (17% CP) diet (Bezerra et al. 2015). Sixth, supplementing a mixture of AAs (containing 0.6% Glu) to a reduced-protein (18% CP) diet for growing broilers (days 6–21 of age) enhanced BW gain and feed efficiency without affecting feed intake, compared with the control group fed an 18% CP diet (Corzo et al. 2005). Interestingly, BW gain or feed efficiency did not differ between the chickens receiving the dietary supplementation with a mixture of AAs and the birds fed a standard 22% CP diet. It should be borne in mind that when dietary protein level is too low (e.g., 13% CP), dietary supplementation with Glu (up to 2.7%) did not improve the growth or egg-laying performance of chickens (Penz and Jensen 1991), which is likely due to the deficiency of other AAs (Wu 2013). Seventh, supplementing 0.54% monosodium glutamate (MSG) to a diet for yellow-feathered broilers between 42 and 63 days of age could enhance meat flavor (Ma et al. 2011). Thus, adequate dietary Glu is needed for maximal growth and feed efficiency of poultry, as well as improvements in egg production and meat quality.

Another application of Glu to poultry feeding during the period of delayed placement of broilers is also beneficial. Under commercial conditions, it may take up to one or two days before newly hatched broiler chicks have access to feed and drinking water on the farm, because of various practical factors, such as hatching time, the processing of chicks, and transportation (Dibner et al. 1998). The delayed feeding of birds generally results in their dehydration, depletion of yolk reserves, distress, and malnutrition, as well as high rates of morbidity and mortality (Bigot et al. 2003; Careghi et al. 2005; Fanguy et al. 1980). Thus, addition of MSG to feed or drinking water may help to improve the gut development and adaptation of neonatal birds.

It is likely that the effects of dietary Glu supplementation are influenced by the composition (particularly the content of Glu and Gln) of the basal diet. For example, dietary supplementation with 0.25–1% Glu between 1 and 49 days of age did not affect growth performance of broilers fed a basal diet containing 22% CP (Khadiga et al. 2009). In addition, Ebadiasl (2011) reported that supplementing 0.5 or 1% Glu to a wheat (68%)- and soybean meal (24%)-based diet between 1 and 35 days of age did not influence the growth performance, gastrointestinal development, jejunum morphology or colon *Clostridium perfringens* counts in male broilers. Wheat is rich in Gln (4.55% Gln on the dry matter basis) (Hou et al. 2019). Thus, 68% wheat in the basal diet would contain 3.09% Gln (i.e., $0.68 \times 4.55\%$). This high amount of Gln from wheat may be sufficient to provide both Gln and Glu for growing chickens, and, therefore, may mask an effect of the supplemental Glu on their growth. Of note, a typical corn- and soybean meal-based starter diet (60% corn and 30% soybean meal; 22% CP) for broilers contains 1.8% Gln and 1.7% Glu, but the content of Glu and Gln in a reduced-protein diet is usually lower (Wu 2013). The beneficial effects of dietary Glu on the growth, production performance, and antioxidative responses of poultry are summarized in Table 7.2.

Table 7.2 Effects of dietary L-glutamate intake or supplementation on the growth and production of poultry

Poultry	Supplemental Glu	Effects	References
Broilers	Purified diet + 10% Glu	Daily weight gain ↑ (4 fold)	Maruyama et al. (1976)
Broilers	Basal diet (21.75% CP) + 0.4, 0.6 or 0.8% Glu; d 1-36	Feed intake ↑ Daily weight gain ↑ and Feed efficiency ↑	Maslami et al. (2019)
Broilers (heat stress, +5 °C)[a]	Basal diet (22-20% CP) + 1% Glu, d 1-21	Intestinal villus height ↑ Weight gain ↑	Porto et al. (2015)
Laying hens	Basal diet (15.2% CP) + 0.34% Glu (vs. 17% CP diet), 140 d	Egg production ↑ No change in egg mass	Bezerra et al. (2015)
Broilers (yellow feather)	Basal diet + 0.54% Glu, d 42-63	Meat flavor ↑	Ma et al. (2011)
Broilers (6–21 d of age)	Low CP (18%) diet + 0, 0.2% Gly or mixed AA[b]	Weight gain ↑ Feed efficiency ↑	Corzo et al. (2005)
Broilers[a] (1–21 d of age)	Low protein (16.2% CP) diet + mixed NEAA[c]	Weight gain ↑ Feed efficiency ↑	Awad et al. (2014)

CP = crude protein; d = days; ↑ = Increase; ↓ = Decrease
[a]Room temperature was 5 °C higher than the thermoneutral temperature
[b]Mixed AA = 0.2% Gly, 0.2% Pro, 0.2% Ala, 0.6% Asp, 0.6% Glu, and 0.3% Leu
[c]Mixed NEAA = 0.8% Gly, 0.43% Pro, 0.33% Ala, 1.21% Glu, and 0.99% Asp

7.4.3 Effects of Dietary Supplementation with Gln in Poultry

7.4.3.1 Effects of Gln on Intestinal Health, Whole-Body Growth, and Feed Efficiency in Poultry

The small intestine is vital for the digestion and absorption of dietary nutrients and for the health and growth of poultry (Bortoluzzi et al. 2018; Moran 2017). Thus, much research has focused on intestinal health, whole-body growth, and feed efficiency in poultry. Yi et al. (2005) reported that dietary supplementation with 1% Gln improved the morphology of the small intestine at 3 and 14 days of age in broiler chickens. Dietary supplementation with Gln also resulted in beneficial effects on enhancing the feed efficiency and weight gain (1–42 days) of broiler chickens subjected to delayed placement (Zulkifli et al. 2016). Nearly all studies demonstrated a beneficial effect of dietary supplementation with 1% Gln on increasing the villus height of the small intestine, improving the intestinal morphology, and augmenting immunity in poultry (Table 7.3). Besides growth performance, dietary supplementation with 0.4% Gln between 1 and 21 days of age enhanced the economic traits of broiler production, as indicated by 14, 10, 16, and 12% improvements in coefficient of variation in BW, BW uniformity, coefficient of variation in breast yield, and breast uniformity, respectively (Ribeiro et al. 2015). It appears that the positive effects of Gln supplementation on broilers are even greater under stress than non-stress conditions. Another application of Gln to poultry feeding is its dietary supplementation to broilers during the period of their delayed placement to improve their survival and growth, as noted previously for Glu.

It is likely that the effects of dietary Gln supplementation are influenced by its dose, the composition (particularly the content of Glu and

Table 7.3 Effects of dietary L-glutamine supplementation on the growth and production of poultry

Poultry	Supplemental Gln	Effects	References
Intestinal growth and health, immunity, health, and feed efficiency			
Broilers (1–28 d of age; EMVC)	Corn-SBM diet (22% CP) + 1% Gln between d 1 and 22	Weight gain ↑ Feed efficiency ↑ Small intestine villus height ↑ Intestinal health ↑ Immunity ↑	Yi et al. (2005)
Broilers (1–21 d of age)	Corn-SBM diet (21.5% CP) + 1% Gln	Small-intestinal villus height ↑ Villus height/crypt depth ratio ↑	Murakami et al. (2007)
Broilers (1–21 d of age)	Corn-SBM diet + 1% Gln	Weight gain ↑ Feed efficiency ↑ Small intestine villus height ↑ IgA ↑ IgG ↑	Bartell and Batal (2007)
Broilers (1–42 d of age)	Corn-SBM diet (20–22% CP) + 1 and 2% Gln	Weight gain ↑ Feed efficiency ↑ Small intestine villus height ↑ Immunity ↑	Soltan (2009)
Broilers (21–42 d of age; challenge with Esp)	Corn-SBM diet (21% CP) + 0.85% AG[a] + 0.85% Thr	Weight gain ↑ Feed efficiency ↑ Small intestine villus height ↑	Fernandes et al. (2018)
Broilers (3–17 d of age) Challenge with ST	Corn-SBM diet (22% CP) + 1% Gln	Weight gain ↑ Feed efficiency ↑ No change in *Salmonella* colonization in the ceca	Fasina et al. (2010)
Broilers (1–21 d of age)	Corn-SBM diet (21.4% CP) + 1% Gln	Duodenum villus height ↑ Villus height/crypt depth ratio ↑	Khempaka et al. (2011)
Turkey poults (21–42 d of age; challenge with Esp) (32–34 °C)	Basal diet + 0 to 0.7% Gln	Weight gain ↑ Feed efficiency ↑ Small intestine villus height ↑	Salmanzadeh and Shahryar (2013)
Broilers (1–28 d of age)	Corn-SBM diet (22% CP) + 1% Gln	Feed efficiency in vaccinated birds ↑	Luquetti et al. (2016)
Ducklings (7–13 d of age; DPV challenge)	Corn-SBM diet (22% CP) + 0.5, 1 and 2% Gln	Weight gain ↑ Feed efficiency ↑ Small intestine villus height ↑ Intestinal health ↑ Immunity ↑	Zhang et al. (2017)

(continued)

Table 7.3 (continued)

Poultry	Supplemental Gln	Effects	References
Broilers (1–42 d of age)	Ovo feeding of 10, 20, 30, 40 and 50 mg Gln in 0.5 ml water	Post-hatching weight gain ↑ Feed efficiency ↑ Small intestine villus height ↑ Muscle growth ↑ Carcass weight ↑	Salmanzadeh et al. (2016)
Broilers (0–21 d of age)	Corn-SBM diet (23.4% CP) + 1 and 2% Gln	Weight gain ↑ Feed efficiency ↑ Small intestine villus height ↑ Amino acid digestibility in the small intestine ↑	Namroud et al. (2017)
Broilers (7–42 d of age)	Corn-SBM diet (20–22% CP) + 0.5 and 1% Gln	Weight gain ↑ Feed efficiency ↑ Small intestine villus height ↑ Mortality of ascites induced by 15 °C ↓	Abdulkarimi et al. (2019)
Broilers (0–24 d of age) NEC challenge	Wheat-barley-SBM diet (22.4–24.1% CP) + 1% Gln	Weight gain ↑ Feed efficiency ↑ Small intestine villus height ↑ Intestinal health ↑ Tissue integrity ↑	Xue et al. (2018)
Growth performance and meat quality under heat stress conditions			
Broilers (1–42 d of age) 28 °C	Corn-SBM diet (20.7% CP) + 0.5 and 1% Gln	Weight gain ↑ Meat quality ↑ Meat color stability ↑	Dai et al. (2009)
Broilers (1–42 d of age) 32 °C	Corn-SBM diet (20–22% CP) + 0.2, 0.5 and 1% Gln	Weight gain ↑ Meat quality ↑ Small intestine villus height ↑	Ayazi (2014)
Broilers (1–42 d of age) 32 °C	Corn-SBM diet (20–22% CP) + 0.25 and 0.5% Gln	Weight gain ↑ Feed efficiency ↑ Small intestine villus height ↑	Jazideh et al. (2014)
Broilers (1–42 d of age) 27–33 °C; 21 d	Corn-SBM diet (20–22% CP) + 0.5, 1, 1.5 and 2% Gln	No effect on growth or carcass yields	Nascimento et al. (2017)
Broilers	Basal diet (24.4- 18.5% CP, d 1–21) + 0.4% Gln	Breast yield ↑ and uniformity ↑	Ribeiro et al. (2015)
Broilers (22–35 d of age) 30–34 °C	Corn-SBM diet (20.7% CP) + 0.5% Gln	Weight gain ↑ Feed intake ↑ Serum insulin ↑ Serum total protein ↑ Tissue integrity	Hu et al. (2016a)
Broilers (35 d of age) 34 °C for 12 h	Corn-SBM diet (20.7% CP) + 1% Gln	Meat water-holding capacity ↑ Meat color ↑ Meat moisture ↑ Drip loss ↓	Hu et al. (2016b)

(continued)

Table 7.3 (continued)

Poultry	Supplemental Gln	Effects	References
Egg production by laying hens			
Laying hens 25–30 °C	Corn-SBM (18.3% CP) + 0.2, 0.4, and 0.8% Gln, 42 days	Egg production ↑ LH↑ FSH ↑ T3 ↑ T4 Small intestine morphology ↑ Oviduct morphology ↑	Dong et al. (2010)
Laying guinea fowl	Corn-SBM (18% CP) + 1% Gln 40 days	Egg production, mass, shell thickness ↑ LH↑ FSH ↑ T3 ↑ T4 ↑ Feed efficiency ↑ Hatchability; Chick birth weight	Gholipour et al. (2017)
Transportation stress			
Broilers (1–21 d of age)	Corn-SBM diet (22% CP) + 1% Gln	Weight gain ↑ Feed efficiency ↑ Small intestine morphology ↑ Hsp70 ↑ Transportation stress ↓	Shakeri et al. (2016)

CP = crude protein; d = day(s); DPV = duck plague virus; EMVC = Vaccination with *Eimeria maxima* on d 0 and challenge with *Eimeria maxima* on d 22; Esp = *Eimeria sp;* FSH = follicle stimulating hormone; Hsp = heat-shock protein; Ig = Immunoglobulin. ↑ = Increase; ↓ = Decrease; LH = luteinizing hormone; NEC = necrotic enteritis; SMB = soybean meal; ST = *Salmonella* Typhimurium; T3 = triiodothyronine; T4 = tetraiodothyronine

Gln) of the basal diet, and management. For example, Nascimento et al. (2017) reported that dietary supplementation with 0.5–2% Gln to broilers between days 1 and 21 of age had no effect on growth performance. However, this study did not include antibiotics or anticoccidials in the basal experimental diets, which can compromise a positive response of broilers that are highly susceptible to performance-impairing pathogens particularly at the early stage of development. In contrast, dietary supplementation with 0.5, 0.75, and 1% Gln to broilers between days 1 and 28 of age (vaccinated with a coccidiosis vaccine immediately after hatching) enhanced immunity acquisition and body-weight gain and could maintain the body weight difference until 42 days of age (Mussini et al. 2012).

7.4.3.2 Effects of Gln on Alleviating Heat or Cold Stress in Poultry

Global warming threatens livestock and poultry production in every region of the world. Because of its anti-oxidative and anti-inflammatory effects (Wu 2009), Gln has been used as a dietary supplement to alleviate heat stress in animals, including broilers (Table 7.3). For example, Olubodun et al. (2015b) reported that dietary supplementation with 0.5–1% Gln augmented small-intestinal villus height, weight gain, and feed efficiency, while reducing post-hatching mortality in 1- to 42-day-old broilers under hot and humid conditions. Similarly, dietary supplementation with 0.5 or 1% Gln to broilers raised under hot conditions (30–34 °C) enhanced

feed intake, serum insulin concentration, tissue integrity, and body-weight gain (Hu et al. 2016a), while improving the water-holding capacity, moisture, and color of meat (Hu et al. 2016b). Interestingly, Fathi et al. (2015) reported that dietary supplementation with 100 ppm Gln to 1- to 42-day-old broiler chickens with cold-induced ascites reduced the rate of mortality and hepatic injury. Thus, adequate dietary Gln is needed for maximal growth and feed efficiency of poultry, as well as meat quality, especially under heat or cold stress conditions.

7.4.3.3 Effects of Gln on Egg Production and Quality

Gln can improve reproductive function in mammals through multiple cell signaling and anti-oxidative mechanisms (Wu 2018a, b). Interestingly, Dong et al. (2010) reported that dietary supplementation with 0.2, 0.4, and 0.8% Gln to laying hens for 42 days improved the morphology of both the small intestine and oviduct and augmented egg production. Supplementation with 1% Gln to laying guinea fowls for 40 days also increased egg yields, mass, and shell thickness, as well as hatchability, chick birth weight, and feed conversion ratio (Gholipour et al. 2017). Gln acts through enhancing the concentrations of follicle stimulating hormone and luteinizing hormone in serum, which are required for egg formation by birds.

7.4.3.4 Effects of Gln on Alleviating Transportation Stress in Poultry

Transportation stress is associated with elevated levels of glucocorticoids (catabolic hormones) in the plasma of animals, including birds. Shakeri et al. (2016) found that long-distance transportation reduced the villus height of the small intestine, body-weight gain, and feed efficiency in broilers between days 1 and 21 of age, and that these adverse outcomes could be effectively alleviated by dietary supplementation with 1% Gln. It is possible that Gln acts through enhancing expression of anti-oxidative genes, including catalase, superoxide dismutase, and glutathione peroxidase (Shakeri et al. 2016).

7.4.4 Effects of Dietary Supplementation with Glu Plus Gln in Poultry

Like the dietary supplementation with Glu or Gln alone, a mixture of both of them in the crystalline form or as a commercial product AminoGut (containing at least 10% Glu and at least 10% Gln) is beneficial for improving the growth performance and health of poultry (Table 7.4). First, broilers supplemented with 1% Glu + Gln between 1 and 13 days of age or 0.5% Glu + Gln between 15 and 21 days of age showed improvements in their growth performance and feed efficiency on days 21 and 42, respectively, compared with the unsupplemented control group (Manvailer et al. 2015). Second, dietary supplementation with 0.4% Glu plus 0.4% Gln between 1 and 21 days of age enhanced the economic traits of broiler production, as indicated by 14, 10, 16, and 12% improvements in the coefficient of variation in BW, BW uniformity, the coefficient of variation in breast yield, and breast uniformity, respectively (Ribeiro et al. 2015). Third, dietary supplementation with 1% Glu plus 1% Gln for 3 days reduced muscle proteolysis and whole-body oxidative stress, while enhancing muscle insulin signaling, in 22-day-old broilers (Furukawa et al. 2020). Fourth, dietary supplementation with 1% AminoGut between 1 and 21 days of age enhanced BW gain and feed conversion ratio at 22–42 days of age (Zulkifli et al. (2016). Fifth, dietary supplementation with 0.5% AminoGut between 1 and 21 days of age attenuated growth reduction in broilers subjected to heat stress (housed in chambers with 34 °C for 5 h from 11 am to 4 pm per day) between 22 and 42 days of age (Olubodun et al. 2015b). Sixth, intestinal growth and development, as well as the regeneration of the intestinal epithelium, on days 7 and 21 of post-hatching were enhanced in *Eimeria acervulina*-infected broilers after being fed a diet supplemented with 1% AminoGut (Pelicia et al. 2015). Seventh, under hot and humid tropical conditions, dietary supplementation with 0.5% AminoGut between 1 and 21 days of age increased

Table 7.4 Effects of dietary supplementation with either L-glutamate plus L-glutamine or AminoGut on the growth and production of poultry

Poultry	Supplement	Effects	References
Broilers	Basal diet (24.4–18.5% CP, d 1–21) + 0.4% Glu + 0.4% Gln	Breast yield ↑ and Uniformity ↑	Ribeiro et al. (2015)
Broilers (22-d-old; heat	Basal diet (20% CP) + 1% Glu plus 1% Gln	Muscle protein degradation ↓ Whole-body oxidative stress ↓ Insulin signaling in muscle ↑	Furukawa et al. (2020)
Broilers[a] (1–42 d of age)	Basal diet + 0.5 to 1% AG[c]	Weight gain ↑ Feed efficiency ↑ Small intestine villus height ↑ Mortality ↓	Olubodun et al. (2015a)
Broilers[b]	Basal diet (21–18% CP) + 0.5% AG[c], d 1–21)	Growth performance on d 22–42 ↑ Small-intestinal villus ↑ Feed efficiency ↑ Post-hatching mortality ↓	Olubodun et al. (2015b)
Broilers (infected with *Eimeria acervulina*)	Basal diet (20.8–19.4% CP) + 1% AG[c], d 1–21)	Intestinal growth and development ↑ Intestinal damage ↓ Regeneration of the intestinal epithelium ↑	Pelicia et al. (2015)
Broilers (hot and humid, maximum 35 °C)	Basal diet (22–20% CP) + 0.5% AG[a] d 1–21	Serum ovotransferin ↑ Intestinal *Escherichia coli* ↓ Mortality ↓ Foot pad dermatitis ↓	Shakeri et al. (2014)

CP = crude protein; Crtl = control diet; d = day(s); Ig = Immunoglobulin. ↑ = Increase; ↓ = Decrease
[a]Raised under hot and humid conditions
[b]Raised under heat stress conditions (29–35 °C vs. 24 °C)
[c]AminoGut (AG) containing a minimum of 10% L-glutamate and 10% L-glutamine
[d]Room temperature was 5 °C higher than the thermoneutral temperature
[e]Mixed AA = 0.2% Gly, 0.2% Pro, 0.2% Ala, 0.6% Asp, 0.6% Glu, and 0.3% Leu
[f]Mixed NEAA = 0.8% Gly, 0.43% Pro, 0.33% Ala, 1.21% Glu, and 0.99% Asp

the serum concentration of ovotransferin and reduced the mortality rate of broilers by a 6% unit (3.9% vs. 9.9% in the control) (Shakeri et al. 2014). At present, the experimental designs of the published studies do not allow us to assess whether dietary Glu and Gln may have a synergetic effect on intestinal development and whole-body protein deposition in broilers. However, the nutritional and physiological functions of dietary Glu and Gln should not be underestimated. In view of their biochemistry and nutrition (Wu 2021), these two AAs may have a synergistic effect on metabolism, anti-oxidative reactions, and lean tissue gain in poultry.

Based on recent research advances, Wu (2014) recommended the dietary provision of Glu and Gln to chickens during different growth phases. For comparison, values on dietary requirements of laying hens are given by He et al. (2021). In practice, dietary intake of Glu and Gln by poultry should be increased under the conditions of environmental stress, inflammation, diseases, and low protein feeding (Wu 2013). The concept that chickens (including laying

hens) and other avian species have dietary requirements for Glu and Gln is now shaping the practice of poultry production worldwide (Olubodun 2015a, b; Wu 2018b; Zulkifli et al. 2016).

7.5 Safety of Glu and Gln Supplementation in Poultry

7.5.1 An Overall Consideration

Poultry have a high capacity to metabolize Glu and Gln via interorgan cooperation. As a quantitatively major tissue, breast muscle extensively oxidizes Glu and Gln to CO_2 (Wu et al. 1991) and plays an important role in the adaptation of poultry to dietary intakes of Glu and Gln. Note that dietary Glu is extensively degraded by the small intestine during its first pass into the portal circulation, supplemental Glu or MSG has a minimal effect on the circulating level of Glu. This is very different than intraperitoneal, intramuscular, or intra-paraventricular nucleus administration of Glu or MSG to birds.

7.5.2 Safety of Glu Supplementation in Poultry

Poultry have a high capacity to metabolize Glu (including oxidizing Glu to CO_2) via interorgan cooperation. Young chicks can tolerate 10% Glu in diets for at least 2 weeks without any adverse effects on feed intake, growth, or health (Maruyama et al. 1976). Similarly, 8- to 14-day-old chicks fed purified diets containing 10 or 12% Glu exhibited normal rates of feed intake, daily weight gains, and feed efficiency, as compared with age-matched chicks fed a conventional corn- and soybean meal-based diet containing 24.5% CP (Sasse and Baker 1973). Supplementing 1% AminoGut to a typical corn- and soybean meal-based diet for 42 days (Olubodun et al. 2015a) or 1.1% MSG to similar basal diets for 21 days (Ma et al. 2011) has been reported to be safe for broilers. This is also observed for 1- to 21-day-old chickens exposed to heat stress when fed a Glu-supplemented diet (Porto et al. 2015). Furthermore, adding 5.3% Glu to the 18%-CP diets of 1- to 45-day-old broilers did not result in any adverse effects (Bezerra et al. 2016). Finally, dietary supplementation with 2.4% Glu to laying hens fed a 16.3% CP diet for 140 days did not adversely influence their feed intake or egg production, compared with the control group fed a 17% crude protein diet (Bezerra et al. 2015). Thus, a safe level of dietary Glu is at least 12% (on the dry matter basis) for chickens.

7.5.3 Safety of Gln Supplementation in Poultry

Poultry have a high capacity to metabolize Gln via interorgan cooperation. As a quantitatively major tissue, breast muscle extensively oxidizes Gln to CO_2 (Wu et al. 1991) and plays an important role in the adaptation of poultry to dietary intake of Gln. Broiler chickens (from birth to market weight) can tolerate 1% supplemental Gln in diets for at least 42 days without any adverse effects on feed intake, growth, or health (Table 7.3). Similarly, 21- to 42-day-old turkey poults do not exhibit any adverse response to dietary supplementation with 0.7% Gln (Salmanzadeh and Shahryar 2013). Moreover, dietary supplementation with 0.8 or 1% Gln for 40–42 days has no negative effects on laying hens (Dong et al. 2010) or laying guinea fowls (Gholipour et al. 2017). Based on reduced feed intake, reduced weight gain, and abnormal impaired intestinal morphology, broilers do not tolerate dietary supplementation with $\geq 2\%$ Gln on the as-fed basis (90% dry matter in the diet) (Bartell and Batal 2007; Khempaka et al. 2011; Soltan 2009). However, in view of intestinal health, growth performance, and feed efficiency, ducklings can tolerate well dietary supplementation with 2% Gln on the as-fed basis (90% dry matter in the diet) (Zhang et al. 2017). Thus, there is a species difference in the sensitivity of poultry to dietary Gln intake.

7.6 Conclusion

Extensive research over the past 20 years has identified Glu and Gln as abundant AAs in poultry diets and bodies. During the first pass through the chicken small intestine into the portal circulation, dietary Glu is extensively catabolized but dietary Gln undergoes limited degradation. In birds, many tissues (including skeletal muscle, kidneys, and lymphoid organs) have a high capacity to oxidize Gln to CO_2. Thus, the endogenous synthesis of Glu and Gln from BCAAs plays an important role in maintaining Glu and Gln homeostasis in the whole body. Both Glu and Gln are essential for the cellular syntheses of proteins and other nitrogenous substances (including GABA, glutathione, aminosugars) with key metabolic and physiological functions, and, therefore, are crucial for the health, survival, growth, development, lactation, and reproduction of poultry. They are truly functional AAs in avian nutrition. Compelling evidence shows that the adequate provision of Glu and Gln from diets is necessary for the maximum growth, anti-oxidative responses, egg production, and feed efficiency in broilers, ducklings, and laying fowl. Thus, poultry have dietary requirements for both Glu and Gln in the current production systems. Dietary levels of 12% Glu and 3.5% Gln (on the dry matter basis) are safe for short- and long-term feeding of birds.

Acknowledgements This work was supported, in part, by Texas A&M AgriLife Research (H-8200) and Japan Society for the Promotion of Science (JSPS) Core-to-Core Advanced Research Networks Program, entitled "Establishment of International Agricultural Immunology Research-Core for a Quantum Improvement in Food Safety."

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Interorgan Metabolism of Amino Acids in Human Health and Disease

8

Patrick J. Ryan, Steven E. Riechman, James D. Fluckey, and Guoyao Wu

Abstract

Amino acids are integral for human health, influencing an array of physiological processes from gene expression to vasodilation to the immune response. In accordance with this expansive range of unique functions, the tissues of the body engage in a complex interplay of amino acid exchange and metabolism to respond to the organism's dynamic needs for a range of nitrogenous products. Interorgan amino acid metabolism is required for numerous metabolic pathways, including the synthesis of functional amino acids like arginine, glutamate, glutamine, and glycine. This physiological process requires the cooperative handling of amino acids by organs (e.g., the small intestine, skeletal muscle, kidneys, and liver), as well as the complete catabolism of nutritionally essential amino acids such as the BCAAs, with their α-ketoacids shuttled from muscle to liver. These exchanges are made possible by several mechanisms, including organ location, as well as the functional zonation of enzymes and the cell-specific expression of amino acid transporters. The cooperative handling of amino acids between the various organs does not appear to be under the control of any centralized regulation, but is instead influenced by factors such as fluctuations in nutrient availability, hormones, changes associated with development, and altered environmental factors. While the normal function of these pathways is associated with health and homeostasis, affected by physical activity, diet and body composition, dysregulation is observed in numerous disease states, including cardiovascular disease and cancer cachexia, presenting potential avenues for the manipulation of amino acid consumption as part of the therapeutic approach to these conditions in individuals.

Keywords

Amino acids · Diets · Diseases · Exercise · Health · Humans · Supplementation

Abbreviations

BCAA	Branched-chain amino acid
BCKA	Branched-chain α-keto acid
EAA	Nutritionally essential amino acid
GSH	Glutathione
NEAA	Nutritionally non-essential amino acid
NO	Nitric oxide
SNAT	Sodium-dependent neutral amino acid transporter

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1332,
https://doi.org/10.1007/978-3-030-74180-8_8

8.1 Introduction

Amino acids are integral to human nutrition and health. These nitrogen containing compounds are not just the building blocks for proteins, but serve a wide range of roles in numerous physiological processes, including cell signaling, gene expression, DNA synthesis, immune response, and nutrient intake and metabolism (Manjarín et al. 2020; Wu 2009). As such, adequate intake, handling, and exchange of amino acids are critical for proper function of virtually all tissues and organ systems, with the disorders associated with their deprivation also being legion.

Although it was once thought that most cells handled amino acids by similar means, research into amino acid nutrition and biochemistry has revealed quite the opposite (Wu 2013a). Each organ within the body is responsible for carrying out specific tasks that are critical to overall homeostasis, including unique roles in the cooperative metabolism of amino acids. This variability in metabolism gives rise to a sophisticated interplay of amino acid exchange among differentiated tissues, with the characteristic synthesis or degradation of amino acids in some organs supplying the needs of others or the participation of multiple organs in a cohesive pathway to synthesize or degrade nitrogenous compounds (Brosnan 2003; Marliss et al. 1971). These processes have vast and wide-ranging implications for amino acids in health and disease, underlying processes from branched-chain amino acid catabolism to creatine biosynthesis. In light of the importance of interorgan cooperativity regarding amino acid metabolism, this review will summarize the important concepts and current knowledge of interorgan amino acid metabolism in physiology, the means by which these processes are regulated, and how the cooperative handling of amino acids by interorgan transport can be disrupted in various disease states.

8.2 Organ-Specific and Interorgan Amino Acid Metabolism

Since amino acids serve a much wider range of functions than just substrates for protein synthesis, their metabolism within the body is of paramount importance, as evidenced by the wide array of enzymes and metabolons available to handle nitrogenous products (Wu 2013a). Far from the previous view that amino acids were passively taken up by the digestive system and solely metabolized by the liver, it is now well-appreciated that amino acid metabolism varies widely across organs, with tissues degrading, metabolizing, and synthesizing amino acids in varied ways based on characteristic expression of cellular enzymes. This heterogeneous expression of enzymes and distinct pattern of amino acid release gives rise to the coordinated interorgan amino metabolism that is crucial for health and homeostasis (Fig. 1).

(a) Small Intestine

As the principal site of nutrient absorption and a major site of amino acid metabolism, the small intestine plays a critical role that ultimately allows dietary amino acids into the bloodstream, providing the only route (short of intravenous infusion) by which the internal organs are supplied with exogenous amino acids (Beaumont and Blachier 2020). However, it should be noted that the first important exchange of nutrient molecules between two unique cell types is not, in fact, between two different organs, but an inter-species metabolic exchange of numerous compounds between the gut microbiome and the enterocytes of the small intestine (Nicholson et al. 2012). These microorganisms generate a number of compounds as a result of their processing of nutrients that can influence host metabolism; among which are the short-chain and branched-chain fatty acids, choline, phenols, polyamines,

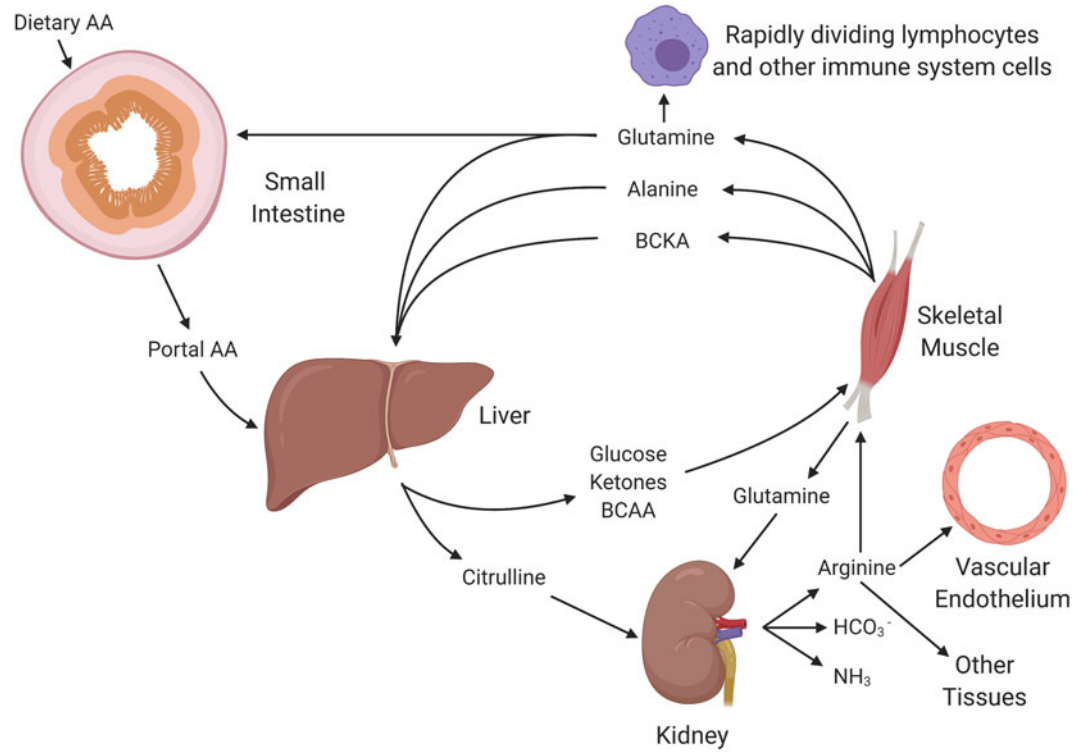

Fig. 1 Interorgan amino acid metabolism. The coordinated and cooperative metabolism and exchange of amino acids and their metabolites is critical to homeostasis and health

and some vitamins, including several of the B-complex vitamins, such as riboflavin (vitamin B_2), biotin (vitamin B_7), folate (vitamin B_9), pyridoxine (vitamin B_6), and cobalamin (vitamin B_{12}) (Nicholson et al. 2012; LeBlanc et al. 2013). While the microbiota in the digestive tract catabolize certain dietary amino acids, thereby affecting the composition of nitrogenous substances absorbed by the gut mucosa, their capacity to supply amino acids to the enterocyte and ultimately to meet the nutritional requirements of the host remains controversial, with some evidence suggesting that the gut microbiota at least contribute lysine to the portal circulation (Dai et al. 2011). However, because non-ruminant species still exhibit deficiencies of amino acid metabolism when fed a protein-free diet, the microbiome is clearly not capable of meeting all of the amino acid demands of the host (Dai et al. 2011). In light of these findings, the contribution of the gut flora to endogenous amino acid concentrations remains an area of active study.

It has been demonstrated, however, that the microbiome influences nitrogen metabolism within the host organism through a less direct means, namely through the production of metabolites from digested amino acids. All of the aforementioned B vitamins play a role in nitrogenous metabolism within the host, including the metabolism of sulfur-containing amino acids, the catabolism of the branched-chain amino acids, and reduction of glutathione, a tripeptide involved in protection against oxidative stress (Stipanuk 2004a; Tong 2013; Ashoori and Saedisomeolia 2014). In this way, it appears that the microbiome, while not directly involved in supplying amino acids to the host, still plays a role in the host's handling of amino acids by supplying the vitamins required for amino acid metabolism.

Although it was once thought that amino acids absorbed by the small intestine entered the portal circulation largely intact, it has now been conclusively demonstrated that this is not the case. Indeed, the enterocytes catabolize nearly all of the dietary glutamate and aspartate taken up from the intestinal lumen, along with roughly two-thirds of the dietary glutamine (Wu 1998). Further, as much as 30–50% of the arginine, methionine, lysine, threonine, glycine, serine, leucine, isoleucine, and valine absorbed in the small intestine are catabolized and unavailable to extraintestinal tissues (Wu 1998). The extensive use of glutamine and glutamate fulfills several functions in the small intestine. Glutamate is the principal energetic substrate for the enterocyte, sparing glucose for use by the rest of the body's tissues and playing a role in gut-brain signaling (Reeds et al. 2000; Brosnan and Brosnan 2013). Glutamine is crucial for many separate processes in the small intestinal mucosa, including maintenance of intestinal integrity via tight junctions and regulation of mucosal cell growth (Krishna Rao 2012; Wang et al. 2015). A key role that both of these amino acids share, however, is serving as a precursor for the de novo synthesis of several critical amino acids produced in the small intestine.

A prime example of the small intestine's role in interorgan amino acid metabolism involves the synthesis of citrulline. The work of Windmueller and Spaeth demonstrated that enterocytes can synthesize citrulline from glutamine, serving as the major source of de novo citrulline synthesis (Windmueller and Spaeth 1981). This citrulline is then transported out of the small intestine into the portal circulation, where it bypasses the liver, before being largely taken up by the kidneys to be used for arginine biosynthesis (Windmueller and Spaeth 1981; Curis et al. 2005). Since this seminal discovery, further investigations have revealed that other amino acids that are catabolized by the small intestine can be used for citrulline synthesis, including proline and glutamate (Wu 1997; Reeds et al. 2000). Because the small intestine is almost the exclusive source of endogenous citrulline in humans, pathologies of the small intestine such as atrophy or resection lead to decrements in citrulline synthesis, ultimately with systemic consequences (Wakabayashi et al. 1994; Crenn et al. 2008).

Unlike other organs, the small intestine generally does not take up amino acids from the circulation (through basolateral transporters) as flux in this direction would be rather at odds with the digestive tract's role in providing the organism with dietary acquired substrate. One exception, however, is glutamine, which is the only amino acid that is both taken up and oxidized by the small intestine in the post-absorptive state (Windmueller and Spaeth 1974; Wu et al. 1994a). This glutamine uptake is made possible through several sodium-dependent cotransporters, the most important being SNAT1, SNAT2, SNAT3, SNAT4, SNAT5, and ATB^0/ASCT2 (Rhoads and Wu 2009). Due in large part to its size, under healthy conditions, the small intestine is one of the principle organs responsible for utilizing plasma glutamine in the post-absorptive state, relying on this amino acid as a fuel source and substrate for continued citrulline synthesis (Watford 2008). The importance of glutamine to intestinal health is further evidenced by studies showing the role of both oral and intravenous infusion of this amino acid in modulating commensal microbiota, innate intestinal immunity, and intestinal integrity (Peng et al. 2004; Ren et al. 2014).

(b) **Liver**

It has been long-established that the liver functions in regulating the circulating levels of substrates such as glucose and amino acids (Hers 1976; Hou et al. 2020), but it should also be noted that it is the first organ that receives blood from the intestinal tract. Therefore, the liver is the first organ exposed to the compounds that the enterocytes release into the circulation, including dietary and synthesized amino acids. With the exception of the branched-chain amino acids (BCAAs), hepatic tissue can catabolize virtually all of the amino acids and is critical for regulating the plasma levels of a number of so-called nutritionally non-essential AA, including aspartate, asparagine, glutamate, glutamine, cysteine,

glycine, serine, and tyrosine from their respective precursors, along with non-proteogenic products such as taurine (Shimomura et al. 2001; Brosnan 2003; Stipanuk 2004b). Taurine provides an illustration of the involvement of the liver in interorgan amino acid metabolism; cysteine, a sulfur-containing amino acid, is critical to protein synthesis in a wide variety of tissues, but is cytotoxic in high concentrations. While cysteine dehydrogenase is expressed in the brain and kidney, its expression (positively regulated by dietary cysteine intake) is the highest in the liver, where it catalyzes the first step of taurine production (Stipanuk 2004b). Thus, the liver serves a two-fold role in cysteine metabolism, both purifying the blood of a potential toxic compound and synthesizing a metabolite with a wide range of functions in health, including immunity and oxidant defense.

There are some notable exceptions to the liver's wide-ranging ability to process amino acids. First are the three aforementioned BCAAs (leucine, isoleucine, and valine), which are largely spared by liver metabolism due to a low level of BCAA transaminase activity (Harper et al. 1984; Brosnan and Brosnan 2006). However, the activity of the branched-chain α-keto acid (BCKA) dehydrogenase complex, the enzyme responsible for handling the product of BCAA transamination and the rate limiting step in overall BCAA catabolism, is much higher in the liver mitochondria than other tissues, making it an important site of oxidation for BCKAs produced elsewhere in the body (Brosnan and Brosnan 2006; Holeček 2018). The non-proteogenic amino acid citrulline also largely bypasses liver metabolism to be converted to arginine in the kidney (Papadia et al. 2018) This is a crucial process, as any arginine taken up by the liver is rapidly catabolized by hepatic type-1 arginase as part of the urea cycle (Curis et al. 2005; Papadia et al. 2018). Production of citrulline from proline, glutamine, or even dietary arginine thus provides a mechanism to spare arginine from liver metabolism, allowing the kidney to resynthesize and supply the rest of the organs with this critical amino acid by resynthesizing it from citrulline.

One of the most critical tasks of the liver is to detoxify the blood of ammonia, the metabolic product of amino acid catabolism. This is achieved via the hepatic urea cycle, characterized by Hans Krebs in the 1930s, where sequential action of several enzymes in the liver mitochondria and cytosol produce urea from ammonia and bicarbonate, utilizing ornithine, citrulline, and arginine as carriers at various stages (Morris 2002). Not only is the urea (or ornithine) cycle the first metabolic cycle discovered in physiology, it also provides a fundamental example of interorgan amino acid metabolism. While intracellular concentrations of ammonia can be relatively high, measurable in the micromolar range, plasma ammonia is quite low in comparison. In humans, elevation of circulating ammonia level from outside the normal range of 20–30 μM is enough to cause vomiting, nausea, and coma, with death occurring at higher concentrations (Wright et al. 2011; Jover-Cobos et al. 2014). In order to keep the plasma levels of ammonia at non-toxic levels, glutamine synthetase in organs such as the brain, kidney, and skeletal muscle can synthesize glutamine from ammonia and glutamate, with the glutamate serving as a carrier of the ammonia molecule (Hakvoort et al. 2017). In this way, extrahepatic tissues can use interorgan cooperativity to shuttle glutamine to the liver mitochondria for oxidation into NH_3 and ultimately into urea, removing a potentially toxic byproduct of amino acid metabolism from the body.

In its position at the center of organism-wide metabolism, one of the principal functions of the liver is to generate glucose (gluconeogenesis) and ketones (ketogenesis) from a number of precursors, including the glucogenic and ketogenic amino acids. These macromolecules are then used as energetic substrates by a wide array of extrahepatic tissues. As the liver largely plays the role of effector in controlling plasma glucose concentration, it obtains a great deal of energy from other sources, so as to "spare" glucose for other organs in the body. This includes amino acids, as up to 50% of the liver's ATP requirement is met by the partial catabolism of amino acids (Brosnan 2000). This catabolism is only partial as the

carbon skeletons procured from breakdown of gluconeogenic amino acids are utilized to produce glucose, thereby providing the liver with necessary ATP while also allowing it to supply other tissues with substrate for ATP production, maintaining glucose availability in the periphery.

The liver also provides an interesting example of functional zonation of amino acid metabolism within an organ. Hepatocytes are divided into two heterogenous groupings: periportal cells, which are associated with the portal vein (90–95% of hepatocytes), and perivenous cells, which are located near the hepatic vein (5–10%). At concentrations lower than 1 mM, extracellular glutamine is converted into glutamate and ammonia by glutaminase in the periportal cells, while the perivenous cells resynthesize glutamine from glutamate and ammonia by the action of glutamine synthase, in an intercellular glutamine–glutamate cycle (Watford 2000). At the cost of 1 mol of ATP per mol of glutamine turnover, this cycle allows the liver to scavenge NH_3 from the plasma, maintaining a low circulating level of ammonia. Further, it allows for NH_3 flux into either urea (under normal pH balance) or glutamine, a neutral amino acid, in order to regulate acid–base balance during conditions that would otherwise lead to acidosis.

Aside from the amino acids, the liver is critical in the interorgan metabolism of several nitrogenous products as well, including glutathione (GSH) and creatine. GSH is a tripeptide composed of glutamate, cysteine, and glycine and serves as the principle intracellular antioxidant in mammalian cells (Lu 2009). While all cell types are capable of synthesizing GSH, the liver is its chief producer and exporter. Plasma content of GSH is rather low when compared to intracellular concentration, largely due to the rapid action of γ-glutamyl transpeptidase on the exterior surface of the liver plasma membrane, which degrades GSH into γ-Glu-$(Cys)_2$. The action of this enzyme plays a key role in interorgan GSH metabolism in two ways. First, degradation of GSH into γ-Glu-$(Cys)_2$ circumvents the extremely unfavorable concentration gradient that exists between extracellular (5–50 μM) and intracellular (0.5–10 mM) GSH, allowing extrahepatic tissues including skeletal and cardiac muscle, the kidneys, and brain, to take up γ-Glu-$(Cys)_2$ and Cys-Gly (derived from $(Cys)_2$) and utilize both these compounds for GSH synthesis. Second, it allows for the transport of cysteine, which at high concentrations can be toxic, by converting into a safe form as γ-Glu-$(Cys)_2$ (Wu et al. 2004). The role of the liver in interorgan creatine metabolism is different, where hepatic cells take up guanidinoacetate released by the kidneys and convert it to creatine via methylation by guanidinoacetate *N*-methyltransferase (Wyss and Kaddurah-Daouk 2000). The newly synthesized creatine is then exported into the plasma, where it is taken up by skeletal muscle (primarily) and other metabolically active tissues such as the brain. Thus, the liver can either serve as the origin for amino acids and related compounds or as part of a more complex network of interorgan cooperation to synthesize or degrade nitrogenous products.

(c) **Kidney**

As mentioned previously, the kidney is not only the primary route for excretion of waste from the body, but is key in the interorgan handling of several amino acids and nitrogenous compounds (Griffith and Meister 1979; Li et al. 2020). Perhaps the best-studied interorgan kidney function is the aforementioned renal-intestinal arginine axis, whereby citrulline released by the small intestine is converted into arginine by the kidney (Dhanakoti et al. 1990; Brosnan and Brosnan 2004). While it may seem circuitous at first glance, the intestinal-renal arginine axis is critical in arginine metabolism, namely by sparing arginine from degradation by hepatic arginase. This mechanism allows for up to 85% of the citrulline released by the intestines to be taken up by the kidney and used for the synthesis of arginine via the sequential action of renal arginosuccinate synthetase and arginosuccinate lyase. This process is critical to whole-body arginine metabolism as arginine production from the kidney amounts to roughly 1.75 g/day of synthesized amino acid in healthy people, and the renal arginine output is sufficient to sustain the needs

of all other tissues, provided there is an adequate supply of citrulline (Brosnan and Brosnan 2004).

One of the main fates of renal arginine is to be used in the synthesis of creatine, which participates in energy metabolism in the brain and skeletal muscle, and also serves as an antioxidant in cells (Wu 2020a). Creatine is synthesized from three amino acids, namely arginine, glycine, and methionine. Approximately 1.7% of the creatine in the body is converted to creatinine per day, a balance that must be replaced by both dietary replenishment of the creatine molecule or its precursors, and by de novo synthesis (Brosnan et al. 2011). Creatine synthesis is initiated in the kidney, where arginine: glycine amidotransferase catalyzes the production of guanidinoacetate and ornithine by transferring the amido group of arginine to the amine group of glycine. Guanidinoacetate is then exported from the kidney, after which it is taken up by the liver, converted into a functional creatine molecule, and released back into the circulation for use by highly metabolically active tissue such as the brain and skeletal muscle. Creatine synthesis, then, adds another layer of complexity upon the interorgan handling of arginine. A representative example of this complexity is citrulline, which is potentially acquired via several distinct catabolic pathways in the small intestine, is released into the portal vein, then bypasses the liver, and is taken up by the kidney. Virtually all the citrulline absorbed by the kidney is then converted to arginine, a significant portion of which is utilized in the synthesis of creatine, a process which must be finished in the liver before the final product can be exported to and utilized by the brain and peripheral tissues (Brosnan et al. 2011). Thus, the integrated cooperation of four distinct organs is required for the de novo production and use of creatine.

(d) **Skeletal Muscle**

The discovery that human skeletal muscles releases a large amount of glutamine (Marliss et al. 1971) led to active research on interorgan metabolism of amino acids over the past 50 years. Although a great deal of the amino acids taken up by the skeletal muscle is incorporated into myofibrillar and other proteins, several enzymatic reactions take place in muscle cells that have important implications for whole-body amino acid metabolism (Wu 2013a). The handling of the BCAAs is a prime example; while they can be catabolized by most other tissues, with the liver being a notable exception, muscle is the major site for transamination of these amino acid in the body, largely due to its size [$\sim$40% and 45% of the body mass in young and adult humans, respectively (Sandoval et al. 2020)]. As such, skeletal muscle is responsible for roughly half of the BCAA catabolism in the organism. A.L. Goldberg and his colleagues were among the first to demonstrate that leucine, isoleucine, and valine can either undergo transamination into their respective branched-chain keto acids (α-ketoisocaproate, α-keto-β-methylvalerate, and α-ketoisovalerate, respectively), with their amino groups being used as a donor to form glutamate from α-ketoglutarate. This glutamate is then either transaminated with pyruvate to produce alanine or amidated to yield glutamine, making muscle a primary source of these amino acids in the post-absorptive state (Odessey and Goldberg 1972; Chang and Goldberg 1978a).

Although the mitochondria and cytoplasm are the primary sites of BCAA transamination in skeletal muscle, the BCKAs produced by this process are not degraded in the muscle due to low levels of BCKA dehydrogenase. Instead, these ketoacids are shuttled to the liver, where hepatic mitochondrial BCKA dehydrogenase decarboxylates the BCKAs to form acyl-CoA, or in the case of 5–10% of the α-ketoisocaproate, β-hydroxy-β-methylbutyrate (HMB). HMB can be used in the liver as a precursor of acetoacetate for cholesterol synthesis or released from the liver where it has a number of effects in other tissues, including skeletal muscle, where it stimulates protein synthesis and muscle growth (Nissen and Abumrad 1997; Zanchi et al. 2011). Thus, the cooperative action of the muscle and liver is critical not only to catabolize the BCAAs, but to the metabolism and function of both these organs (Fig. 2).

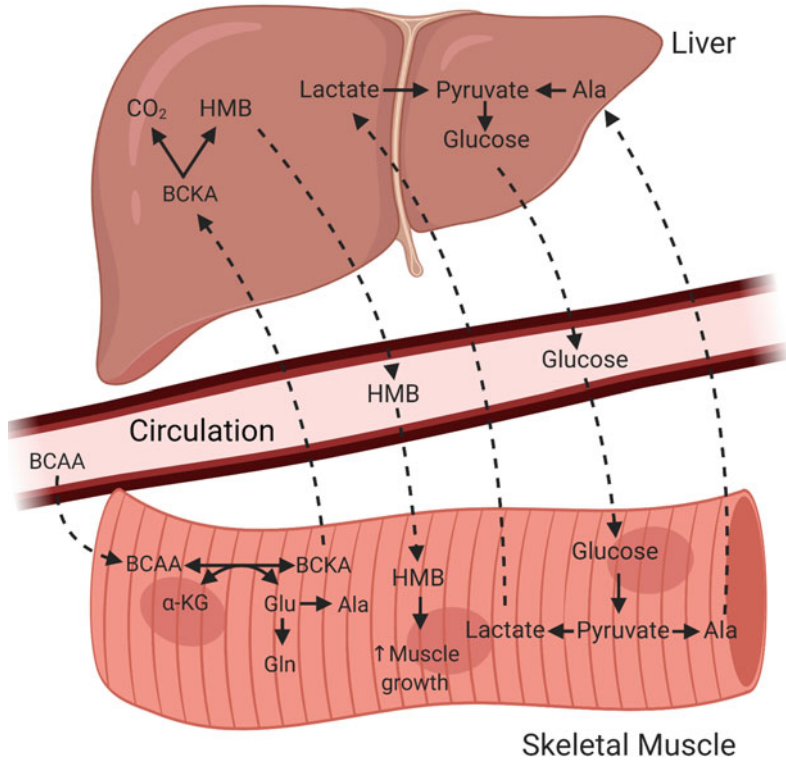

Fig. 2 Cooperative AA Metabolism Between Liver and Muscle. The liver and skeletal muscle engage in multiple interorgan amino acid exchanges, including: the cooperative metabolism of the branched chain amino acids (BCAA) into branched chain keto acids (BCKA) and ultimately β-hydroxy-β-methylbutyrate (HMB) and the glucose-alanine (Cahill) cycle, which allows for clearance of muscle pyruvate and transport of ammonia to the liver using alanine as a carrier

Beyond BCAA oxidation, the skeletal muscle and liver engage in other forms of interorgan amino acid metabolism. Based on its metabolic activity and fiber type, skeletal muscle can either catabolize glucose into pyruvate for oxidation in the Krebs cycle in the mitochondria or into lactate, which can then be released into the circulation and taken up and converted to pyruvate by more oxidative muscle fibers, or circulated to the liver where it is utilized for gluconeogenesis in the Cori cycle. A similar phenomenon occurs in the glucose-alanine (the Cahill cycle), where the muscle uses blood glucose to produce ATP and pyruvate, with the pyruvate being transaminated with glutamate to form alanine (Felig 1973; Chang and Goldberg 1978b). This alanine is then converted to glucose and urea in the liver. Although there is no net production of glucose, the glucose-alanine cycle performs several important functions, including transporting ammonia to the liver in a non-toxic form, removing pyruvate from active skeletal muscle to allow continued flux through the mitochondrial electron transport chain and effectively converting muscle glycogen (which cannot be directly exported from muscle) to glucose in the liver via the recycling of end-products of glycolysis (Sarabhai and Roden 2019). This is physiologically significant for converting muscle glycogen into glucose in the body, because skeletal muscle lacks glucose-6-phosphatase.

(e) Other Organs

While the tissues listed above are the principal components of interorgan cooperativity in amino

acid handling, other cell types [e.g., endothelial cells (Durante 2020), as well as cells in the brain (He and Wu 2020), lungs (Chen et al. 2020), endocrine organs (Flynn et al. 2020), the reproductive tract (Gao 2020), the immune system (Ren et al. 2020), the skin (Solano 2020), and other sense organs (Wu 2020b)] are involved in specialized patterns of amino acid metabolism as well. The glutamine–glutamate shuttle in the brain is one interesting examples of interorgan amino acid handling by different cell types within the same tissue. Glutamate is a well-documented excitatory neurotransmitter in the brain, as well as a potential source for anaplerotic replenishment of tricarboxylic acid cycle intermediates (Meldrum 2000; Schousboe et al. 2014). After being released into the synaptic cleft by neurons, it can be rapidly taken up by neighboring astrocytes, where cell-specific glutamine synthetase converts the glutamate into glutamine. This glutamine is then transported back to the neuron, where neuronal phosphate-activated glutaminase converts it back to glutamate, where it can again be utilized as a neurotransmitter (Bélanger et al. 2011). The glutamine–glutamate shuttle, then, serves multiple functions, namely, to clear the synaptic cleft of an excitatory glutamate, preventing excitotoxicity, while also replenishing a critical neurotransmitter.

As the negative consequences of protein deprivation on the immune system have been well-documented, it should come as no surprise that a wide array of amino acids are used in immune cell function. Of these, the interorgan fluxes of glutamine and arginine are perhaps the most important (Li et al. 2007; Grohmann and Bronte 2010). As mentioned previously, skeletal muscle is the major source of the glutamine in the body, the bulk of which is produced through BCAA transamination. Lymphocytes, macrophages, and neutrophils oxidize glutamine in a process termed *glutaminolysis* to yield glutamate, and lesser amounts of aspartate, alanine, lactate, and pyruvate, providing these cells with substrate for nucleotide synthesis, GSH production, and nitric oxide (NO) generation (Ganeshan and Chawla 2014; Arts et al. 2016). The production of NO is reliant on the degradation of arginine to citrulline by NO synthase and important for immunity. As such, there is evidence to suggest that high concentrations of arginine (2 mM) may be needed for the maximum synthesis of NO by lymphocytes, macrophages, and monocytes (Wu et al. 2009). The demand of immune cells for glutamine and arginine, therefore, can put added stress on skeletal muscle and the kidneys, which are the main endogenous sources of these amino acids, respectively. While under conditions of eustress, muscle and renal output of these products is sufficient to meet interorgan demand, but under more stressful conditions, the supplementation of glutamine and arginine in the diet may be required to sustain a maximal immune response (Wu et al. 2009; Tan et al. 2009).

Finally, lactating tissue provides an interesting perspective on amino acid metabolism (Kim and Wu 2009; Wu et al. 2018). While virtually every other organ in the body is dedicated to preserving the health and homeostasis of the organism, the mammary glands are unique in that, and their role is to support the development of the offspring utilizing the resources of the mother. Thus, the role of the lactating tissue in supplying glutamine, proline, glycine, 4-hydroxyproline, and many more amino acids and proteins may be more accurately termed an "inter-organismal" amino acid metabolism, as this milk is the only source of nutrients for early neonatal growth (Li and Wu 2018; Wu et al. 2019). In order to support both milk production and to ensure an adequate amount of nutritive support to the neonate, the mammary glands actively degrade an extensive number of amino acids, including arginine and BCAAs (Kim and Wu 2009). This arginine serves multiple purposes promoting increased blood flow to the mammary tissue via NO production and providing polyamines that the neonate will use for growth, and proline that can later be converted back to arginine in the neonate's small intestine, in an arginine–proline cycle (Wu et al. 2011).

8.3 Regulation and Control of Interorgan Amino Acid Metabolism

Despite decades of investigation into the cooperative handling of amino acids between organs, a centralized control mechanism for interorgan amino acid availability has yet to be identified. Instead, the handling of amino acids in various tissues is affected by a range of inputs, including nutrient availability, changes across the developmental span, hormones, and environmental factors. Although they are not under any apparent central control, the integrated handling of amino acids among the various systems of the body in response to these various perturbations serves to reinforce the importance of interorgan amino acid metabolism.

Nutrient Availability

Perhaps the only constant affecting all organs involved in amino acid trafficking is substrate availability. Both animal- and plant-source foods contain large amounts of BCAAs, glutamate, and glutamine, whereas the content of proline and glycine is much lower in plant- than in animal-sourced foods (Hou et al. 2019; Li and Wu 2020). Although many amino acids can be synthesized within the animal (the so-called nutritionally non-essential amino acids, NEAA), a number of them cannot, and so must be supplied exogenously (the nutritionally essential amino acids, EAA). For instance, the three BCAAs cannot be synthesized de novo by any organ in the human body, and so must be replenished from the diet or risk perturbations in the downstream signaling pathways they participate in, including skeletal muscle and liver metabolism (Brosnan and Brosnan 2006). On the other hand, while arginine can be produced de novo, the kidney is entirely dependent upon the release of citrulline from the small intestine for arginine synthesis; if this supply of citrulline is disrupted, renal arginine production will be severely reduced (Brosnan and Brosnan 2004). In this instance, the categorization of amino acids as either essential or non-essential can be somewhat a misnomer. For example, because arginine is synthesized from citrulline in the kidney and some other tissues, it has largely been categorized as an NEAA. However, under several pathological conditions, such as metabolic disease, endothelial dysfunction, and wound healing, endogenous arginine synthesis is insufficient to match physiological demand, and as such must be provided through the diet (Wu et al. 2009). This has led to the development of the category of "functional amino acids," those that the organism is capable of producing, but are needed in greater quantities in times of stress or altered growth in order to support interorgan amino acid metabolism (Wu 2013b).

Development

As discussed previously, the characteristic expression of enzymes in a tissue is what gives rise to diverging abilities to process and metabolize amino acids. However, even within a cell type, this expression can change based on developmental status or external stressors. This phenomenon is readily apparent in shifts in porcine enterocyte enzyme expression after birth. Neonates are entirely dependent upon their mother's milk for nutrients, which, as discussed previously, is rich in proline and glutamine, but remarkably deficient in arginine (Wu et al. 1994b; Wu 1995). As such, the enterocytes of baby pigs express an armamentarium of enzymes capable of synthesizing arginine from proline and glutamine, including P5C synthase, carbamoyl phosphate synthase, and arginosuccinate lyase. This allows the intestine of the pig to sustain its need for arginine during rapid post-partum growth, at least until renal arginosuccinate lyase can be expressed and the renal arginine-citrulline axis can be established. After early growth, the expression of these enzymes in the enterocyte wanes, and arginine production from citrulline is largely taken over by the kidney.

Endocrine

There should be little surprise that the array of hormones involved in controlling the body's physiological processes will also have an effect on amino acid metabolism. As hormones serve to integrate and coordinate the response of tissues, their effects on interorgan amino acid metabolism are generally in line with their overall physiological functions. For example, insulin governs a wide range of cellular reactions to feeding, including the regulation of protein synthesis, and system A amino acid uptake (Pause et al. 1994; Hyde et al. 2002), in addition to its role in facilitating glucose disposal. Growth hormone has similar effects (Møller and Jørgensen 2009). In contrast, stress hormones such as glucocorticoids attenuate protein synthesis in peripheral tissues, increasing the availability of amino acids for release to the circulation and use for gluconeogenesis in the liver, while also increasing BCKA dehydrogenase expression to facilitate the use of the BCAAs as energetic substrates (Shah et al. 2000; Rose and Herzig 2013). Glucagon acts in a similar fashion, promoting hepatic uptake of amino acids. At first glance, it may seem odd that insulin and glucagon have the same effective effect on amino acid availability, namely the uptake of plasma amino acids by tissues (Flakoll et al. 1994). However, an organ-specific action of these hormones explains their functional difference; insulin is responsible for the deposition of amino acids as proteins in the periphery, namely the skeletal muscle, while glucagon promotes hepatic uptake of amino acids for use in gluconeogenesis. In this way, these two hormones balance the flow of amino acids in response to nutrient and endocrine signals.

Environment

Changes in an organism's environment can also lead to alterations and compensations in interorgan amino acid metabolism. For example, under conditions of chronic acidosis, plasma glutamine is increased by up to two-fold, primarily due to skeletal muscle protein breakdown and consequent glutamine release (Taylor and Curthoys 2004). While the kidney does not normally filter large amounts of glutamine, under prolonged acidosis, up to 20% of the glutamine in the plasma is filtered into the lumen of the nephron, where much of it is absorbed into the proximal tubule. This glutamine is then utilized by the tubule epithelial cells to carry out gluconeogenesis via mitochondrial glutaminase and glutamine dehydrogenase, and cytosolic phosphoenolpyruvate carboxylase, the expression of which, is induced by acidosis (Welbourne 1987). This gluconeogenesis has the benefit of producing two molecules of bicarbonate, which are then released back into the plasma, restoring pH balance.

Another perturbation that alters amino acid metabolism is physical exercise. Along with the changes in energetics that come with muscular contraction (resulting in increased pyruvate availability for alanine synthesis), vasodilation of the muscle vasculature (mediated largely by NO derived from arginine) results in increased blood flow and therefore amino acid delivery to muscle (Wagenmakers 1998). As a consequence, muscular transamination of BCAAs, as well as alanine and glutamine release, increases during exercise. Further, exercise results in reductions in liver amino acid output, increased glucogenesis and ketogenesis (at higher intensities and durations), and increases in the expression of the hepatic BCKA dehydrogenase enzyme (Shimomura et al. 2004). These changes have been observed to be independent of the increased concentration of amino acids from the muscle, indicating that physical exercise may play a role in regulating interorgan amino acid metabolism independently of changes in amino acid flux (Argilés et al. 2016).

8.4 Health and Disease

The contribution of each organ to its role in interorgan amino acid metabolism is crucial to homeostatic metabolism and physiology. As may be expected, disruption in these and other carefully balanced processes carries severe implications for health which manifest with a number of pathological conditions.

a. **Cachexia**

Dysregulation in metabolic activity and accelerated, unchecked cell growth are at the core of cancer pathology, so it is not surprising that amino acids plays a large role in cancer cell metabolism. As cancer cells are undergoing rapid growth and division, they require a proportionate increase in substrates to sustain these processes. Glutamine serves this purpose by serving as a substrate for protein and polyamine synthesis, as well as conversion into other amino acids, while also being used as an important energetic and anaplerotic substrates (DeBerardinis and Cheng 2010). Cancers in later stages of growth also have a high demand for arginine as a precursor for protein and nucleotide synthesis, although in earlier stages of development, arginine can be useful for halting tumor growth (Wu et al. 2009).

This demand for glutamine has consequences for tissues distinct from the tumor and its tissue of origin, namely the skeletal muscle (Fig. 3). One of the most devastating consequences of cancer pathology is skeletal muscle cachexia, a wasting disease characterized by severe reductions in skeletal muscle mass, resulting in severe decrements in quality of life, and ultimately serving as the cause of death in 1/3rd of cancer cases (Tisdale 2009). As the muscle is the major source for endogenously produced glutamine, the high demand for this amino acid is primarily met by protein degradation in striated tissue, leading to extensive proteolysis and atrophy as the tumor continues to grow and demand more energetic substrates (Parry-Billings et al. 1991). Experiments in rats have demonstrated that glutamine supplementation can halt muscle atrophy and improve markers of health in cancer models (Yoshida et al. 2001). Another demand upon skeletal muscle may in part be due to the nature of cancer metabolism: As described by Otto Warburg, cancer cells rely extensively on non-oxidative glycolysis, producing a large amount of lactic acid as a consequence of their elevated metabolic rate. In order to adjust to this chronic acidosis, the kidney requires glutamine to produce bicarbonate and restore pH balance (Drochioiu 2008; Tisdale 2009). In support of this idea, it should be noted that cachexia is not exclusive to cancer, but can also be seen in conditions such as diabetes and chronic kidney disease, both of which result in metabolic acidosis. There are reports that dietary supplementation with arginine, glutamine, BCAAs, or a mixture of casein and whey protein is beneficial for well-being in cancer patients (Table 8.1).

b. **Obesity and Diabetes**

Also not surprising, interorgan amino acid handling can be disrupted in metabolic disease. As hyperinsulinemia is concomitant with obesity and metabolic syndrome, alterations in the processes that insulin regulates occur as well, such as irregularities in BCAA metabolism. Because insulin suppresses the activity of BCKA dehydrogenase in the liver, which is the rate limiting step in BCAA oxidation, chronically high levels of insulin will suppress this activity further, leading to elevated plasma levels of the BCAAs and their metabolites as their degradation cannot be completed (Adams 2011; Lynch and Adams 2014). It is currently not known whether this increase in BCAA concentrations is causal of insulin resistance and metabolic disturbance or a consequence, as BCAAs have also been demonstrated to have beneficial effects on liver and skeletal muscle metabolism. In contrast, plasma levels of other amino acids, particularly arginine and glucogenic amino acids (such as glutamine and aspartate) are decreased in obese and diabetic animals (Wijekoon et al. 2004; Gancheva et al. 2018). The decrease in the content of these amino acids likely arises from several mechanisms, including increased glutamine uptake by the small intestine and kidneys. Interestingly, both supplementation with glutamine and arginine show promise for the treatment of type-2 diabetes (Table 8.2), obesity (Table 8.3), and other related metabolic problems (Table 8.4).

c. **Liver Failure**

As it lies at the center of so many metabolic processes, disorders of the liver, whether due to

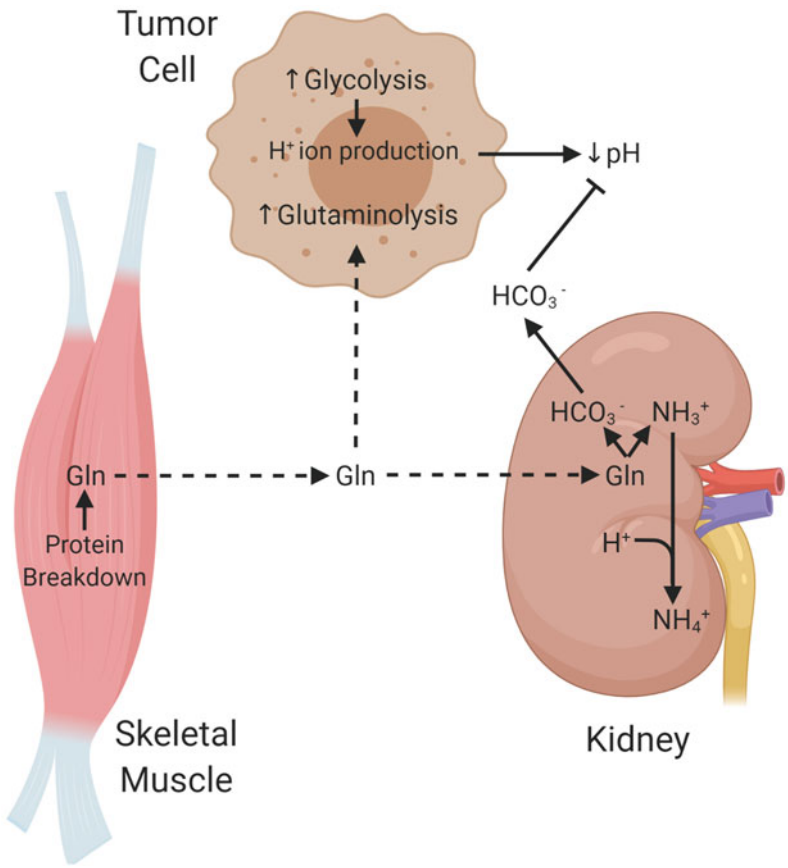

Fig. 3 Glutamine metabolism in cancer cachexia. Rapidly-dividing tumor cells have drastic consequences for interorgan amino acid metabolism. Chief among these is glutamine, which is required both by the cancer cell for energy and growth, and by the kidney, which must balance the reduced pH brought about by metabolically active cancers. As the skeletal muscle is one of the chief suppliers of glutamine, these demands can lead to severe protein breakdown in the muscle, resulting in the loss of mass and strength that is characteristic of cancer cachexia

cirrhosis or steatosis, pose major problems for amino acid metabolism. In contrast to complications from obesity, liver disease results in reduced levels of circulating BCAAs, while the concentration of circulating aromatic amino acids increases (Morgan et al. 1982). In this case, the perturbation to BCAA metabolism does not occur in the liver, although it does arise there. Because damage to liver tissue reduces the amount of functional tissue capable of urea synthesis, other tissues must compensate to detoxify circulating ammonia. Based on its prevalence throughout the body and ability to detoxify ammonia through glutamine synthesis, skeletal muscle is key in adapting to altered ammonia metabolism (Wright et al. 2011). One of the consequences of liver failure is hyperammonemia due to the reduction in ureagenesis to remove ammonia from the blood. As a result, the skeletal muscle responds by increasing ammonia uptake and transamination of BCAAs and ammonia into glutamine, a strategy which can help stabilize plasma ammonia levels in patients with adequate muscle mass, but becomes untenable with muscle wasting, and further deprives other tissues of BCAAs. The kidney also

Table 8.1 Effects of amino acid supplementation on humans and animals with cancer

Supplement	Effect	Model Organism	Duration	Dosage
Arginine, Glutamine, β-hydroxy-methyl-butyrate (HMB)	Increased body weight and fat-free mass (May et al. 2002)	Patients with advanced (stage IV) solid state tumors	24 weeks	14 g arginine, 14 g glutamine, 3 g HMB per day
BCAA (Muto et al. 2005)	Reduced risk of liver cancer (Muto et al. 2005)	Men and women with decompensated cirrhosis	2 years	4 g BCAAs (952 mg isoleucine, 1904 mg leucine, 1144 mg valine) 3x/day
Carnitine	Weight gain and improvement of global health status (Kraft et al. 2012)	Men and women with pancreatic cancer	12 weeks	4 g/day
Glutamine	Maintenance of gut integrity in patients undergoing radiochemotherapy (Yoshida et al. 2001) Decreased postoperative complications and hospital stay after colorectal surgery (Oguz et al. 2007)	Esophageal cancer patients Men and women diagnosed with colorectal cancer	4 weeks At least 5 days pre and postsurgery	30 g/d 1 g/kg/day
Histidine	Reduced tumor (leukemia) size in combination with methotrexate treatment (Kanarek et al. 2018)	Severe combined immunodeficient mice	5 days	400 μl of 46 mg/ml histidine solution
Whey protein	Increased body weight, fat-free mass, and reduced chemotherapy toxicity (Cereda et al. 2019)	Advanced cancer patients candidate to or receiving chemotherapy	3 months	20 g/day
Whey and casein protein enriched with 10% leucine	Increased muscle protein synthesis (Deutz et al. 2011)	Male cancer patients	Immediate response (5 h)	11.9 g whey, 24.2 g casein, 4.16 g leucine

attempts to respond by reducing glutamine release into the blood and increasing ammonia excretion in urine (Wright et al. 2011).

d. **Traumatic Injuries**

A large body of literature supports the idea that amino acids are crucial to recovery from various forms of exogenous trauma. The main pattern of altered amino acid metabolism after a major injury is similar across various types of trauma, whether blunt trauma or burns: skeletal muscle proteolysis and increased BCAA catabolism, followed by the release of glutamine and alanine (Wilmore 2001; Herndon and Tompkins 2004). Evidence also suggests that arginine is critical to the wound healing process. These amino acids must again largely be supplied by the skeletal muscle and kidney, although exogenous supplementation may also be necessary to meet metabolic demands (Wu et al. 2009).

8.5 Conclusions and Future Directions

Based on the significant cellular infrastructure that has evolved to regulate them, amino acids, their metabolites, and the pathways that regulate them are a clearly critical component of human

Table 8.2 Effects of amino acid supplementation on humans and animals with type 2 diabetes

Supplement	Effect	Model Organism	Duration	Dosage
Arginine	Reduced body mass, fat mass, fasting glucose, and triglycerides; increased nitric oxide production (Fu et al. 2005) Decreased fat mass and waist circumference with maintenance of fat-free mass, reduced plasma insulin, fructosamine, and triglycerides, improved endothelial function (in conjunction with exercise and hypocaloric diet) (Lucotti et al. 2006) Decreased fat mass and waist circumference with maintenance of fat-free mass, improved glucose tolerance (Monti et al. 2012)	Zucker diabetic fatty rat Obese type 2 diabetic subjects Human patients with metabolic syndrome and risk of type 2 diabetes	10 weeks 3 weeks 18 months	1.25% arginine in drinking water 8.3 g/day 6.4 g/day
Arginine, Glutamine, β-hydroxy-methyl-butyrate	Improved wound healing (Sipahi et al. 2013)	Type 2 diabetic humans	4 weeks	7.4 g arginine, 7.4 g glutamine, 1.3 g HMB given twice per day
BCAA	Reduced HbA1c, with no effect on body weight or adiposity (Guo et al. 2010)	NONcNZO10/LtJ (RCS10) and B6.Cg-A^y/J (A^y) mice	8 months	1.5% leucine in drinking water
Carnosine	Reduced serum triglycerides and stabilized atherosclerotic plaques (Brown et al. 2014)	Streptozotocin-induced diabetic apoE$^{-/-}$ mice	20 weeks	2 g/L in drinking water
Cysteine + Glycine	Increased glutathione concentration and reduction in plasma markers of oxidative stress (Sekhar et al. 2011)	Uncontrolled type 2 diabetic humans (HbA1c: 8–10%)	2 weeks	0.81 mmol/kg/day cysteine (given as *n*-acetylcysteine) 1.33 mmol/kg/day glycine
Glutamine	Reduced HBA1C, fasting blood glucose, and body fat (Mansour et al. 2015) Reduced HbA1c and fructosamine (Samocha-Bonet et al. 2014)	Type 2 diabetic humans Type 2 diabetic humans	6 weeks 4 weeks	30 g/day 30 g/day
Histidine	Decreased waist circumference and fat mass, reduced HOMA-IR and inflammatory markers (Feng et al. 2013)	Obese women with metabolic syndrome	12 weeks	4 g/day
Taurine	Reduced glycemia and mortality rate (Di Leo et al. 2004) Ameliorated diabetic retinopathy (Yu et al. 2008)	Streptozotocin-induced diabetic rats Streptozotocin-induced diabetic rats	2 years 12 weeks	5% w/w enriched taurine diet 1.2% w/w enriched taurine diet

Table 8.3 Effects of amino acid or protein supplementation on humans and animals with obesity

Supplement	Effect	Model Organism	Duration	Dosage
Arginine	Decreased body weight and fat mass, increased muscle mass, reduced plasma glucose, triglycerides, and leptin (Jobgen et al. 2009) Decreased body weight, waist circumference, BMI, and waist-to-hip ratio (Hurt et al. 2014)	Diet induced obese rats (Sprague–Dawley) Obese women	12 weeks 12 weeks	1.51% arginine in drinking water 3 g arginine 3x/day
Carnosine	Normalized adipokine content (Baye et al. 2018)	Overweight and obese patients	12 weeks	1 g/ 2 × per day
Glutamine	Decreased body weight and waist circumference (Laviano et al. 2014) Decreased body weight and reduced plasma insulin and glucose (Opara et al. 1996)	Obese and overweight women C57BL/6 J mice (high fat diet),	4 weeks 5 weeks	0.5 g/kg body weight/day 2.87% of total energy consumed
Histidine	Reduced inflammation (Sun et al. 2014)	High fat diet induced obese rats (Sprague–Dawley)	4 weeks	0.375 g/kg body weight/day (low dose) 1.975 g/kg body weight/day (high dose)
Taurine	Reduced inflammation (C-reactive protein and TBARS) (Rosa et al. 2014)	Obese women	8 weeks	3 g/day
Whey Protein	Decreased body weight and fat mass, reduced plasma ghrelin and insulin (Baer et al. 2011) Reduced blood triglycerides, total cholesterol, LDL, insulin, and HOMA-IR (Pal et al. 2010)	Obese men and women Overweight and obese men and women	23 weeks 12 weeks	See reference for amino acid composition 27 g whey protein 2x/day

physiology and health. As our understanding of the importance of amino acids to cellular processes has evolved, it has become clear that these nitrogenous compounds serve as more than being substrates for the synthesis of proteins. Rather, amino acids participate in virtually every process in the cell and organism, from energy metabolism and cell growth to the regulation of blood flow. As this knowledge of their ubiquity in cell physiology has progressed, so too has the appreciation for how amino acids are handled in the body. To date, it is clear that interorgan cooperation is both crucial for maintaining the availability and metabolism of amino acids and the processes that utilize them. This realization has proven beneficial not only in the basic science of nutrition, but has led to advances in clinical medicine and therapeutics for a variety of diseases and disorders. The future of research in interorgan amino acid metabolism looks to be just as exciting, with developments and discoveries of new knowledge about how amino acids flow between organs in health and disease, opening new avenues in the sciences of nutrition, supplementation, and medicine.

Acknowledgement All figures made with Biorender.com.

Table 8.4 Effects of amino acid or protein supplementation on humans and animals with vascular disease

Supplement	Effect	Model Organism	Duration	Dosage
Arginine	Decreased body weight, waist circumference, and systolic blood pressure (decreased diastolic blood pressure only observed in high arginine group), reduced HbA1c and serum triglycerides (only in high arginine group) (Dashtabi et al. 2015) Reduced blood pressure (meta-analysis) (Dong et al. 2011)	Obese humans Healthy and diseased patients	8 weeks 2–24 weeks	1 g or 2 g of arginine 3x/day 4–24 g/day
β-Alanine and Histidine	Increased functional capacity and strength (combined with exercise training) (Stefani et al. 2020)	Male Wistar rats subject to chronic heart failure	8 weeks	250 mg/kg/day
BCAAs	Increased life span, cardiac and skeletal muscle biogenesis, improved exercise performance, and reduced reactive oxygen species production (D'Antona et al. 2010)	Male B6.129S2 mice	3 months (exercise performance and ROS study)	1.5 mg/g body weight/day
Whey protein	Decreased blood pressure and improved vascular function (Fekete et al. 2016)	Healthy men and women	8 weeks	28 g whey protein 2x/day
Whey or soy protein	Reduced systolic blood pressure (He et al. 2011)	Prehypertensive or stage 1 hypertensive men and women	8 weeks	40 g/d whey or soy protein

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9 Amino Acids and Their Metabolites for Improving Human Exercising Performance

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Abstract

Achieving adequate nutrition for exercising humans is especially important for improving both muscle mass and metabolic health. One of the most common misunderstandings in the fitness industry is that the human body has requirements for dietary whole protein and that exercising individuals must consume only whole protein to meet their physiological needs. This view, however, is incorrect. Instead, humans at rest or during exercise have requirements for dietary amino acids (AAs), and dietary protein is a source of AAs in the body. The requirements for AAs must be met each day to avoid a negative nitrogen balance in individuals with moderate or intense physical activity. By properly meeting increased requirements for AAs through increased intake of high-quality protein (the source of AAs) plus supplemental AAs, athletes can improve their overall athletic performance. AAs or metabolites that are of special importance for exercising individuals include arginine, branched-chain AAs, creatine, glycine, taurine, and glutamine. The AAs play vital roles as both substrates for protein synthesis and molecules for regulating blood flow and nutrient metabolism. The functional roles of AAs include the maintenance of cell and tissue integrity; stimulation of mechanistic target of rapamycin and AMP-activated protein kinase cell signaling pathways; energy sources for the small intestine, cells of the immune system, and skeletal muscle; antioxidant and anti-inflammatory reactions; production of neurotransmitters; modulation of acid–base balance in the body. All of those roles are crucial for the overall goal of improving exercise performance. Therefore, adequate intakes of proteinogenic AAs and their functional metabolites, especially those noted in this review, are essential for optimal human health (including optimum muscle mass and function) and should be a primary goal of exercising individuals.

Keywords

Skeletal muscle · Exercise nutrition · Amino acids

Abbreviations

AA	Amino acid
AMPK	AMP-activated protein kinase
BCAA	Branched-chain amino acid
MTOR	Mechanistic target of rapamycin
NO	Nitric oxide

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1332,
https://doi.org/10.1007/978-3-030-74180-8_9

9.1 Introduction

Amino acids (AAs) are organic substances that contain both amino and acid groups (Wu 2013). Those AAs that are substrates for intracellular protein synthesis are called proteinogenic AAs. In humans, proteins (large polymers of AAs linked via peptide bonds) fulfill both structural (e.g., cytoskeletal, membrane and extracellular matrix components) and non-structural (e.g., transport, biochemical reactions, and gene expression) functions. Plant- and animal-source foods contain proteins, small peptides, and free AAs (AAs that are not present in the peptide form), but differ in their composition of AAs (Hou et al. 2019; Li et al. 2011) and protein quality (Moughan 2003). Animal proteins generally have higher digestibility coefficients and biological values than plant proteins. As for all animals, humans have daily nutrient requirements that must be met in order to survive and thrive. Humans have daily basal requirements for AAs in their diets that must be met through either the diet or de novo synthesis in the body (Wu 2013). Often, even if an individual has the ability to synthesize a specific AA from appropriate precursors, the AA may not be synthesized in adequate amounts to meet daily requirements for optimal health. In such cases, these AAs must be provided in the form of dietary protein or as a dietary supplement (Hou et al. 2015). Arginine, glutamine, glycine, and taurine are the prototypes in human nutrition (McNeal et al. 2018; Wang et al. 2013; Wu 2020).

There is a common misconception, especially within fitness communities, that humans must meet a "protein requirement" each day, and this is commonly referred to by athletes as "hitting macro targets." Humans do not have a requirement for whole protein, as whole protein has no nutritive value until it is hydrolyzed in the gastrointestinal tract into its constituent AAs (Reeds et al. 2000; Wu 2016; Young 1987). When whole protein is digested, the resulting tripeptides, dipeptides, and free AAs are absorbed through apical membrane transporters (peptide transporter-1 and individual AA transporters) into the enterocytes (columnar absorptive epithelial cells) of the small intestine. Within those cells, tripeptides and dipeptides are hydrolyzed by peptidases into free AAs, so that the peptides have nutritive value in the body. Those absorbed AAs that are not catabolized by the enterocytes are released from their basolateral membrane into the blood stream (Wu 2018). Once in the bloodstream, the individual AAs take on a variety of functions. Some AAs (e.g., arginine, glycine, and glutamine) play a greater role than simply contributing to increasing muscle protein mass because they have a regulatory capacity (such as stimulating protein synthesis and inhibiting proteolysis in skeletal muscle, as well as reducing obesity) (Jobgen et al. 2009a; Manjarín et al. 2020; Simmons et al. 2020). Non-proteinogenic AAs, such as taurine, citrulline, and ornithine fulfill essential functions (e.g., anti-oxidative, metabolic, and regulatory roles in the body) (Hou and Wu 2017; Wu 2013). For example, taurine is essential for the structure, integrity, and function of the eyes of humans (Wright et al. 1986), and dietary taurine plays an important role in whole-body energy homeostasis to exert an anti-obesity effect in mammals (Kim et al. 2019). Requirements for AAs must be met for optimal health and optimal exercise performance in humans.

In athletes, or any exercising individual, the need for replenishing AAs each day is much greater than for a sedentary individual (Simmons et al. 2016). This is because muscle protein in exercising individuals is being broken down, and AAs are being oxidized, at a much higher rate than in a sedentary person (Rennie et al. 1981). In highly active individuals, supplementation with a high-quality protein (such as whey or casein), crystalline free AAs, or both is paramount for meeting daily AA requirements. Meeting those requirements is of great benefit to the individual, as nutritional status can directly affect exercise performance and the recovery of skeletal muscle following strenuous activity (McGlory and Phillips 2016). Therefore, for an exercising individual, it is paramount to know the effects of functional AAs, and not just those that contribute directly to increasing muscle protein mass, to more effectively improve well-being and exercise performance.

9.2 Exercise Training and Protein Supplementation

Exercise involves the physical contraction of skeletal muscles, which are attached to bones through tendons (connective tissue). Skeletal muscle (made up of multinucleated myocytes) moves and supports the skeleton and produces force. There are 640 known skeletal muscles in humans. During exercise, muscle protein catabolism and muscle pain occur at a greater rate compared to that when resting (Byrne et al. 2004; Evans 2004). This will lead to a negative balance between the rate of protein synthesis is slower than the rate of protein breakdown within the body. The degree of this negative balance will depend upon the type or intensity of exercise being done. In general, exercises are classified as (1) endurance (aerobic) exercises to increase heart rate (e.g., jogging, yard work, swimming, biking, dancing, and playing tennis); (2) strength exercises [resistance exercises (e.g., weight-lifting) and anaerobic exercises] to enhance skeletal muscle mass and strength; (3) balance exercises to prevent falls (e.g., standing on one foot and Tai Chi); and (4) flexibility exercises to stretch skeletal muscles (e.g., stretch of shoulder and upper arm; yoga). There is evidence that even moderate exercise (e.g., 55% of an individual's VO_2 max) will increase muscle protein catabolism in the whole body by 25% (Lemon et al. 1997). This will increase an individual's daily protein requirement above 1 g/kg body weigh/day, which tends to be the standard recommendation for sedentary adults (Wu 2016). During exercise (approximately 45% of maximum O_2 consumption for 90 min), total urea excretion (urine + sweat losses) increases by 100% above pre- and post-exercise values, with 30% of the total urea excretion during exercise in the form of sweat losses (Calles-Escandon et al. 1984). Therefore, if the amount of protein ingested is not increased, the rate of protein breakdown will continue to exceed the rate of protein synthesis, resulting in the loss of skeletal muscle mass and a state of negative nitrogen balance within the body. The concept of negative nitrogen balance was well established in a 1981 study in which healthy adults performed intense daily exercise for three weeks and were only permitted to consume 1 g of whole protein/kg body weight/day. These adults exhibited a negative nitrogen balance on each day of the three-week study (Young and Torún 1981). Furthermore, healthy exercising individuals must take care to ingest increased amounts of daily protein based upon the type of exercise they are doing. For those individuals participating in endurance training, 88% more protein will be required each day than that for non-exercising individuals (1.37 g/kg body weight/day vs 0.73 g/kg body weight/day) (Tarnopolsky et al. 1988). Comparatively, for those individuals participating in strength training, there is an even greater need to increase protein intake, up to 104% more protein must be ingested each day, compared to a sedentary individual (1.41 g/kg body weight/day vs. 0.69 g/kg body weight/day) (Tarnopolsky et al. 1992). This is likely because the individuals with strength training have a greater rate of muscle protein breakdown than those with endurance training in which the primary goal is usually to improve cardiovascular performance. The dramatic increases in protein requirements are actually indicative of an increased daily demand for AAs, which makes dietary supplementation with AAs a beneficial option. Dietary supplementation is a much easier method for meeting increased requirements for all AAs through consumption of high-quality protein, specific AAs, or both.

The goals of exercising individuals typically are to enhance strength, function, and mass of skeletal muscle, in addition to improving overall body health and personal well-being (Evans 2004). Physical measurements for these goals and overall muscle health include fatigue, muscle soreness, the rate of repair of muscle protein, and energy metabolism. Keeping these metrics in mind, there are ways of observing and measuring whether an exercising individual is meeting their requirements for increased dietary protein. When exercising subjects consume adequate post-exercise levels of protein (as described

previously for both endurance and strength training), the protein balance of the entire body is positive, instead of negative (Rennie and Tipton 2000). This means that the rate of protein synthesis is greater than the rate of protein degradation in the whole body (including skeletal muscle). As shown in Fig. 9.1, rates of protein synthesis in skeletal muscle increase most dramatically shortly after exercise and taper off over the following 48 h and return to resting levels at the end of 48 h (Phillips et al. 1997). Of note, the increase in muscle protein synthesis for 48 h post-workout can occur after only one workout (Rasmussen and Phillips 2003).

Results of studies have shown that dietary supplementation with both protein (the source of AAs) and free AAs has an appreciable beneficial effect on lean muscle mass. For example, when exercising individuals consumed adequate levels of protein (14 g of high-quality protein) plus 6 g of free AAs for 10 weeks at 1 h before and one hour after resistance training, lean muscle tissue mass and muscle strength increased (Willoughby et al 2007). Lean tissue mass increased by a total of 2.92 kg, and an additional 0.32 kg of lean tissue was added specifically to the thigh muscles. Muscle strength increased by 21% and 28% for the bench press and leg press exercises, respectively (Willoughby et al 2007). In 2004, a similar study compared two high-quality proteins, whey and casein (Tipton et al. 2004). Exercising individuals consumed 20 g of whey or casein immediately following exercise. Acute ingestion of either protein after exercise resulted in a similar increase in the net positive balance of skeletal muscle protein (Tipton et al. 2004).

Results of published studies indicate that supplementing exercising individuals with either high-quality protein or a mixture of free AAs increases muscle protein mass and exercise performance. For example, as with the consumption of 40 g whey protein (Moore et al. 2009; Witard et al. 2014), ingestion of 40 g of a mixture of individual AAs also increases muscle protein synthesis in human adults at rest and after resistance exercise (Tipton et al. 1999). These findings indicate that free AAs can be supplemented in place of protein to achieve equivalent results. Further research is necessary to understand which AAs are most beneficial for supplementation to the diets of exercising individuals (including athletes) for both structural (increasing muscle mass) and functional (performance) improvements. Some of those AAs are highlighted in the following sections.

9.3 Arginine

L-Arginine is a basic AA present in physiological fluids of humans and is required for the syntheses of protein, nitric oxide (NO), polyamines, creatine, agmatine, and homoarginine (Wu and Morris 1998). NO is a major vasodilator that relaxes the smooth muscle cells of the blood vessels, thereby increasing their size (diameter) and leading to reduced blood pressure. Thus, NO is especially important during strenuous exercise for increasing the delivery of nutrients and oxygen, as well as the removal of waste products from muscle and other organs and tissues. By storing energy as phosphocreatine (see below), creatine plays a crucial role in energy metabolism in the brain and skeletal muscle (Wu 2020). For this reason, creatine is important to exercising individuals. Arginine also stimulates mechanistic target of rapamycin (MTOR) to increase protein synthesis and activates AMP-activated protein kinase (AMPK) (Wu 2013). Activated AMPK stimulates fatty acid oxidation, an important endogenous source of energy during aerobic exercise (Jobgen et al. 2006). Arginine is also important in the urea cycle for the detoxification of ammonia (Wu 2013). This is very important in exercising individuals, as skeletal muscle protein is degraded at such an increased rate that there is an increased risk for the accrual of degradation waste products such as ammonia (Wu and Morris 1998). The NO-dependent and independent roles of arginine are summarized in Table 9.1.

Mariotti et al. (2013) reported that dietary supplementation with 3 g arginine within the physiological range increased whole-body NO synthesis during an 8-h period by nearly 100% in healthy adults. There is also evidence that dietary

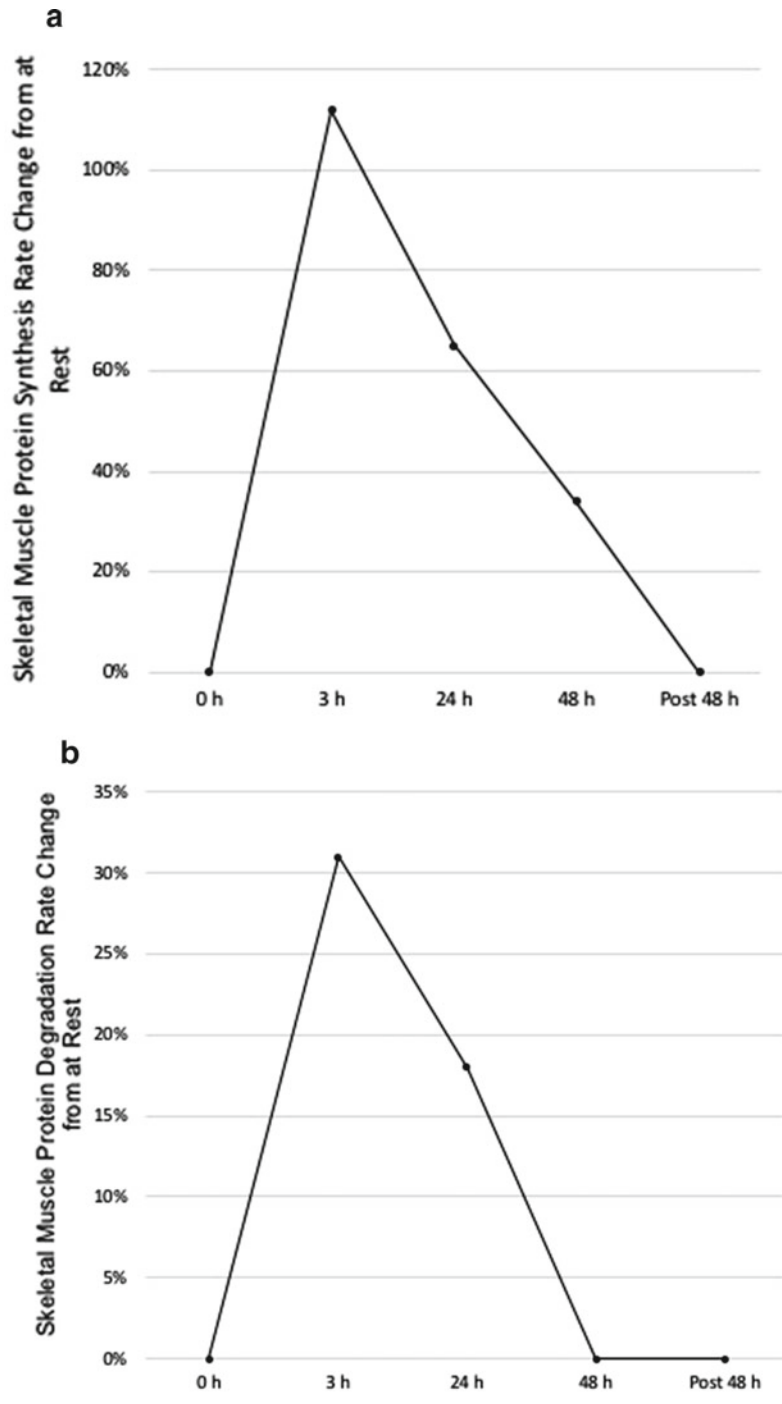

Fig. 9.1 Changes in protein synthesis (Panel **a**) and protein degradation (Panel **b**) in the skeletal muscle of adult humans after a resistance exercise bout, compared with the value at rest. Study subjects (who were moderately active (recreational cycling and running)) were fasted overnight before protein synthesis, and protein degradation were measured at rest (0 h). During the study, the subjects consumed meat-free diets. Adapted from Phillips et al. (1997)

supplementation of 6.6 g arginine augmented the rate of forearm blood flow in healthy adults by 28% and 25% at 120–125 and 180–185 min post administration, respectively (Monti et al. 2013). As a beneficial regulator of glucose metabolism, ammonia detoxification, and anti-oxidative reactions (Jobgen et al. 2006, 2009b), intravenous infusion of L-arginine increased glucose

Table 9.1 NO-dependent and independent roles of L-arginine in humans

NO-dependent actions	NO-independent actions
↑GC and smooth muscle cell relaxation	↑EC membrane polarization and transport activity
↑EC proliferation & angiogenesis	↑Extracellular and intracellular pH
↑Vasodilation and the flow of blood to tissues	↑Expression of GTP-CH1 for BH4 synthesis
↑Transport of water across the cell membrane	↑Expression of GSH-synthetic genes in cells
↑Glucose transport and oxidation by tissues	↑MTOR cell signaling and protein synthesis
↑Brown adipose tissue development	↑Brown adipose tissue development
↑Oxidation of fatty acids in tissues	↑Production of agmatine and homoarginine
↑Anti-inflammatory responses	↑CO production and signaling
↑Killing pathogens (e.g., bacteria and viruses)	↑Beneficial bacteria in the intestine
↓Endothelin-1 release by EC	↑Release of insulin, GH, glucagon and prolactin
↓Leukocyte adhesion to blood vessel wall	↑Synthesis of ornithine, creatine, Pro and PA
↓Platelet aggregation within blood vessel	↑Plasmin generation and fibrinogenolysis
↓Superoxide production by EC	↓Leukocyte adhesion to non-EC matrix
↓Expression of cell adhesion molecules	↓FA profile, oxygenation and viscosity of blood
↓Expression of monocyte chemotactic peptides	↓Angiotensin-converting enzyme activity
↓Proliferation of vascular smooth muscle cells	↓Release of O_2^- and H_2O_2 and lipid peroxidation
↓EC mitochondrial injury and apoptosis	↓Formation of TXB_2, fibrin and platelet–fibrin
↓Obesity and dyslipidemia	↓Homocysteine in blood
↓Cardiovascular disease (including hypertension)	↓Lactate, ammonia, and ketone bodies in blood

Abbreviations: *BH4* tetrahydrobiopterin; *CO* carbon monoxide; *EC* endothelial cell; *FA* fatty acid; *GC* guanylyl cyclase; *GH* growth hormone; *GSH* glutathione; *GTP-CH1* GTP-cyclohydrolase-1; *MTOR* mechanistic target of rapamycin; *NO* nitric oxide; *PA* polyamines; *Pro* proline; *TXB_2* thromboxane B_2 The symbols ↑ and ↓ denote increase and decrease, respectively

clearance during prolonged exercise in humans (McConell et al. 2006). Likewise, the intravenous administration of arginine-HCl (3 g over 30 s) reduced exercise-induced increases in the concentrations of both lactate and ammonia in the plasma of healthy adults by 13.4% and 17.0%, respectively (Schaefer et al. 2002). Thus, L-arginine supplementation may improve endurance performance. In support of this view, Santos et al. (2002) reported that the oral administration of 3 g of L-arginine enhanced the capacity for resistance to muscle fatigue in adults. Likewise, Bailey et al. (2010) found that supplementation with L-arginine decreased the O_2 cost of low-intensity exercise and increased the time to exhaustion in severely intense exercise. Similarly, compared to the placebo, dietary supplementation with L-citrulline (6 g/day for 7 days; an effective precursor of L-arginine) enhanced exercise tolerance and total work done during an aerobic performance trial in adults (Bailey et al. 2015). Consistent with this result, L-citrulline supplementation (2.4 g/days for 7 days) reduced the time needed to complete a 4 km cycle, compared to the placebo group (Suzuki et al. 2016). Furthermore, acute oral administration of 8 g citrulline malate enhanced athletic anaerobic performance by 53% and decreased muscle soreness 40% in men (Pérez-Guisado and Jakeman 2010). In a recent meta-analysis of 15 published studies, Viribay et al. (2020) indicated that the oral administration of arginine could improve both aerobic and anaerobic performance. These authors further recommended: (1) acute supplementation with 0.15 g/kg body weight between 60–90 min before aerobic or anaerobic exercise to achieve its beneficial effect; (2) chronic supplementation

with 1.5–2 g arginine/day for 4–7 weeks in the case of aerobic exercise or 10–12 g/day for 8 weeks in the case of anaerobic performance.

9.4 Branched-Chain Amino Acids (BCAAs)

Some of the most commonly supplemented AAs for athletes in strength training, at all levels, are BCAAs. They are leucine, isoleucine, and valine. These AAs are not synthesized de novo by mammalian cells and, therefore, must be included in human diets. Very good sources of BCAAs are high-quality proteins such as whey protein and those in meat (Li and Wu 2020; Rezaei et al. 2016; Wu et al. 2016). For example, 40 g of whey protein contains 10.2 g of BCAAs (about 25% of whey protein is BCAAs; Rezaei et al. 2016). The BCAAs are among the most popular supplements for athletes in strength training.

As highly abundant AAs in skeletal muscle, BCAAs are necessary for enhancing protein mass in this tissue. However, BCAAs play another vital role beyond providing structural components of muscle protein. BCAAs are also substrates for the synthesis of AAs such as glutamate, glutamine, and alanine in skeletal and cardiac muscles (Chang and Goldberg 1978; Tischler and Goldberg. 1980). Additionally, leucine stimulates the MTOR cell signaling pathway to promote muscle protein synthesis (Wu 2009). Therefore, BCAAs have consistently been reported in many studies to improve muscle strength and performance in exercising humans (Gualano et al. 2011; Matsumoto et al. 2009; Mittleman et al. 1998; Ra et al. 2018; Shimomoura et al. 2010). Interestingly, the necessary dosage of BCAAs to achieve desired results is relatively low. For example, BCAA supplementation at either 0.1 g/kg body weight/day or 5–10 g/day is adequate for improving exercise performance and delaying onset of muscle soreness in adults (Matsumoto et al. 2009; Ra et al. 2018; Shimomura et al. 2010).

9.5 Creatine

Another dietary supplement for athletes in strength training is creatine. It is not an AA, but is synthesized from three AAs (glycine, arginine, and methionine) in humans through inter-organ metabolism (Wu 2013). Creatine plays many important functional roles in human health, especially in skeletal muscle function and performance. While humans do have the necessary enzymes for synthesizing creatine de novo, as is shown in Fig. 9.2, it is essential that adequate amounts of the necessary precursor AAs are provided in the diet. In exercising individuals with increased rates of creatine turnover, it is not likely that their physiological requirements for creatine will be met by de novo synthesis. Therefore, creatine must be supplied in the diet, either by consuming animal-source products such as meat and milk or via AA supplementation. Creatine is not present in plant foods (Hou et al. 2019). That being said, it should be noted that vegan athletes will not likely meet and maintain adequate creatine levels if they do not consume animal products (Brosnan and Brosnan 2007). These individuals may be deficient in de novo synthesis of creatine possibly due to inadequate intakes of glycine, arginine, and methionine. While all athletes must take special care to meet their increased requirements for creatine, vegan athletes definitely need to supplement their diets with creatine or AAs essential for its synthesis. While creatine is synthesized de novo via the renal-hepatic axis, it is found primarily in skeletal muscle and, to a much lesser extent, in the brain. A 70-kg adult human has approximately 120 g of creatine plus creatine phosphate in their body, with 95% of that being stored in the skeletal muscle (Brosnan and Brosnan 2007). Creatine is stored in skeletal muscle primarily as creatine phosphate, which principally acts as an energy reserve for adenosine triphosphate (ATP) replenishment in the muscle during strenuous exercise when ATP is being depleted rapidly. Creatine is converted to creatine phosphate by creatine kinase when

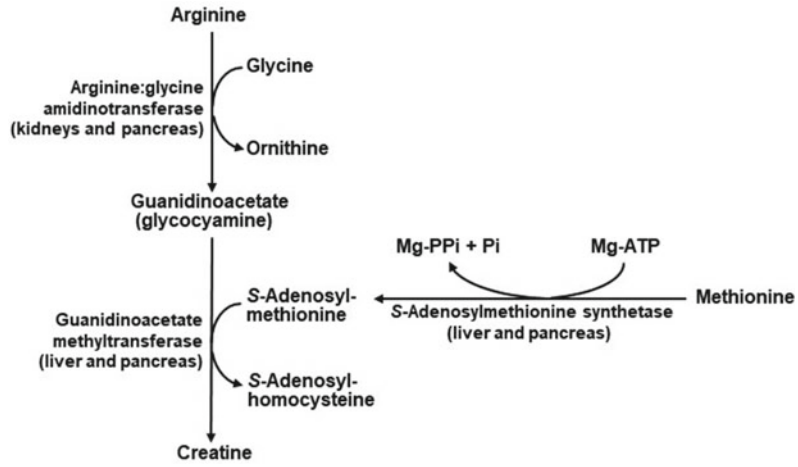

Fig. 9.2 Creatine synthesis via inter-organ metabolism of amino acids in humans. This metabolic pathway requires arginine, glycine, and methionine. Adapted from Wu (2013)

skeletal muscle is at rest. Through the action of this enzyme, the energy from the γ-phosphate bond in ATP is transferred to one mol of creatine, forming creatine phosphate, for storage in vivo, as shown below (Wu 2018).

$$\text{ATP} + \text{Creatine} \leftrightarrow \text{Phosphocreatine} + \text{ADP} + \text{H}^{+}$$
(Creatine Kinase)

This reversible reaction is vital for the rapid replenishment of ATP after skeletal muscle carries out strenuous activity and the concentration of ADP increases, driving the reaction toward ATP formation and dephosphorylation of creatine phosphate. When the muscle is at rest and the intramuscular concentration of ATP is higher than that of ADP, the reaction is driven toward formation of the more stable creatine phosphate, so that the energy reserve is maintained. The concentration of creatine phosphate is usually 3- to 4-times greater than the concentration of ATP in human skeletal muscle, thus providing the muscle with a large reservoir to draw from during intense exercise (Brosnan and Brosnan 2007). Put another way, in a resting state, about two-thirds of creatine in the body exists as creatine phosphate, and only one-third is present as creatine (McGilvery and Murray 1974). Thus, although creatine is not a source of cellular energy, this AA metabolite helps to store energy as phosphocreatine in animal tissues, particularly the brain and skeletal muscle.

There is also a constant and consistent loss of creatine each day. Creatine plus creatine phosphate is lost at a rate of 1.7% of whole-body creatine per day, as a metabolite called creatinine (Brosnan and Brosnan 2007). As opposed to the creatine kinase reaction, the spontaneous reaction that forms creatinine is irreversible, and so, creatinine is not used to form creatine. Therefore, it is the remaining 98.3% of creatine that is continually recycled each day to act as a reservoir for storing energy for ATP via creatine kinase. As the overall creatine loss each day is at a very low basal level, only minimal creatine supplementation is necessary to achieve total muscle saturation with creatine. As shown in Fig. 9.3, the oral administration of 3 g of creatine monohydrate (the common creatine supplement form) each day for 28 days was adequate for total skeletal muscle saturation (142 mmol/kg of dry matter) on day 28 in men who weighed 76 kg (Hultman et al. 1996). For comparison, in individuals who did not receive supplementation, the amount of creatine in skeletal muscle is 114 g or 122 mmol/kg of dry matter (Hultman et al. 1996).

In exercising humans, the rate of creatine phosphate utilization by skeletal muscle is

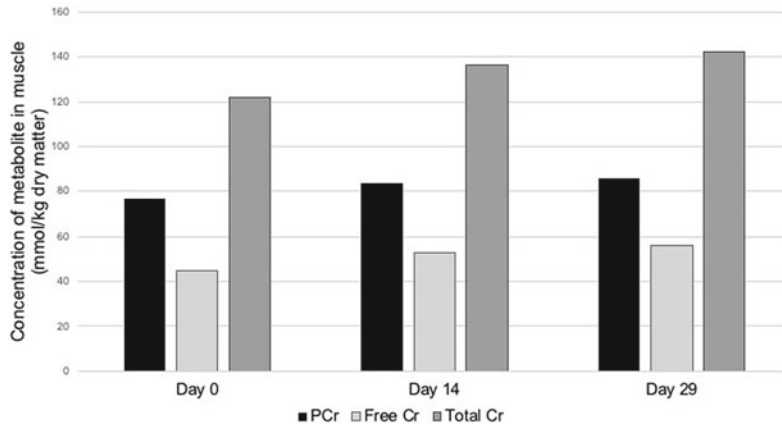

Fig. 9.3 Increases in intramuscular concentrations of phosphocreatine (PCr), free creatine (Free Cr), and total creatine (Total Cr) in adult men in response to a daily supplement of 3 g creatine monohydrate per day for 28 days. Adapted from Hultman et al. (1996)

approximately 23 mmol of creatine phosphate/L of muscle water/min or 483 mmol creatine phosphate/whole-body skeletal muscle/min. A 70-kg exercising individual is expected to have approximately 21 L of muscle water, and with the creatine recycling mechanism via creatine kinase, ingesting only 3 g of creatine monohydrate can help to improve performance for up to 1 h during strenuous exercise.

With regard to creatine supplementation, the theory of "more is better" permeates the fitness community and athletic circles. More is not always better when it comes to any dietary supplement. Besides placing an undue load on the body to remove excess creatine, it is also a waste of resources if continually increasing creatine intake beyond a saturation concentration does not improve athletic performance. Based on the current understanding of creatine metabolism, continuous supplementation every day is not necessary to achieve creatine saturation in skeletal muscle (Wu 2020). This is directly contradictory to the current belief in fitness communities that recommended creatine supplements upwards of 5 g of creatine per daily serving, with some supplements providing as much as 20 g of creatine per day for "loading phases." Again, based on creatine biochemistry, such high doses are not necessary. Creatine does play a vital role as a supplement for exercising athletes, but it does so at relatively lower doses (e.g., 3 g of creatine monohydrate/day) that provide significant contributions to energy storage within skeletal muscle and, therefore, improves athletic performance (Kreider et al. 2017).

9.6 Glycine

As for all AAs, humans have a daily requirement for glycine (Wang et al. 2013). When calculating total glycine intake, all forms of glycine must be considered, including protein-bound glycine and free glycine, as both contribute to overall glycine nutrition. Glycine can be synthesized by enzymes in the human body, but the rate of synthesis may not be adequate to meet the increased demands of exercising individuals either due to inadequate ingestion of precursor AAs or low activities of key enzymes in the biochemical pathway.

Adequate glycine intake is vital to human nutritive status, as glycine plays many roles throughout the body, and its metabolites contribute even further to overall health (Jackson 1991; Razak et al. 2017; Wang et al. 2013). Without good health, athletes cannot perform at their peak level. Some of the direct roles of glycine include the synthesis of collagen, an effector in cell signaling pathways as a primary ligand and as a neurotransmitter in the central nervous system, and as an antioxidant and anti-inflammatory co-factor (Wu 2013). These functions directly affect athletic performance. Collagen synthesis and health are important for the

maintenance of tissues that undergo repetitive loading and unloading motions, such as strenuous strength training exercises. Additionally, increased muscle protein breakdown has the potential for increased oxidative damage, and therefore, glycine's antioxidant role can directly improve the health of these tissues. Further, the indirect roles of glycine through its metabolites are even more numerous. Some of the glycine metabolites of note for exercising individuals are serine, creatine, heme, and glutathione. Serine, an AA that can be synthesized from glycine, is important for protein synthesis, one-carbon metabolism, and gluconeogenesis, which are all vital pathways for improving both muscle mass and athletic performance during exercise. Creatine has been discussed previously, and one mol of glycine is totally incorporated into one mol of creatine (using 1.0 g glycine for the synthesis of 1.74 g creatine each day) (Wu and Morris 1998). Heme is also synthesized from glycine and is a component of hemoglobin and myoglobin which are especially important for carrying oxygen-rich blood to skeletal muscles (and other tissues) from the lungs during endurance-type exercises. Finally, glutathione is synthesized from glycine, and it is a free radical scavenger and an antioxidant, similar to glycine (Wu et al. 2004). Again, antioxidant activity is important in athletes in whom the presence of damaged and recovering skeletal proteins are greatly increased. An expansive list of the actions of glycine and its metabolites is summarized in Table 9.2.

Glycine, similarly to creatine, intake is considerably reduced in vegan and vegetarian athletes due to plant-based products being very deficient in glycine (Hou et al. 2019; Jackson et al. 1991; Li and Wu 2020; Razak et al. 2017; Wang et al. 2013). Therefore, challenges faced with meeting creatine intake for vegan and vegetarian athletes also exist regarding glycine intake. Because of this, supplementation with AA is likely to be necessary in vegan athletes, who must take special care to be sure that their

Table 9.2 Physiological functions of glycine and its metabolites in humans

Direct action of glycine	
Direct action of glycine	Protein synthesis (particularly accounting for 1/3 of amino acids in collagen and elastin); cell signaling; inhibition of calcium influx through activation of the glycine-gated channel in the cell membrane; inhibitory neurotransmitter in the central nervous system; co-agonist with glutamate for N-methyl-D-aspartate receptor receptors; antioxidant; anti-inflammation; one-carbon metabolism; conjugation with bile acids
Functions of glycine metabolites	
Serine	Protein synthesis, one-carbon metabolism, and gluconeogenesis; conversion into choline via a series of reactions requiring methionine; conversion into ethanolamine through formation of phosphatidylserine; synthesis of D-serine (a neurotransmitter) in the brain
Porphyrins and heme	Hemoproteins (e.g., hemoglobin, myoglobin, catalase, and cytochrome c); production of carbon monoxide (a signaling molecule); storage of iron
Bilirubin	Natural ligand of aryl hydrocarbon receptor in the cytoplasm
Creatine	Antioxidant; antiviral; antitumor; energy metabolism in heart, skeletal muscle and brain; neurological and muscular development and function
Glutathione	Free radical scavenger; antioxidant; cell metabolism (e.g., formation of leukotrienes, mercapturate, glutathionylspermidine, glutathione-nitric oxide adduct and glutathionylproteins); signal transduction; regulation of gene expression; apoptosis; cellular redox; immune response
Nucleic acids	Coding for genetic information; gene expression; cell cycle and function; protein synthesis; lymphocyte proliferation; facilitation of wound healing
Heme	An essential component of hemoglobin, myoglobin, and heme-containing enzymes

Adapted from Wang et al. (2013)

increased daily requirement for AA for carrying out exercise is met. An excellent source of glycine and its AA precursors for human diets is meat (Wu et al. 2016).

9.7 Taurine

Taurine is one of the most important non-proteinogenic AAs for exercise nutrition. A 70-kg person has ~70 g taurine, with the skeletal muscle, heart, retina, and placenta containing 15–20, 28–40, 20–35, and 20–35 mM taurine, respectively (Wu 2020). This β-amino acid is important for the maintenance of human health and especially for peak athletic performance. Taurine can be synthesized in the liver of most mammals, including humans (Greenstein and Winitz 1961; Meister 1965). If not synthesized de novo, taurine requirements must be met by adequate dietary intake. Taurine is most abundant in animal products such as dairy, meat, and fish, but is absent from plant-source foods (Hou et al. 2019; Li and Wu 2020; Wright et al. 1986).

Taurine plays many valuable roles in the body, including osmoregulation, retinal function, and conjugation with bile acids for proper emulsion of dietary fat (Danielsson and Sjövall 1975; Oja and Saransaari 2013). Also, similar to glycine and its metabolite serine, taurine has antioxidant and anti-inflammatory properties of special importance to exercising individuals whose muscles are being damaged at a greater rate than for sedentary individuals (Colovic et al. 2018). Therefore, antioxidants and anti-inflammatory metabolites are especially crucial for maintaining health in athletes. Because of the physiological functions of taurine, it must be included as a beneficial nutrient in diets. Additionally, the full effects of taurine on an individual's health can only be of maximum benefit if the proper amounts and composition of AAs exist in the diet (Wu 2013). The physiological functions of taurine in humans are summarized in Table 9.3.

Taurine, similar to other AAs and metabolites, such as glycine and creatine, is of special importance to vegan and vegetarian athletes, who must ensure adequate intake of taurine along with their requirements for all proteinogenic AAs (Wu 2016). Most other athletes should have no problem meeting increased taurine requirements if they consume sufficient amounts of animal-source foods (such as meat, milk and eggs). Acute or chronic oral administration of 1 to 6 g taurine per day to exercising adults who consume regular diets improves their endurance performance (Waldron et al. 2018). This can be

Table 9.3 Physiological functions of taurine in humans

Physiological function	Comment
Conjugation with bile acids to form bile salts in the liver	Facilitates intestinal absorption of dietary lipids (including lipid-soluble vitamins); eliminates cholesterol in bile via the fecal route
A major antioxidant, anti-inflammatory, and anti-apoptotic factor	Protects humans from heat stress, oxidative stress, and tissue injury
A physiological stabilizer of cell membranes	Maintains the integrity of cells and tissues
A regulator of Ca^{2+} signaling and cell metabolism	Plays an important role in glucose and energy homeostasis; reduce obesity
Retinal photoreceptor activity	Maintains the normal function of the eyes
A significant contributor to osmoregulation	Maintains water and ion balances in cells
A key component of nerve and muscle conduction networks	Maintains the normal function of the central and peripheral nervous systems, as well as skeletal and cardiac muscles
N-Halogenation with hypochlorous acid (HOCl) and hypobromous acid (HOBr)	Formation of taurine chloramine and taurine bromamine to kill pathogens (e.g., bacteria, fungi, parasites, and viruses)

explained by an important role of taurine in vascular endothelial function at rest and after resistance exercise (Ra et al. 2019).

9.8 Glutamine

Glutamine can be ingested from the diet in the free form or peptide-bound form in whole proteins (Wu 2013). Of particular note, glutamine is abundant in meat, milk, wheat flour, and potatos (Hou et al. 2019). Most (~70%) of dietary glutamine is utilized by the small intestine primarily via oxidative pathways in humans (Wu 2013). Interestingly, glutamine is the most abundant AA in the plasma (0.5–0.6 mM) and skeletal muscle (20–25 mM) of humans. Thus, the circulating glutamine is synthesized de novo from glutamate and ammonia by glutamine synthetase mainly in skeletal muscle (Curthoys and Watford 1995). However, it is unlikely that this conditionally essential AA will be synthesized in adequate amounts in exercising individuals, as the requirements for glutamine and related AAs will greatly increase with exercise. Furthermore, glutamine is degraded in skeletal muscle (Wu et al. 1991), and this process is likely enhanced during exercise as an intramuscular source of both energy and NH_3 to buffer H^+.

Glutamine has many roles in overall human health that demonstrate the importance of its adequate intake. The direct effects of glutamine are on protein synthesis, nitrogen balance, and energy metabolism. Glutamine also stimulates the MTOR cell signaling pathway, the master regulator of protein synthesis, and, therefore, augments the rate of protein synthesis in cells (Wu 2013). This is important for athletes because protein synthesis is vital for increasing skeletal muscle mass, as well as for the repair and replacement of tissues damaged during exercise. Glutamine also acts as a nitrogen reservoir which is important for maintaining proper nitrogen balance in athletes who tend to enter states of negative nitrogen balance when not consuming adequate amounts of protein (Wu 2016; Young and Torún 1981). Glutamine also plays a role in the synthesis of nicotinamide adenine dinucleotide (NAD) and its phosphorylated form (NADP) that are required for the biochemical pathways involved in the oxidation of glucose, fatty acids, and AAs (Wu 2018). Glutamine is also a precursor for many metabolites that contribute to membrane receptors, angiogenesis, and overall homeostasis in the body. Some of these important metabolites include aminosugars, arginine, glutamate, and aspartate. Arginine is vital for the whole-body homeostasis and function, as noted previously.

Two other metabolites of glutamine that play important roles during exercise are glutamate and aspartate. Both of those metabolites are components of the malate shuttle which is important for energy metabolism to meet the increased energy demands of exercising individuals. Additionally, these two AAs contribute to the synthesis of alanine that is a vital substrate for gluconeogenesis and the provision of increased energy to meet demands of exercise. Furthermore, glutamate and aspartate, along with glutamine, are the major metabolic fuels for the small intestine of mammals (Wu 2013). A summary of physiological and nutritional functions of glutamine are presented in Table 9.4. Of note, there is evidence that glutamine is an anti-fatigue AA in sports nutrition (Coqueiro et al. 2019).

9.9 Summary and Perspectives

There is a common misconception among fitness communities that humans have a requirement for whole protein. This is a false concept as it is individual AA requirements that must be met. Whole protein does not have a nutritive role until it is broken down to its constitutive AAs and small peptides via digestive enzymes in the small intestine and then absorbed into the bloodstream (Watford and Wu 2018). If an exercising human does not meet their increased demands for AAs each day, they run the risk of entering into a negative nitrogen balance when rate of breakdown of body protein exceeds the rates of protein synthesis. A negative nitrogen balance will have disastrous effects for an athlete looking to achieve peak exercise performance. Meeting the

Table 9.4 Physiological functions of glutamine in humans

Direct action of glycine	
Direct action of glutamine	Protein synthesis; regulation of protein turnover through cellular MTOR signaling; regulation of cell volume; gene expression; immune function; a major fuel for rapidly proliferating cells; inhibition of apoptosis; syntheses of purine, pyrimidine, ornithine, citrulline, arginine, proline, and asparagine; nitrogen reservoir; synthesis of NAD(P)
Functions of glutamine metabolites	
Arginine	Precursor for the synthesis of nitric oxide (a major vasodilator) and creatine (required for brain and muscle energy metabolism); activation of MTOR and AMPK signaling pathways to enhance protein synthesis and fatty acid oxidation; antioxidant; regulation of insulin secretion; ammonia detoxification; enhancer of immune function
Glutamate and aspartate	Excitatory neurotransmitters; components of the malate shuttle; cell metabolism; ammonia detoxification; major fuels for enterocytes; synthesis of alanine (a major substrate of gluconeogenesis)
Glucosamine-6-phosphate	Synthesis of aminosugars and glycoproteins; inhibition of nitric oxide synthesis; anti-inflammation; angiogenesis; cell growth & development
Ammonia	Renal regulation of acid–base balance; synthesis of glutamate and carbamoyl-phosphate

Adapted from Wu (2013)
AMPK AMP-activated protein kinase; *MTOR* mechanistic target of rapamycin.

requirements for all AAs is vital for total health; however, some AAs are particularly important to achieve peak athletic performance. Failure to meet increased requirements for AAs, such as BCAAs, glycine, taurine, and glutamine and AA metabolites (e.g., creatine), severely decrease the exercise performance of individuals. Some AAs are vital for physiological functions that directly affect athletic performance, such as the activation of MTOR for increasing protein synthesis to increase skeletal muscle mass. Additionally, as intensive exercise damages tissues and proteins, the antioxidant and anti-inflammatory properties of AAs are very important. For those functions to be efficient, BCAAs must be provided in adequate amounts to provide the structural components of skeletal muscle protein synthesis. Another vital role of AAs in exercise is to support muscle energy metabolism (Kreider et al. 2017) and blood flow (Wu and Meininger 2009). For example, phosphocreatine directly provides a pool of ATP for skeletal muscle to draw from during strenuous activities, glutamine and glycine influence energy metabolism through gluconeogenesis, and the synthesis of glutathione (an antioxidant), respectively, and NO serves as a major vasodilator in the body. Because all of these vital functions require an adequate supply of AAs, there are severe consequences of not meeting the increased requirements for these nutrients by athletes. However, to properly supplement the exercising human with AAs, there must be a thorough understanding of the impact of various AAs to ensure that AA supplements are correctly formulated to avoid an undesirable nitrogen overload in the body. Overall, proteinogenic AAs, non-proteinogenic AAs, and bioactive AA metabolites (e.g., polyamines and glutathione) all play important functional roles beyond protein synthesis. These roles directly impact human health, and exceptional care should be taken to meet these requirements to ensure optimal performance in all exercising individuals. All functional AAs are crucial for optimum exercise performance and health in humans.

Acknowledgements Work in our laboratory was supported by Texas A&M AgriLife Research #H8200.

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Role of L-Arginine in Nitric Oxide Synthesis and Health in Humans

10

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Abstract

As a functional amino acid (AA), L-arginine (Arg) serves not only as a building block of protein but also as an essential substrate for the synthesis of nitric oxide (NO), creatine, polyamines, homoarginine, and agmatine in mammals (including humans). NO (a major vasodilator) increases blood flow to tissues. Arg and its metabolites play important roles in metabolism and physiology. Arg is required to maintain the urea cycle in the active state to detoxify ammonia. This AA also activates cellular mechanistic target of rapamycin (MTOR) and focal adhesion kinase cell signaling pathways in mammals, thereby stimulating protein synthesis, inhibiting autophagy and proteolysis, enhancing cell migration and wound healing, promoting spermatogenesis and sperm quality, improving conceptus survival and growth, and augmenting the production of milk proteins. Although Arg is formed de novo from glutamine/glutamate and proline in humans, these synthetic pathways do not provide sufficient Arg in infants or adults. Thus, humans and other animals do have dietary needs of Arg for optimal growth, development, lactation, and fertility. Much evidence shows that oral administration of Arg within the physiological range can confer health benefits to both men and women by increasing NO synthesis and thus blood flow in tissues (e.g., skeletal muscle and the corpora cavernosa of the penis). NO is a vasodilator, a neurotransmitter, a regulator of nutrient metabolism, and a killer of bacteria, fungi, parasites, and viruses [including coronaviruses, such as SARS-CoV and SARS-CoV-2 (the virus causing COVID-19). Thus, Arg supplementation can enhance immunity, anti-infectious, and anti-oxidative responses, fertility, wound healing, ammonia detoxification, nutrient digestion and absorption, lean tissue mass, and brown adipose tissue development; ameliorate metabolic syndromes (including dyslipidemia, obesity, diabetes, and hypertension); and treat individuals with erectile dysfunction, sickle cell disease, muscular dystrophy, and pre-eclampsia.

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1332,
https://doi.org/10.1007/978-3-030-74180-8_10

Keywords

Arginine · Blood flow · Disease · Health · Metabolism · Nitric oxide · Skeletal muscle

Abbreviations

AA	Amino acid
ADMA	Asymmetric dimethylarginine
AMPK	AMP-activated protein kinase
Arg	L-arginine
BH4	Tetrahydrobiopterin
BW	Body weight
COVID-19	Coronavirus disease-2019
eNOS	Endothelial nitric oxide synthase
GTP-CH1	GTP cyclohydrolase-I
NHANES	National health and nutrition examination survey
iNOS	Inducible nitric oxide synthase
NMMA	N^G-monomethylarginine
MTOR	Mechanistic target of rapamycin
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
PPAR	Peroxisome proliferator-activated receptor
SARS-CoV	Severe acute respiratory syndrome coronavirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCD	Sickle cell disease

10.1 Introduction

L-arginine (Arg) is a basic amino acid (AA) present in the physiological fluids of humans. This nutrient is required for the synthesis of protein and other low-molecular-weight substances, including nitric oxide (NO), creatine, polyamines, homoarginine, and agmatine (Bollenbach et al. 2019; Tsai and Kass 2009; Wu and Morris 1998). The oxidation of one guanidino nitrogen of Arg by NO synthase (NOS) to generate NO occurs in virtually all mammalian cells, including endothelial cells, macrophages, neuronal cells, muscle cells, adipocytes, enterocytes, and renal epithelial cells (Stuehr et al. 2001). Three distinct isoforms of NOS [neuronal NOS (nNOS; NOS1), inducible NOS (iNOS; NOS2), and endothelial NOS (eNOS; NOS3)] have been identified. They are encoded by different genes and differ in molecular, catalytic, and immunological properties, cellular distribution, regulation of activity, and sensitivity to inhibitors (Alderton et al. 2001). All NOS isoforms require tetrahydrobiopterin (BH4), NADPH, FAD, FMN, and heme for NO synthesis (Fig. 10.1), whereas eNOS and nNOS also require calcium and calmodulin for their enzymatic activities (Förstermann and Sessa 2012). Available evidence shows that NO synthesis in mammals is regulated by dietary factors (Wu and Meininger 2002). For example, Arg, taurine, polyunsaturated fatty acids, calcium, vitamin C, phytoestrogens, and polyphenols stimulate, while glutamine, lysine, saturated fatty acids, fructose, cholesterol, and homocysteine inhibit, NO synthesis by eNOS and nNOS (Wu and Meininger 2002). This illustrates the complexity of NO production and has important implications for understanding the responses of humans to dietary Arg supplementation with regard to NO production by specific cells and the whole body. The main objective of this article is to highlight the role of Arg in NO synthesis and health in humans.

10.2 The Arg Paradox in Mammalian NO Synthesis

The K_M value (substrate concentration for half-maximal rate) of eNOS for Arg is only 2.9 μM (Pollock et al. 1991), which is much lower than the intracellular concentration of Arg (500–3000 μM) in almost all cell types studied (including endothelial cells) except hepatocytes (<50 μM) (Wu 2013). These observations imply that eNOS may be saturated with intracellular Arg and that endothelial NO synthesis may not respond to alterations in extracellular Arg concentrations. However, available evidence shows that: (a) increasing extracellular concentrations of Arg from 0.1 mM (physiological level in the plasma of overnight fasted humans) to 10 mM

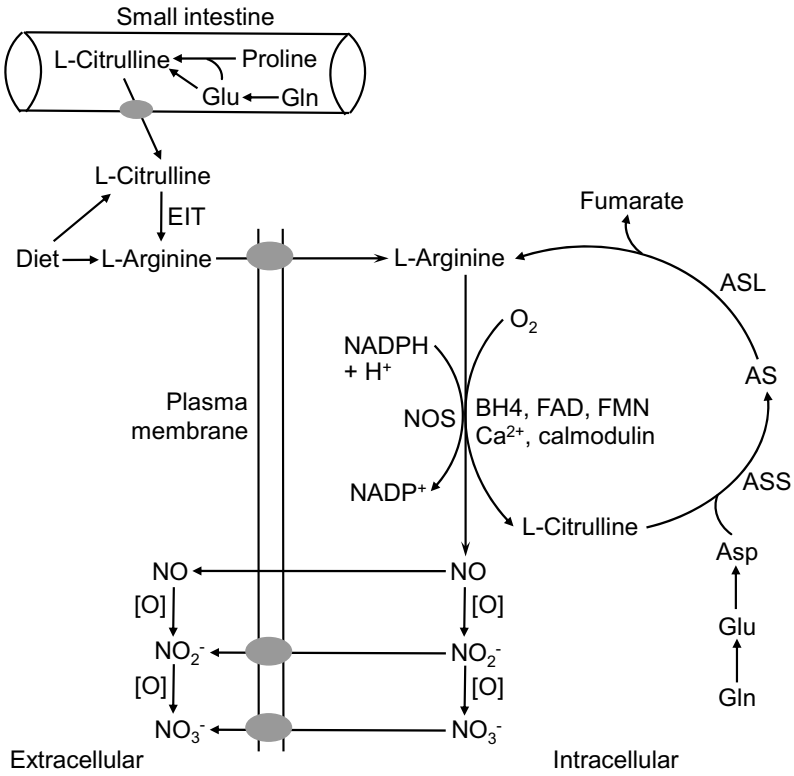

Fig. 10.1 Sources of L-arginine and its conversion into nitric oxide (NO) in humans. The diet provides L-arginine and/or L-citrulline. Enterocytes of the small intestine synthesize L-citrulline from dietary L-glutamine (Gln), L-glutamate (Glu), and proline, as well as Gln in arterial blood. The small intestine releases L-citrulline, which is utilized by extraintestinal tissues and cells (EIT), such as the kidneys, endothelial cells, and macrophages, for the production of L-arginine via argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) in the presence of L-aspartate (Asp). L-arginine is oxidized by NO synthase (NOS) to form NO and L-citrulline. All isoforms of NOS require tetrahydrobiopterin (BH4), NADPH, FAD, FMN, and calmodulin for catalytic activity, and endothelial NOS (eNOS, also known as NOS3) also requires Ca^{2+} for catalytic activity. The NOS-derived L-citrulline is recycled into L-arginine, and this is known as the arginine-citrulline cycle in mammalian cells. NO has a short half-life and is rapidly oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) in the presence of oxygen

dose-dependently increases NO production by cultured endothelial cells in the presence of a physiological concentration of glutamine (Arnal et al. 1995; Closs et al. 2000; Li et al. 2001); and (b) elevating physiological concentrations of Arg in plasma by >25% through oral administration of Arg enhances systemic and vascular NO production in healthy adult humans and other mammals (Kohli et al. 2004; Maxwell and Cooke 2001; Wu and Meininger 2002). This "Arg paradox" for NO synthesis has been explained by a number of theories, which include (a) the competitive inhibition of eNOS by endogenous inhibitors [e.g., asymmetric dimethylarginine (ADMA)] (Tsikas 2017; Tsikas et al. 2000); and (b) the stimulation of BH4 production by Arg in endothelial cells through enhancing the expression of the GTP cyclohydrolase-I (GTP-CH1) gene (Shi et al. 2004).

We found that the addition of 2 μM N^G-monomethylarginine (NMMA) or ADMA (about twice their concentrations in the plasma of normal rats) to culture medium containing physiological concentrations of AAs (including 0.2 mM Arg and 0.5 mM glutamine) only moderately reduced NO synthesis in the coronary endothelial cells of normal rats (by about 7%), as compared with 1 μM NMMA or ADMA (physiological

concentrations in the plasma of normal rats) (Kohli et al. 2004). By contrast, the addition of 10 μM sepiapterin (a substrate for BH4 synthesis via the salvage pathway) increased the intracellular concentration of BH4 by 4.8-fold and NO synthesis in the coronary endothelial cells of normal rats by 168% (Kohli et al. 2004). Likewise, the overexpression of the GTP-CH1 gene in the coronary endothelial cells of diabetic BB rats enhanced NO production by 23-fold and augmented acetylcholine-induced aortic relaxation by 73% (Meininger et al. 2004). These results indicate a crucial role of BH4 in the synthesis of NO from Arg in endothelial cells that could have important implications for cardiovascular health.

10.3 Physiological Functions of Arg and NO in Mammals

Arg has crucial roles in nutrition, physiology, and metabolism (Table 10.1). First, as a major building block for protein, Arg represents 14% of total nitrogen in body protein. In addition, there are multiple pathways for Arg catabolism to generate NO, ornithine, polyamines, proline, glutamate, agmatine, creatine, homoarginine, and agmatine with enormous physiological significance (Agostinelli 2020; Wu et al. 2013a). Quantitatively, a large amount of dietary Arg (2.3 g/day) is used to synthesize creatine (a substance that is highly abundant in skeletal muscle and brain and is crucial for energy metabolism in the excitable tissues; Wu 2020) in healthy adult humans (Wu and Morris 1998). Thus, Arg requirements by the fetus, young animals, and adults are particularly high and adequate Arg intake is crucial for health. In support of this view, animal studies have shown close links between abnormal Arg metabolism and the pathogenesis of compromised pregnancy or aberrant fetal programming (Wu et al. 2013a; Herring et al. 2018; Hsu and Tain 2019). Second, Arg is required for maintaining hepatic urea synthesis in an active state (Morris 2006). Consequently, humans (particularly infants) within defects in Arg synthesis via the intestinal-renal axis must be supplemented with Arg to prevent ammonia toxicity and hyperammonemia-induced death. Third, Arg stimulates the secretion of growth hormone and insulin, promotes the production of polyamines (putrescine, spermidine, and spermine) and homoarginine, and also activates cellular mechanistic target of rapamycin (MTOR) and focal adhesion kinase cell signaling pathways in mammals, thereby playing an important role in regulating protein synthesis and degradation, cell proliferation and migration, wound healing, spermatogenesis, sperm quality, conceptus survival and growth, and the production of milk proteins (Bazer et al. 2015; Hou et al. 2016; Ma et al. 2018; Rhoads et al. 2004, 2007; Wu et al. 2009). Fourth, Arg inhibits the expression of pro-oxidative and lipogenic genes, while increasing the expression of genes related to mitochondrial biogenesis [e.g., peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α and AMP-activated protein kinase (AMPK), and glutathione synthase], development of brown adipose tissue, anti-oxidative responses, and increased oxidation of energy substrates (e.g., fatty acids and glucose) in a cell-specific manner (Fu et al. 2005; Jobgen et al. 2009a, b; Ma et al. 2017; McKnight et al. 2010). Arg may have a synergistic effect with interferon tau [a protein for pregnancy recognition in ruminant (Bazer et al. 2020)] to enhance the mass of brown adipose tissue in obese animals (Posey et al. 2019).

Compelling evidence shows that dietary supplementation with Arg is beneficial in improving immunity, neurological function, wound healing, nutrient absorption, mitochondrial biogenesis, skeletal muscle function, and insulin sensitivity (Chen et al. 2010; Flynn et al. 2002; Hoang et al. 2013; McKnight et al. 2010; Rhoads and Wu 2009; Wu et al. 2009). There are also reports that oral administration of Arg can reduce hyperglycemia, dyslipidemia, obesity, high blood pressure, atherosclerosis, fertility (e.g., improved sperm quality in men, as well as improved ovulation and egg quality in women), embryonic and fetal deaths, pre-eclampsia, infections, and intestinal inflammation (Alexander and Supp 2014; Dorniak-Wall et al. 2014; Hadi et al. 2019; Mariotti 2020; Maxwell and Cooke 2001;

Table 10.1 Roles of L-arginine in growth, health, and disease

Roles of L-arginine	Effect	Mediators
Cardiovascular health and disorders		
Vascular vasodilatation	↑	NO
Coronary and peripheral arterial diseases	↓	NO
Heart failure, stroke, and ischemia/reperfusion injury	↓	NO
Sickle cell anemia and vasculopathy	↓	NO
Endothelial dysfunction in patients with CVRF		
Aging and hyperhomocysteinemia	↓	NO
Diabetes, hypertension, and smoking	↓	NO
Hypercholesterolemia and high-fat feeding	↓	NO
Hormone secretion		
Growth hormone, glucagon, insulin, and prolactin	↑	NO and ornithine
Placental lactogen and progesterone	↑	NO and ornithine
Immune function		
B-cell maturation and antibody production	↑	NO, PA, and PS
Killing pathogens (bacteria, fungi, parasites, and virus)	↑	NO
T-cell proliferation and cytokine production	↑	NO, PA, and PS
Metabolism		
BAT growth and energy-substrate oxidation	↑	cGMP, PA, cAMP, and NO
Cell signaling (AMPK, MTOR, and cGMP)	↑	NO and Arg
Lactogenesis and neonatal growth and development	↑	Arg, NO, MTOR, PA, and proline
Mitochondrial biogenesis and function	↑	cGMP, PA, and NO
Expression of anti-oxidative genes	↑	mTOR, arginine, and NO
Transport of glucose by skeletal muscle	↑	cGMP and NO
Oxidation of fatty acid and glucose	↑	cGMP, NO, and homoarginine
Protein synthesis and muscle growth	↑	MTOR, PA, and creatine
Water transport across the cell membrane	↑	NO, cGPM, and cAMP
Ammonia detoxification via the urea cycle	↓	Arg, NAG, and ornithine
Obesity, insulin resistance, and dyslipidemia	↓	AMPK, Arg, and NO
Orotic aciduria and gout	↓	NAG and ornithine
Production of ROS and oxidative stress	↓	Arg, creatine, GSH, PA, and NO
Protein degradation and apoptosis; muscle atrophy	↓	MTOR, NO, and autophagy
Expression of anti-oxidative genes	↓	Arginine and NO
Reproduction		
Embryo implantation, survival, and growth	↑	NO, PA, PS, and mTOR
Fetal survival, growth, and health	↑	NO, PA, PS, and MTOR
Ovulation, ovarian steroidogenesis, and oocyte quality	↑	NO and PA
Placental angiogenesis, growth, and function	↑	NO, PA, PS, and MTOR
Spermatogenesis, sperm quality, and male fertility	↑	NO, PA, and PS
Uterine contractility and preterm labor	↓	NO

(continued)

Table 10.1 (continued)

Roles of L-arginine	Effect	Mediators
Erectile dysfunction in men	↓	NO
Pre-eclampsia in human pregnancy and animal models	↓	NO
Brain function	↑	Creatine, NO, and PS
Skeletal-muscle function		
Blood flow	↑	NO
Exercise performance	↑	NO and creatine
Lean muscle mass	↑	Net PS (increased PS, decreased PD)
Tissue injury and repair		
Cystic fibrosis and lung injury	↓	NO, PA, and proline
Gastrointestinal, liver, and muscle injury	↓	NO, PA, proline, and FAK
Necrotizing enterocolitis in infants	↓	NO, PS, PD, and PA
Renal disease with systemic hypertension	↓	NO
Severe malaria, ulcers, and mitochondrial myopathy	↓	NO
Tissue integrity, wound healing, and angiogenesis	↑	NO, PA, proline, and PS
Tumor growth		
Tumorigenesis at early stages	↓	NO
Tumorigenesis at late stages	↑	PA, proline, ornithine, and PS

The symbols "↑"and "↓" denote enhancement and inhibition (or prevention), respectively. *AMPK* AMP-activated protein kinase; *BAT* brown adipose tissue; *CVRF*, cardiovascular risk factors; *FAK* focal adhesion kinase; *GSH* glutathione; *MTOR* mechanistic target of rapamycin (protein kinase); *NAG* N-acetylglutamate; *NO* nitric oxide; *PD* protein breakdown; *PA* polyamines; *PS* protein synthesis; *ROS* reactive oxygen species. Adapted from Flynn et al. (2002) and Wu et al. (2009, 2013a)

Stanislavov and Rohdewald 2014; Wu 2013a). Studies with young pigs have revealed a beneficial effect of Arg supplementation on increasing muscle growth and reducing the incidence of weaning-induced diarrhea (Shan et al. 2012; Wu et al. 2010). In the face of the global obesity epidemic, much attention has been directed to the use of Arg to improve metabolic health in individuals. The proposed biochemical mechanisms for the effect of Arg in reducing obesity are illustrated in Fig. 10.2.

The actions of Arg on the vasculature and other tissues are mediated by both NO-dependent and independent mechanisms (Förstermann and Sessa 2012; Wu and Meininger 2002). Specifically, NO inhibits leukocyte adhesion, platelet aggregation, superoxide generation, the expression of vascular cell adhesion molecules and monocyte chemotactic peptides, proliferation of smooth muscle cells, and the release of endothelin-1 (a vasoconstrictor). Thus, NO is a vasodilatory, anti-atherogenic, anti-proliferative, and antithrombotic factor in the cardiovascular system. In addition to its role in vascular vasodilatation, NO also regulates neurotransmission, host immunity, nutrient and energy metabolism, and whole-body homeostasis (Bronte and Zanovello 2005; Ignarro et al. 1999; Ren et al. 2018). In this regard, it is noteworthy that NO kills pathogens, such as bacteria, fungi, parasites, and viruses [including coronaviruses, such as severe acute respiratory syndrome coronavirus (SARS-CoV; Åkerström et al. 2005) and coronavirus disease-2019 (COVID-19) virus, also known as SARS-CoV-2; Akaberi et al. 2020)]. Physiological levels of NO exert their effects by stimulating guanylyl cyclase to produce cGMP (Ignarro et al. 1999), activate cell signaling pathways (Jobgen et al. 2006), inhibit the activity of aconitase (an enzyme of the Krebs cycle), and regulate gene expression to modulate nutrient metabolism (Nisoli et al. 2003).

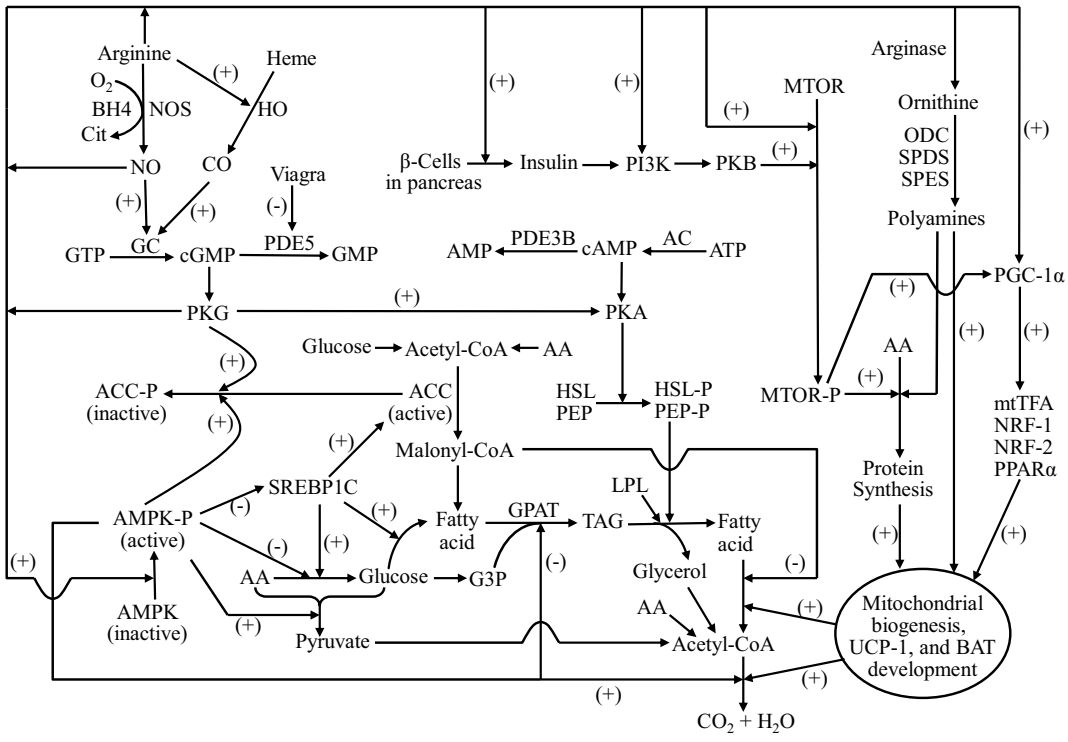

Fig. 10.2 Proposed mechanisms whereby L-arginine enhances substrate oxidation and reduces adiposity via cGMP, cAMP, and AMP-activated protein kinase (AMPK) signaling pathways in humans. L-arginine increases the expression of key proteins and enzymes (e.g., AMPK and PGC-1α) responsible for mitochondrial biogenesis in brown adipose tissue and substrate oxidation in insulin-sensitive tissues (e.g., skeletal muscle, liver, and white adipose tissue). In addition, L-arginine enhances cAMP production and cAMP-activated hormone-sensitive lipase activity in white adipose tissue. Through these multiple mechanisms, L-arginine stimulates lipolysis in white adipose tissue to release fatty acids, and increases the oxidation of fatty acids to CO_2 in the liver and skeletal muscle, thereby reducing excess fat mass in obese subjects. Abbreviations: AA, amino acids; AC, adenylyl cyclase; ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; BH4, tetrahydrobiopterin; Cit, citrulline; GC, guanylyl cyclase; G3P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; HO, heme oxygenase; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; MTOR, mammalian target of rapamycin; mtTFA, mitochondrial transcription factor A; NO, nitric oxide; NOS, nitric oxide synthase; NRF, nuclear respiration factor; ODC, ornithine decarboxylase; PDE5, phosphodiesterase 5; PDE3B, phosphodiesterase 3B; PEP, perilipins; PGC-1α, peroxisome proliferator-activated receptor (PPAR-) coactivator 1α; PKA, cAMP-dependent protein kinase A; PKG, cGMP-dependent protein kinase G; PPAR, peroxisome proliferator-activated receptor α; SPDS, spermidine synthase; SPES, spermine synthase; SREBP-1c, sterol regulatory element binding protein-1c; TAG, triacylglycerols. Reproduced, with permission, from Wu (2013). Amino Acids: Biochemistry and Nutrition. CRC Press, Boca Raton

10.4 Dietary Requirements of Humans for Arg for Optimal Health

Food contains various amounts of Arg primarily in the form of protein (Table 10.2). By contrast, free Arg and free L-citrulline are highly abundant in watermelon (e.g., 1150 and 2014 mg/L in watermelon pomace juice, respectively; Wu et al. 2007). Peptide (protein)-bound Arg is abundant in meat, eggs, soybean, peanuts, and pistachio nuts (Hou et al. 2019; Li and Wu 2020). About 40% of dietary Arg is metabolized in the small intestine during the first pass into the portal blood in humans (Castillo et al. 1993) and animals (Wu

Table 10.2 Content of total (free plus peptide-bound) amino acids in staple foods commonly consumed by US adults

Food	Arginine	Cysteine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Proline	Valine
Corn grain	4.34	2.17	2.68	3.85	12.8	2.85	2.26	11.3	5.072
Peanut	35.5	4.15	6.98	10.8	18.1	9.98	3.43	16.5	12.7
Pistachio nut	23.8	3.56	5.30	10.2	16.9	11.9	3.80	12.9	13.2
Potato	5.15	1.14	1.70	3.19	4.72	5.13	1.53	3.02	4.98
Soybean	37.2	7.67	12.7	23.9	40.0	32.6	6.43	28.4	24.6
Sweet potato	3.08	1.63	1.18	3.31	4.88	3.61	1.22	2.64	4.46
Wheat flour	7.20	3.04	3.54	4.92	10.0	3.64	2.48	23.2	6.24
White rice	7.28	1.66	2.20	3.74	7.26	2.33	2.02	5.41	5.19
Meat[b]	52.4	11.2	31.7	41.1	66.7	72.0	25.3	32.9	47.4
Chicken egg	30.7	10.7	12.0	24.3	41.5	33.4	14.9	18.3	29.9
Dairy product	5.60	1.36	4.64	8.14	15.9	12.4	4.46	18.4	10.1

[a]Taken from Hou et al. (2019), Wu (2021), and Wu et al. (2016b). Values are expressed as mg/g of dry matter. All the amino acids presented in this table are L-isomers
[b]Loin cuts of beef
DM, dry matter.

2009; Wu et al. 2016a). Results of the third National Health and Nutrition Examination Survey (NHANES) indicate that mean Arg intake for the US adult population is 4.4 g/day, with 25, 20, and 10% of people consuming <2.6 (suboptimal), 5 to 7.5, and >7.5 g/person/day, respectively (King et al. 2008). Consistent with the NHANES data, we found that dietary Arg intakes by healthy US adult men and women were 4.6–6.8 and 4.3–5.0 g/person/day, respectively (McNeal et al. 2018) based on data from our study that included a food frequency questionnaire and estimates of the nutritional analyses (www.Harvardsffq.date). These amounts are equivalent to approximately 84 and 96 mg Arg/kg lean body mass/day in men and women, respectively.

While it was traditionally believed that Arg was adequately synthesized in adult humans and was not needed in their diets (see Hou et al. 2015 for review), a deficiency of Arg in the diet severely impairs the reproductive functions of adult men and women (Wu et al. 2000, 2009, 2013a), while potentially causing cardiovascular dysfunction (Kamada et al. 2001; Wu and Meininger 2000, 2009) and skeletal-muscle abnormalities including atrophy (Burns et al. 1981; Hnia et al. 2008). Although starvation can lead to a reduction in the circulating level of Arg in humans, it is a much rarer cause of low Arg than elevated Arg catabolism (Morris et al. 2017). One condition especially linked to the enhanced degradation of Arg via the arginase pathway is hemolysis, which accompanies various NO-consuming diseases, including malaria, thalassemia, and SCD (Morris et al. 2017). Although less than 1% of dietary Arg is used for whole-body NO synthesis in healthy adult humans, an adequate amount of dietary Arg is required to maintain NO homeostasis in the body (Wu and Morris 1998).

Human infants or adults do not synthesize sufficient Arg from glutamine/glutamate and proline via the intestinal-renal axis (Becker et al. 2000; Rhoads et al. 2005; Wu et al. 2000). Thus, both newborn and adult humans, as well as non-primate mammals (e.g., swine), do have dietary needs for Arg for optimal growth, development, health, lactation, and fertility (Wu et al. 2013b). Much evidence indicates that healthy adult humans can well tolerate oral administration of 6 g supplemental Arg/day beyond Arg intake from regular diets (Maxwell and Cooke 2001; McNeal et al. 2016; Shao and Hathcock 2008).

Healthy adults with normal gastrointestinal function can even tolerate 30 g Arg (provided as Arg-HCl) per day in equally divided doses for at least 90 days (McNeal et al. 2018). Thus, although Arg had long been thought to be a nutritionally nonessential AA (IOM 2005), this term is now recognized as inaccurate in nutritional science (Hou and Wu 2017).

10.5 Determination of NO Synthesis by Cells and Whole-Body NO Synthesis

NO has a short half-life in oxygenated aqueous solution (e.g., <5 s in cell culture medium and in blood) (Baylis and Vallance 1998). Thus, it is technically difficult to directly measure NO that is synthesized from Arg in isolated cells or in the body. However, NO is oxidized to form nitrite and then nitrate in cells and blood. Because nitrite and nitrate are stable at neutral pH (e.g., 7.0 and 7.4), they can be used as an indicator of NO synthesis in a biological system if the exogenous provision of nitrite plus nitrate is low or negligible (Tsikas et al. 2006). Of note, nitrite and nitrate are quantitatively excreted in the urine of humans and other mammals, with the amount of nitrate being approximately 100 times more abundant than nitrite (Jobgen et al. 2007). For example, approximately 52% of the nitrate that enters the blood circulation appears in the urine (Houben et al. 2010). In healthy adults (the mean BW of 69 kg) consuming 0.8 g high-quality protein/kg BW per day (dietary nitrate intake = 180 μmol), endogenously synthesized nitrate that appears in the urine is 510 μmol/day (Green et al. 1981). Besides the nitrate formed from the Arg-derived NO, the diet (foods, drinking water, and beverages), ambient air, and intestinal bacteria are sources of the nitrate in the urine (Fig. 10.3). The average US adult ingests 1600 μmol nitrate daily from the typical diet (Green et al. 1981). Thus, when the diet is not controlled for nitrite or nitrate, nitrite plus nitrate in the plasma and urine are not a valid indicator of systemic NO synthesis in the body (Baylis and Vallance 1998; Evans et al. 2004).

Several methods are available to assess NO synthesis in humans, depending on the availability of analytical instruments and study objectives. These techniques include the: (1) conversion of [guanidino-^{15}N]arginine into [^{15}N]nitrite plus [^{15}N]nitrate (stable isotope approach) (Tsikas et al. 2006; Siervo et al. 2011); (2) accumulation of nitrite plus nitrate in the 24-h urine if exogenous provision of nitrite plus nitrate is low or negligible (Jobgen et al. 2007); (3) endothelium-dependent vasodilation of a blood vessel (e.g., brachial artery; Clarkson et al. 1996); (4) rate of NO-mediated blood flow in a blood vessel (e.g., brachial artery) (Monti et al. 2013); and (5) changes in systolic or diastolic blood pressure (Siani et al. 2000).

10.6 Effects of Oral Arg Supplementation on NO Production in Humans

The half-life of Arg in the blood of healthy humans is about 1 h (Bode-Boger et al. 1998; Schwedhelm et al. 2007). This knowledge is important for the frequency and dosing of Arg administration to effectively maintain an elevated concentration of Arg in plasma, as well as for the measurement of Arg in plasma under fed or fasting conditions. Some reports indicated that oral Arg supplementation did not increase NO production in the whole body of healthy adults (e.g., Alvares et al. 2012a; Fayh et al. 2013; Liu et al. 2009; Vanhatalo et al. 2013). However, a careful analysis of those studies led to the following common concerns. First, the diets (including food, drinking water, and beverages) consumed by the study subjects were not controlled for nitrite or nitrate; and therefore, the concentrations of nitrite plus nitrate in the serum (or plasma) or urine were not valid indicators of whole-body NO synthesis or endothelial NO synthesis. High dietary intakes of nitrite and nitrate may mask a stimulatory effect of dietary Arg on whole-body NO synthesis in subjects (Fig. 10.3). Second, the authors did not determine dietary intake of any AAs (including Arg) and other nutrients that are known to affect

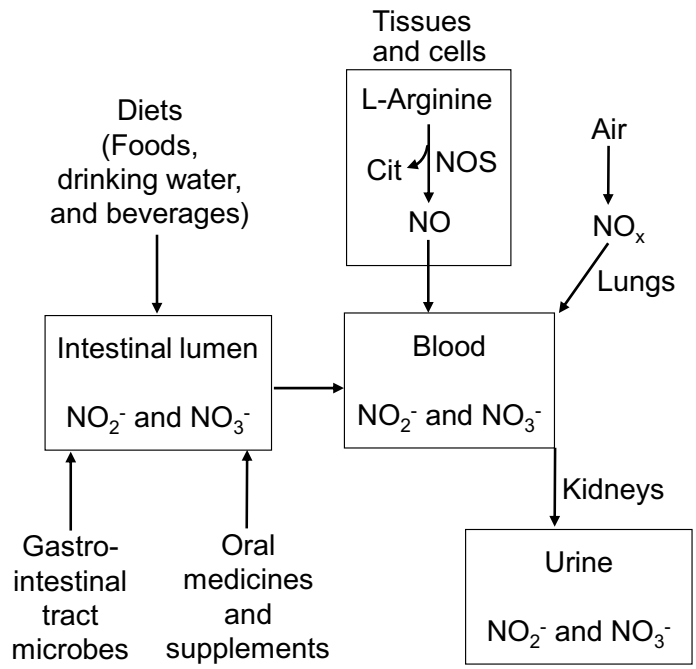

Fig. 10.3 Exogenous and endogenous sources of nitrite and nitrate in the blood and urine of humans. Nitric oxide (NO) synthase produces a relatively small amount of NO in tissues and cells. NO is oxidized primarily in the blood to nitrite and nitrate. Nitrogen oxide (NO_x) may be inhaled through the lungs and may appear as nitrite and nitrate in the blood. Other sources of nitrite and nitrate in the body are diets (including drinks), oral medicines and supplements, and microbes in the gastrointestinal tract. Diet that is not controlled for nitrite or nitrate is the major source of blood and urinary nitrite and nitrate. Levels of nitrite and nitrate in the plasma and urine, when measured as an indicator of whole-body NO production, may mask a stimulatory effect of dietary arginine intake on endogenous NO synthesis in subjects consuming diets with high amounts of nitrite and nitrate

endothelial NO synthesis (Wu and Meininger 2002). For example, Arg, taurine, polyunsaturated long-chain fatty acids, vitamin A, vitamin C, vitamin E, folic acid, and calcium stimulate, but lysine, glutamine, glucosamine, and saturated long-chain fatty acids) inhibit, endothelial NO synthesis (Wu and Meininger 2002). Third, 24-h urine collection was not performed by these authors (e.g., Alvares et al. 2012a; Fayh et al. 2013; Liu et al. 2009; Vanhatalo et al. 2013) and, thus, the total amounts of nitrite plus nitrate in the urine excreted by the study subjects could not be determined. Finally, very large variations in the measured metabolites existed among the study subjects, and the sample size may not be sufficient to detect statistical significance. Meaningful conclusions from studies critically depend on their sound designs.

Well-designed studies involving tracers and bioassays support the notion that oral Arg supplementation increases NO production in the whole body and vasculature of healthy adults. For example, in the study by Mariotti et al. (2013), nine healthy young men in a post-absorptive state (after an overnight fast) consumed a liquid meal containing 50 g AAs (including 1.73 g Arg and [^{15}N-^{15}N-guanido] arginine as a tracer) supplemented with or without 3 g Arg, followed by the collection of blood samples every hour and of urine samples every 2 h for 8 h. [^{15}N]Arginine in plasma, [^{15}N]urea in plasma and urine, and [^{15}N]nitrate in urine were determined using mass spectrometry. Data were analyzed using a compartmental modeling approach to calculate the conversion of Arg into urea and NO in the whole body. Results of this

study support the earlier notion that Arg metabolism via the arginase and NOS pathways is highly complex in mammals, including humans (Wu and Morris 1998). Specifically, about 40% of dietary Arg is metabolized in the small intestine and cannot enter the systemic circulation, whereas Arg is synthesized in the kidneys, endothelial cells, and other cell types from citrulline that is formed in enterocytes from dietary glutamine, glutamate, and proline as well as arterial glutamine (Wu and Morris 1998). Furthermore, there is an Arg-citrulline cycle to convert the Arg-derived citrulline into Arg via argininosuccinate synthase and lyase at the expense of aspartate and ATP in all mammalian cells, including macrophages and endothelial cells (Morris 2006; Wu 2013). Thus, dietary Arg and plasma Arg have different metabolic pathways. Using an integrative methodology, Mariotti et al. (2013) provided strong evidence that dietary supplementation with 3 g Arg within the physiological range increased whole-body NO synthesis by nearly 100% in healthy adults.

In another study (Monti et al. 2013), seven healthy subjects (2 males and 5 females; a mean BW of 62 kg) consumed a diet containing 1900 kcal/day energy, 68 g/day protein (including 2.6 g Arg), and other necessary nutrients. Subjects underwent three different tests in random order with at least 14 days between tests. For a test, the subjects consumed either Arg-enriched biscuits (providing 171 kcal energy, 21.9 g digestible carbohydrates, 3.6 g protein, 6.6 g Arg, 7.5 g fat, and 4.3 g dietary fiber per biscuit), placebo biscuits, or 6.6 g Arg powder after an overnight fast. All subjects drank 250 mL natural water. Blood samples were taken at 0, 30, 60, 90, 120, 180, and 240 min for analysis of Arg, nitrite plus nitrate, and cGMP. Forearm blood flow (a bioassay for NO synthesis by endothelial cells and NO bioavailability) was measured using the strain-gauge venous occlusion plethysmography every 60 min for 240 min. Results of this study indicated that dietary supplementation of 6.6 g Arg in the form of a biscuit or pure powder similarly increased concentrations of Arg (at 90–240 min), nitrite plus nitrate (at 120 min), and cGMP (at 60 min) in the plasma of healthy subjects by 55%, 42%, and 48%, respectively, when compared to the placebo group. Additionally, the rate of forearm blood flow in the subjects supplemented with Arg powder was increased by 28% and 25% at 120–125 and 180–185 min post-administration, respectively. The values for skeletal-muscle blood flow were similar between Arg-enriched biscuit and Arg-powder groups.

In support of findings from the above in vivo studies, results of a cross-sectional study involving 2771 men and women indicated that dietary Arg intake was positively associated with serum concentrations of NOx, and this association was significantly influenced by sex, age, body mass index, and hypertension status (Mirmiran et al. 2016). Likewise, the oral administration of 5 g Arg/day substantially increased the urinary excretion of nitrite plus nitrate in patients with erectile dysfunction (Chen et al. 1999). Furthermore, dietary supplementation with 10 g Arg to patients with coronary artery disease for 3 or 6 months enhanced whole-body NO synthesis (as indicated by increased urinary levels of nitrate; Schneider et al. 2015). Taken together, these findings support the conclusion that dietary Arg supplementation increases NO synthesis by both healthy adults and individuals with cardiovascular dysfunction (Wu and Meininger 2002).

10.7 Effects of Oral Arg Supplementation on Enhancing Blood Flow to Skeletal Muscle in Humans

In postnatal mammals (including humans), deoxygenated blood flows from the body into the right atrium and then into the right ventricle. The right ventricle pumps blood through the pulmonary artery into the lungs for gas exchange. Oxygenated blood from the lungs flows into the left atrium and then the left ventricle, and finally, through the aorta and its branches, to various parts of the body. The rate of blood flow depends on blood volume and the diameter of the blood vessel. Namely, blood flow = V × A, where V = blood volume and A = the cross-sectional

area of the blood vessel. Vasodilators (e.g., NO, adenosine, and nitroglycerin) relax the smooth muscle of the blood vessels, thereby increasing the diameter of the lumen of blood vessels and blood flow.

There is evidence that oral Arg supplementation increases blood flow to skeletal muscle in healthy adults, as reported for rats (Ohta et al. 2007). For example, Alvares et al. (2012b) found that acute Arg supplementation augmented blood volume in muscle, which is an indicator of improved blood flow to skeletal muscle. In addition, as noted previously, Monti et al. (2013) demonstrated that oral administration of 6.6 g Arg enhanced the rate of blood flow to the forearm of adult humans. Similar results were reported by Marchesi et al. (2001). In this study, seven young (23±3 years; mean ± SD) healthy males, physically active, without cardiovascular risk conditions, consumed breads and grains daily, as well as fish and poultry weekly. Olive oil was the principal lipid, and their total lipid consumption ranged between 25 and 35% of energy intake, with saturated fats providing no more than 8% of total calories. After an overnight fast, a high-lipid load was administrated and then flow-mediated vasodilation of the brachial artery in the upper arm was measured at baseline and 2, 4, and 6 h. This provided data on muscle blood flow before Arg supplementation. Thereafter, the study subjects continued to consume the same diets and received oral administration of 6 g Arg (2 g in each dosing, three times a day) for 10 days. On the 11th day, after an oral lipid load, flow-mediated vasodilation of the brachial artery in the upper arm was measured at baseline and 2, 4, and 6 h. Of particular note, the rate of muscle blood flow was markedly reduced at 2 and 4 h after an oral lipid load, which was associated with an increase in plasma concentrations of triglycerides. Compared with the value at 2 h after an oral lipid load, Arg supplementation increased the rate of muscle blood flow by twofold at 4 and 6 h. Importantly, compared with the pre-supplementation value, oral administration of Arg greatly enhanced the rate of muscle blood flow by nearly 100%. These results indicate that the oral administration of Arg into healthy subjects consuming a high-lipid diet enhances muscle blood flow. Oral administration of 3 g Arg along with 15 g of nutritionally essential AAs to older men (about 70 years of age) improved microvascular perfusion in skeletal muscle, as compared with the subjects of similar age receiving 15 g of the nutritionally essential AAs without Arg (Mitchell et al. 2017). This is consistent with the conclusion that the oral administration of Arg can improve both aerobic and anaerobic performance in humans (Bailey et al. 2010; Santos et al. 2002; Viribay et al. 2020).

Some researchers reported that oral Arg supplementation did not increase blood flow to skeletal muscle in humans (Fahs et al. 2009; Fayh et al. 2013; Tang et al. 2011). A careful analysis of these studies reveals their methodological deficiencies. For example, very large variations in muscle blood flow existed among the study subjects. However, the authors did not do a statistical power calculation to identify an appropriate sample size based on the known variation in blood flow to muscles in humans. Thus, the sample size in all the studies was not sufficient to detect statistical significance or draw a definite conclusion. Furthermore, an inappropriate control was used in the studies, For example, in the study of Tang et al. (2011), the authors used glycine as the isonitrogenous control, which is inappropriate in nutritional studies because glycine regulates glutathione synthesis in cells (including endothelial cells), improves anti-oxidative capacity (Wang et al. 2013), enhances NO synthesis by nNOS, and stimulates muscle protein synthesis (Li et al. 2009; Sun et al. 2016). Additionally, the ratio of Arg:lysine in the protein drink used in the Tang's study was 10:1.3 = 7.7:1.0. This Arg:lysine ratio, which is twice the upper limit of 3.5:1.0 in the lumen of the small intestine, can inhibit lysine transport by the small intestine and induce an imbalance among basic AAs in the body (Wu et al. 2009). Furthermore, the pH of this Arg-supplemented solution (2.5% Arg) would yield a pH > 11.0 (Jobgen et al. 2009), thereby severely disturbing the acid–base balance in the intestine. Overall, the interpretation of the results from those studies

is seriously confounded, and no definite conclusion can be drawn to negate a potential effect of Arg on blood flow to muscles in healthy adults.

10.8 Effects of Oral Arg Supplementation on Enhancing Blood Flow to the Corpora Cavernosa of the Penis

NO relaxes the smooth muscle of corpora cavernosa in the penis, thereby contributing to the development and maintenance of an erection of the penis (Rajfer et al. 1992). There are reports that oral Arg supplementation enhanced blood flow to the corpora cavernosa of the penis. For example, in a randomized, double-blind, placebo-controlled study, 46 men with confirmed organic erectile dysfunction received oral administration of either 5 g Arg/day or placebo for 6 weeks (Chen et al. 1999). The amount of Arg or placebo was divided into three equal doses daily. Before and after the study, the patients completed a questionnaire related to sexual drive, erectile function, and overall sexual satisfaction. Results indicated that 31% of patients taking the Arg supplement reported a significant subjective improvement in erectile function (Chen et al. 1999). Interestingly, all the patients who reported subjective improvements initially had low urinary excretion of nitrite plus nitrate; and the values had doubled by the end of the study. Thus, dietary Arg supplementation may be more effective for ameliorating erectile dysfunction in patients with alterations in the endothelial Arg-NO pathway and a reduction in vascular NO availability.

In another study, 15 impotent men received oral administration of 2.8 g/day Arg or placebo (microcrystalline cellulose) for a 2-week period (Zorgniotti and Lizza 1994). The placebo was given first. The patients were asked to keep a diary of their sexual behavior. Forty percent of men (6/15) in the treatment group reported improvement in erectile function, compared to none in the placebo group (0/15). The positive responders were younger and had a better penile vascular structure than the non-responders. In support of this finding of improved erectile function, a meta-analysis of 10 different studies with a total of 540 patients indicated that oral administration of 1.5–5 g Arg could improve erectile function, as compared with the placebo group (Rhim et al. 2019). Likewise, dietary supplementation with 1.5 g L-citrulline per day augmented erectile hardness in patients with mild erectile dysfunction. As previously mentioned, L-citrulline can be converted into Arg for the synthesis of NO. These participants were given 1.5 g L-citrulline per day, and researchers noted that the supplement was safe and psychologically well-tolerated by the study participants, while improving their sexual function (Cormio et al. 2011). Similar results have been reported for oral administration of Arg plus Pycnogenol (an extract isolate from the French maritime pine bark; containing antioxidants such as procyanidins, bioflavonoids, and phenolic acids) or a combination of Arg, L-citrulline, Pycnogenol, and roburins [belonging to a class of tannins known as ellagitannins (antioxidants) that can be metabolized by intestinal bacteria to generate bioactive molecules called urolithins (Stanislavov and Rohdewald 2015)]. Thus, dietary supplementation with Arg or L-citrulline alone or in their combination with other bioactive substances may be beneficial for treating erectile dysfunction in men.

10.9 Effects of Oral Arg Supplementation on Alleviating Oxidative Stress and Associated Vascular Complications in Human Diseases

NO deficiency in the vasculature and other tissues occurs under a variety of diseased conditions (including obesity, diabetes, and hypertension) and in response to smoking, hypercholesterolemia, and oxidative stress, because of reduced NOS enzymatic activity and increased oxidation of NO by oxidants (Maxwell and Cooke 2001; Wu and Meininger 2009).

A severe deficiency of BH4 in endothelial cells is the major factor contributing to the impaired NO synthesis in the vasculature under diseased conditions (Hoang et al. 2013; Shi et al. 2004). This can be explained by reduced expression of GTP-CH1 and increased oxidation of BH4. Oxidative stress also reduces the availability of NADPH (another essential cofactor for NOS) in cells (Wu and Meininger 2009).

In sickle cell disease (SCD), painful crises are known to be associated with vaso-occlusion and low levels of NOx in the blood (Eleutério et al. 2019). Oral administration of Arg has been reported to be beneficial for the treatment of this disease. For example, Arg supplementation (1 g/day for 6 weeks) has been found to improve liver tests (indicated by decreased serum alanine aminotransaminase, and aspartate aminotransaminase activities) and reduce serum malondialdehyde levels (an index of oxidative injury) in individuals with SCD (Jaja et al. 2016). Notably, Morris et al. (2013) conducted a double-blinded placebo-controlled trial to determine the effects of Arg supplementation on 38 US children with SCD in vaso-occlusive crisis. The patients were randomized to receive oral Arg (100 mg/kg BW thrice daily) or placebo, and opioid requirements were recorded. The authors found a 54% reduction in total parenteral opioid use and 51% lower pain scores in the Arg-treated group. Importantly, in a similar clinical trial of Nigerian children with sickle cell crisis, oral Arg [100 mg Arg-HCl/kg body weight (BW), 3 times per day] reduced analgesic use and shortened time to crisis resolution (Onalo et al. 2021). Furthermore, in an adult SCD trial in Brazil, Arg (500 mg) plus hydroxyurea were used to increase urinary NOx over a 4 month time period; in that study, there were no changes in the number of crises or hospitalizations; however, there was a reduced frequency of pain episodes at 4 months (Eleutério et al. 2019). Interestingly, human red blood cells contain arginase (Yang et al. 2013), which degrades Arg and therefore, can reduce NO production (Morris 2006). Due to hemolysis and the injury of tissues, patients with SCD usually have an elevated arginase activity in their blood to degrade Arg (Morris et al. 2005). Therefore, oral or intravenous administration of Arg may not be highly effective to increase the circulating level of Arg in these subjects. This problem can be alleviated through the use of L-citrulline, which is effectively converted into Arg via argininosuccinate synthase and lyase in humans (Wu and Morris 1998).

One other condition in which Arg supplementation may be beneficial, although evidence is limited, is in the prevention of pre-eclampsia. This condition is associated with reduced uteroplacental perfusion, which leads to activation of mechanisms promoting maternal vasoconstriction and reduced blood flow to the placenta. The concentrations of Arg in the plasma of women with pre-eclampsia are very low (only about 20% of the value for healthy subjects; D'Aniello et al. 2001), possibly owing to reductions in maternal Arg intake and Arg synthesis coupled with increased Arg catabolism in maternal and fetal tissues. Of note, the oral administration of Arg (3.3 g twice per day; 6.6 g/day) plus antioxidant vitamins can reduce the incidence of pre-eclampsia, whereas antioxidant vitamins alone have no significant effect (Vadillo-Ortega et al. 2011). After reviewing seven randomized clinical trials, Dorniak-Wall et al. (2014) concluded that Arg supplementation may have a role in the prevention and/or treatment of pre-eclampsia.

In obese and diabetic patients, the concentration of Arg in plasma is reduced due to enhanced expression of arginase to degrade Arg and reduced activity of Arg-synthetic enzymes (Wu et al. 2009). In the heart and endothelial cells, Arg upregulates the expression of GTP-CH1, thereby increasing the generation of BH4 from GTP to stimulate NO synthesis (Shi et al. 2004; Wu et al. 2007). Furthermore, dietary Arg supplementation enhances the expression of antioxidative genes and glutathione-synthetic genes for glutathione production and concentrations in tissues, including the liver and white adipose tissue (Jobgen et al. 2009a, b). In contrast to the diseased state, the concentrations of Arg and cofactors of NOS (e.g., BH4 and NADPH) in cells are relatively constant and are adequate for NO production in normal individuals (Meininger and Wu 2011). Therefore, patients with

endothelial dysfunction respond more sensitively to dietary Arg supplementation than healthy subjects (Bode-Böger 2006; Clarkson et al. 1996; Förstermann and Sessa 2012; Luiking et al. 2012; McKnight et al. 2010; McNeal et al. 2009; Wu and Meininger 2000; Wu et al. 2000). For example, the oral administration of Arg reduced systolic and diastolic blood pressure (2.2–5.4 and 2.7–3.1 mmHg, respectively) in hypertensive adults, while decreasing the incidence of hospital-acquired infections by 40% and the length of stay in the hospital for surgical patients (McRae 2016). In addition, a meta-analysis of 11 double-blind, placebo-controlled clinical studies among 387 participants revealed reductions in both systolic and diastolic blood pressure (5.39 and 2.66 mmHg, respectively) among adults consuming 4–24 g supplemental Arg per day (Dong et al. 2011). Furthermore, dietary supplementation with Arg can maintain lean body mass, while reducing systolic blood pressure and serum glucose concentrations, in overweight or obese women, and can lower serum concentrations of free fatty acids (a risk factor cardiovascular disorders) in both men and women with a body mass index greater than 30 kg/m^2 (McNeal et al. 2018).

Duchenne muscular dystrophy is a disease resulting from mutations in dystrophin, a structural protein in muscle; eventually, these children develop profound weakness owing to muscle damage during contraction, inflammatory myopathy, necrosis, and fibrosis (Blake et al. 2002). This disease is associated with reduced NO synthesis in the whole body (Hörster et al. 2015). In one therapeutic study, 7 children aged 7–10 years with Duchenne muscular dystrophy were treated with Arg and metformin (which activates nNOS in human skeletal muscle) to increase intramuscular NO availability (Hafner et al. 2016). Sixteen weeks later, there was a decrease in muscle oxidative stress and reduced resting energy expenditure; more importantly, 4 of 5 ambulatory children had an increase in walking distance and motor function score (Hafner et al. 2016). A combined use of L-citrulline (3 × 5 g/day) plus metformin may also be promising to enhance NO synthesis and alleviate clinical syndromes in adults with muscular dystrophy (Hanff et al. 2018).

10.10 Effects of Oral Arg Supplementation on Enhancing Immunity in Humans and Mitigating the Current Global COVID-19 Pandemic

Dietary supplementation with Arg within the physiological range has long been known to enhance cell-mediated and humoral immunities (including the development and maturation of T-cells and B-cells, the production of cytokines and antibodies, and the phagocytosis of macrophages and NO generation) to kill invading pathogens in both humans and non-primate animals (Li et al. 2007; Popovic et al. 2007; Ren et al. 2013). Growing evidence supports this notion (Ren et al. 2018; Satoh et al. 2020). Thus, because NO is a killer of viruses (including SARS-CoV and SARS-CoV-2) and also maintains blood flow and oxygen supply to tissues (including the lungs, kidneys, and brain) and their functions, and because Arg-derived polyamines are essential for the recovery of tissues from injury, Arg along with overall AA nutrition is expected to play an important role in the prevention and treatment of COVID-19. Notably, AAs (e.g., L-glutamine and glycine) other than Arg are also known to reduce inflammation and enhance immunity in mammals (Li et al. 2007). Oral administration of an Arg-based nutritional cocktail would be highly significant, because as of May 15, 2021, more than 161 million COVID-19 cases have been confirmed with more than 3.35 million deaths attributed to COVID-19 worldwide since the disease was identified in December 2019 (Wikipedia 2021). There is a suggestion that a depletion of Arg can provide an effective therapeutic approach for individuals with COVID-19 as for cancer patients (Grimes et al. 2021). However, a deficiency of Arg will severely impair not only immune responses to

viruses but also blood flow to vital organs and whole-body homeostasis in the body. Thus, we propose that improving Arg nutrition through dietary supplementation of Arg and its precursor (e.g., L-citrulline) along with other AAs (e.g., L-glutamine and glycine) or consuming foods rich in Arg [e.g., beef (Wu et al. 2016b) and nuts (Hou et al. 2019)] or both Arg and L-citrulline [e.g., watermelon (Wu et al. 2007)] can reduce risk for coronaviruses and mitigate the current global COVID-19 pandemic. Clinical research is warranted to test this novel hypothesis. We are excited to note that a clinical trial is being conducted to evaluate short- and long-term effects of adding Arg to standard therapy in patients with COVID-19 (SARS-CoV-2; Fiorentino 2021).

10.11 L-Citrulline as an Effective Precursor of Arg in Humans

In humans, L-citrulline is synthesized de novo from glutamine/glutamate and proline in their enterocytes (Wu and Morris 1998). It is worthy to note that L-citrulline can successfully replace Arg for treating patients affected by the impaired absorption of Arg (e.g., humans with lysinuric protein intolerance) (Kamada et al. 2001). L-citrulline does not share the same transporter with Arg across the plasma membrane and the intracellular conversion of L-citrulline into Arg consumes ammonia in the form of aspartate (Wu and Meininger 2000). In mammals (including humans), L-citrulline is effectively used for Arg synthesis by the kidneys, endothelial cells, macrophages, smooth muscle cells, and other cell types (Wu and Morris 1998). Thus, urinary L-citrulline excretion accounts for <1% of the citrulline load (0.18 g/kg BW/day) filtered by kidneys in healthy adults (Rougé et al. 2007). Citrulline supplementation may be a more effective way to increase Arg concentration in plasma and the whole-body NO status, for example, in SCD, because L-citrulline is readily converted into Arg within cells. Furthermore, our studies with rats, pigs, and sheep have shown that L-citrulline has a much longer half-life than Arg in the blood and exogenously administered L-citrulline can sustain elevated concentrations of Arg in the plasma for a much longer period than exogenously administered Arg (Lassala et al. 2009; Wu 2021). This may also be true for humans.

10.12 Conclusion

In summary, humans have dietary requirements for Arg to meet functional needs, because this AA is inadequately synthesized in the body. There are myths about Arg nutrition in humans due to both insufficient knowledge and severely confounded results from inadequately designed clinical experiments (including inappropriate controls and a small sample size). Compelling evidence shows that oral administration of Arg within the physiological range can confer benefits in increasing NO synthesis in the vasculature, blood flow to muscles, and sexual function in humans. Furthermore, dietary supplementation with Arg can enhance immune and anti-oxidative responses, as well as gastrointestinal and pulmonary functions, ammonia detoxification, fertility, embryonic survival, wound healing, brown adipose tissue development, fatty acid and glucose oxidation, and lean tissue mass. Arg supplementation can beneficially ameliorate metabolic syndromes (including dyslipidemia, obesity, diabetes, and hypertension) and treat patients with SCD, muscular dystrophy, and pre-eclampsia. Finally, Arg-derived NO can kill pathogens, including the virus that causes COVID-19. The scientific evidence available to date supports the notion that Arg or L-citrulline offers promise in improving the health and well-being of both men and women.

Acknowledgements We thank International Council of Amino Acid Science (Brussels, Belgium) for the financial support of our research on arginine safety and metabolism in adult humans.

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Composition of Amino Acids in Foodstuffs for Humans and Animals

11

Peng Li, Wenliang He, and Guoyao Wu

Abstract

Amino acids (AAs) are the building blocks of proteins that have both structural and metabolic functions in humans and other animals. In mammals, birds, fish, and crustaceans, proteinogenic AAs are alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. All animals can synthesize de novo alanine, asparagine, aspartate, glutamate, glutamine, glycine, proline, and serine, whereas most mammals (including humans and pigs) can synthesize de novo arginine. Results of extensive research over the past three decades have shown that humans and other animals have dietary requirements for AAs that are synthesizable de novo in animal cells. Recent advances in analytical methods have allowed us to determine all proteinogenic AAs in foods consumed by humans, livestock, poultry, fish, and crustaceans. Both plant- and animal-sourced foods contain high amounts of glutamate, glutamine, aspartate, asparagine, and branched-chain AAs. Cysteine, glycine, lysine, methionine, proline, threonine, and tryptophan generally occur in low amounts in plant products but are enriched in animal products. In addition, taurine and creatine (essential for the integrity and function of tissues) are absent from plants but are abundant in meat and present in all animal-sourced foods. A combination of plant- and animal products is desirable for the healthy diets of humans and omnivorous animals. Furthermore, animal-sourced feedstuffs can be included in the diets of farm and companion animals to cost-effectively improve their growth performance, feed efficiency, and productivity, while helping to sustain the global animal agriculture (including aquaculture).

Keywords

Diets · Foodstuffs · Functional amino acids · Health · Metabolism · Nutrition

Abbreviations

AA	Amino acids
AASA	An amino acid that is synthesizable de novo in animal cells
BCAA	Branched-chain amino acid
EAA	Nutritionally essential amino acid

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1332, https://doi.org/10.1007/978-3-030-74180-8_11

IOM Institute of medicine
NRC National research council
SAA Sulfur-containing amino acids

11.1 Introduction

Amino acids (AAs) are constituents of proteins and peptides in foodstuffs for humans and animals. Free AAs (e.g., taurine and β-alanine) with physiological and nutritional significance are also present in various kinds of foods (Wu 2018, 2021). The 20 canonical proteinogenic AAs are alanine (Ala), arginine (Arg), asparagine (Asn), aspartate (Asp), cysteine (Cys), glutamate (Glu), glutamine (Gln), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), and valine (Val). All animals are not capable of forming the carbon skeletons of Cys, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr, and Val, and therefore cannot synthesize de novo these 11 AAs; these AAs were traditionally defined as nutritionally essential AAs (EAAs; Wu 2013). De novo synthesis of Arg occurs in most mammals (including humans, pigs, and rats) but not in cats, ferrets, mink, tigers, birds, and perhaps carnivorous fish (Li et al. 2021c; Wu 2021). Plant and animal proteins differ in the composition and amounts of AAs, as well as their digestibilities (Wu 2021). A proper balances of dietary AAs is essential for optimum growth, development, and health in both humans and animals. Thus, a database on the composition of AAs in foodstuffs is much needed to facilitate the formulation of animal diets and to guide the consumption of foods by freely living individuals.

Although the content of AAs that are synthesizable de novo in animal cells (AASAs) had long been considered to be nutritionally nonessential and ignored in chemical analysis and dietary requirements (IOM 2005; NRC 2012), these AAs are both nutritional and physiological essential for mammals (Hou et al. 2015; Manjarin et al. 2020; Zhang et al. 2021), birds (He et al. 2021a), fish (Jia et al. 2021; Li et al. 2020a), and crustaceans (Li and Wu 2020a; Li et al. 2020b). All animals can synthesize Asp, Asn, Glu, Gln, Gly, Pro, and Ser, while converting Met into Cys, Arg into Pro, and Phe into Tyr. The term "nutritionally nonessential AAs" has now been recognized as a misnomer in nutritional sciences (Hou and Wu 2017). Humans and animals do have dietary requirements for both EAAs and AASAs (Hou et al. 2016; Wu 2016; Wu et al. 2013). Some nonproteinogenic AAs (e.g., citrulline, ornithine, taurine, 4-hydroxyproline, and β-alanine) have important physiological functions (Wu 2013), and taurine is essential for retinal function and integrity Wu 2020b). Over the past decade, we have determined the composition of AAs in staple foodstuffs commonly consumed by humans (Hou et al. 2019; Shanely et al. 2020) and animals (Li et al. 2011; Li and Wu 2020a, b). The major objective of the present article is to highlight these data for dietary formulation and nutritional research.

11.2 Analysis of AAs in Foodstuffs

AAs occur in foodstuffs as the constituents of proteins and peptides, and also in the free form (Wu 2013). A scheme for the preparation of free AAs, peptides, and proteins from foods, cells, or tissues using 1.5 M $HClO_4$ and centrifugation is outlined in Fig. 11.1. Many methods have been used to analyze free AAs, including gas, ion-exchange, and liquid chromatographies, as well as mass spectrometry (Silvestre 1997; Thannhauser et al. 1998; Tsao and Otter 1999; Wu 1993; Wu and Knabe 1994). To date, liquid chromatography is more widely used than gas chromatography for AA analysis. Most chromatographic procedures involve pre- or post-column derivatization with reagents that give rise to fluorescent or colorimetric AA derivatives for appropriate detection. Among liquid chromatography methods, high-performance liquid chromatography (HPLC) involving the pre-column derivatization with *o*-phthaldialdehyde (OPA) is most popular for the analysis of free AAs in foods, cells, and tissues (Dai et al. 2014).

To analyze AAs in proteins and peptides, the polymers must first be completely hydrolyzed by appropriate acid, alkaline, or enzymatic methods.

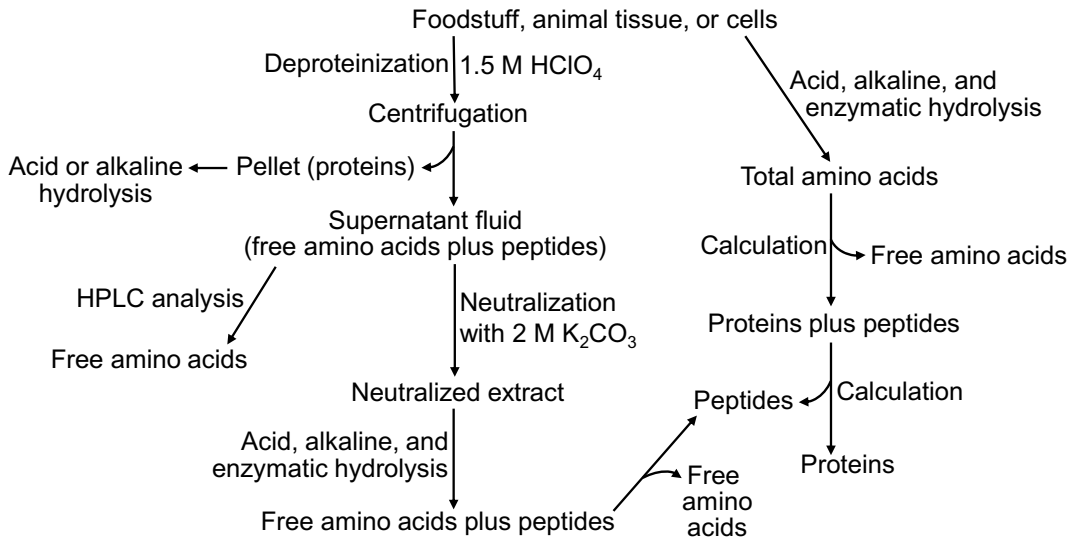

Fig. 11.1 Workflow of the HPLC analysis of free amino acids (AAs), as well as AAs in peptides and proteins. A sample (e.g., foodstuff, animal tissue, or cells) is deproteinized with 1.5 M $HClO_4$, (perchloric acid, PCA), followed by centrifugation (600× *g* for 15 min) and the collection of the supernatant fluid for the HPLC analysis of its free AAs. Peptides in the neutralized supernatant fluid are hydrolyzed to free AAs through an acid, alkaline, or enzymatic method for the calculation of the amounts of AAs in peptides, namely the differences in AAs before and after the peptide hydrolysis. To determine the amounts of AAs in proteins, the sample (e.g., foodstuff, animal tissue, or cells) undergoes acid, alkaline, or enzymatic hydrolysis, followed by the HPLC analysis of total AAs. Through the subtraction of free AAs and AAs in peptides, the amounts of AAs in proteins can be calculated

The standard method for the acid hydrolysis of proteins involves 6 M HCl at 110 °C for 24 h (Dai et al. 2014). This procedure results in the complete conversion of Gln and Asn into Glu and Asp, respectively, as well as the complete destruction of Trp (Wu et al. 1999). By contrast, there is little loss of Trp when proteins are hydrolyzed with alkaline solution (e.g., 4.2 M NaOH at 105 °C for 20 h in the presence of 1% thiodiglycol). Furthermore, a mixture of proteases and peptidases (e.g., pronase E, prolidase, pyroglutamate aminopeptidase, carboxypeptidase A, and aminopeptidase M) are now routinely used to analyze Glu, Gln, Asp and Asn in proteins (Li and Wu 2020a; b). Figure 11.2 outlines the workflow for protein hydrolysis and the subsequent analysis of AAs by our HPLC method involving pre-column derivatizaion and fluorescence detection of AA-OPA derivatives (Wu and Meininger 2008).

11.3 Composition of AAs in Foodstuffs Consumed by Humans

A healthy adult human with minimal, moderate, and intense physical activity requires a dietary intake of 1.0, 1.3, and 1.6 g protein per kg body weight per day, respectively (Wu 2016). To meet this nutritional goal, one must have knowledge of AA composition in foods. Thus, we analyzed total (free plus peptide (protein)-bound AAs in plant-sourced foods consumed by humans: corn grains, peanuts, pistachio nuts, potatoes, soybean, soy protein concentrate, sweet potatoes, wheat flour, and white rice (Hou et al. 2019). Results are summarized in Table 11.1. Data on the content of total AAs in animal-sourced foods, such as the milk of humans, cows, and sows, as well as beef, chicken eggs, casein, gelatin, and

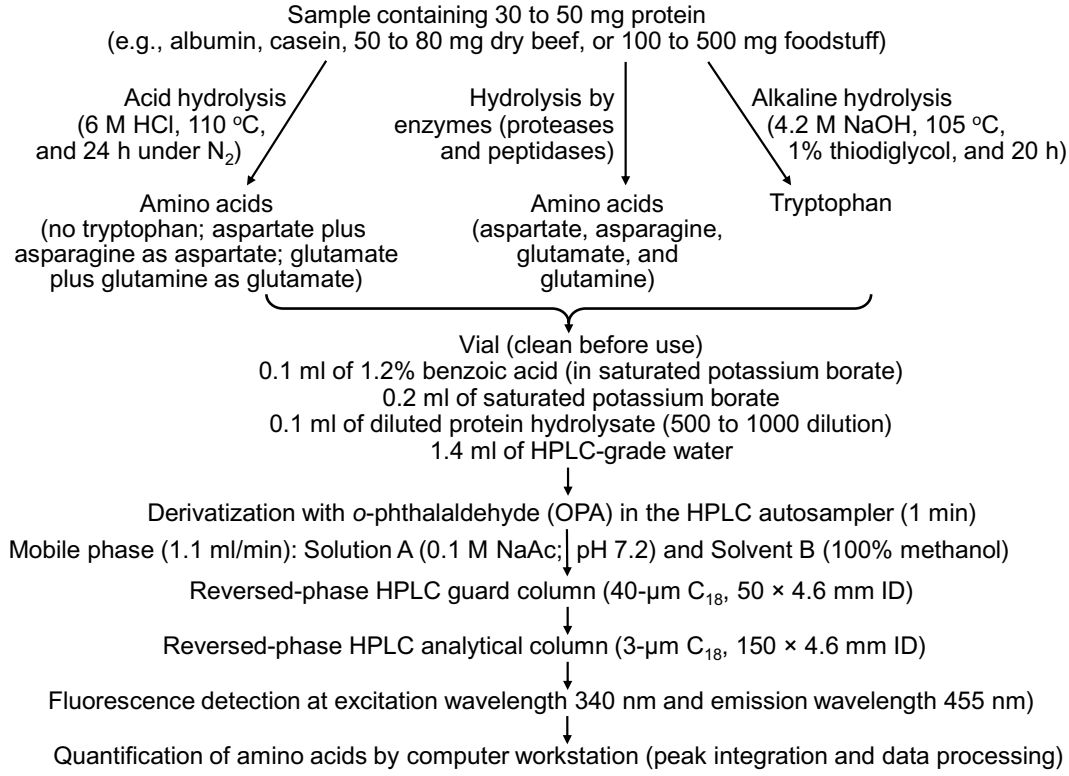

Fig. 11.2 Schematic of HPLC analysis of amino acids (AA) in proteins of animal tissues and foods by using pre-column derivatization with *o*-phthaldialdehyde (OPA). The HPLC analysis requires multiple steps, including sample preparation, automated derivatization of AA with OPA, injection of sample into the column, elution with mobile phase, fluorescence detection, and data processing. All the procedures are performed at room temperature (20–25 °C). Use of an autosampler is necessary for accurate and reproducible determination of AA. The internal standard (ethanolamine) can be omitted when the external standard calibration is used to determine the concentration of AA in protein hydrolysates. Solvent A = 0.1 M sodium acetate buffer (pH 7.2), 0.5% tetrahydrofuran and 9% methanol; Solvent B = methanol. NaAc, sodium acetate

whey protein are summarized in Table 11.2. These values can be used as references when dietary guides are recommended to individuals. This will help readers to readily recognize any highly inconsistent data on AA composition in human foods that have been published in the literature or presented at scientific and trade meetings.

11.3.1 Plant-Sourced Foods for Humans

In most of plant-sourced foods, Glu plus Gln represents a high proportion of total AAs, Gly and Pro are relatively deficient, His is the fourth least abundant AA, Trp is the least abundant AA, and Cys or Met is either the second or third least abundant AA in food, depending on its type (Sarkar et al. 2021; Wu 2021). Except for sweet potatoes and wheat flour, Glu plus Gln accounts for about 18–20% of total AAs in the plant-sourced foods. In sweet potatoes and wheat flour, Glu plus Gln contribute to about 10% and 30% of total AAs, respectively. In wheat flour, the ratio of total Gln to total Glu (g/g) is l6.25–1.00. Because of the role of Gln in the small intestine (Beaumont and Blachier 2020; Ren et al. 2020), wheat flour is an excellent source of Gln for protecting the gut from oxidative stress and inflammation. The

Table 11.1 Content of total amino acids (peptide-bound plus free amino acids) in plant-sourced foods for humans

AA	Corn grain	Peanut	Pistachio nut	Potato	Soybean	Soybean concentrate protein	Sweet potato	Wheat flour	White rice
Ala	7.97	12.4	11.3	2.82	22.9	36.1	4.80	5.42	4.58
Arg	4.34	35.5	23.8	5.15	37.2	60.8	3.08	7.20	7.28
Asp	4.74	14.2	9.89	4.05	37.3	58.7	3.34	3.58	4.27
Asn	3.88	19.7	10.2	19.2	24.6	37.1	12.0	4.47	4.60
Cys	2.17	4.15	3.56	1.14	7.67	14.3	1.63	3.04	1.66
Glu	7.13	28.6	18.4	1.01	52.6	87.6	5.46	2.80	7.38
Gln	11.8	29.0	26.9	16.3	43.0	72.6	2.20	45.5	7.96
Gly	4.43	19.0	11.7	2.74	24.0	37.6	3.52	6.31	3.95
His	2.68	6.98	5.30	1.70	12.7	21.8	1.18	3.54	2.20
Hyp	0.04	0.79	0.59	0.08	0.78	0.14	0.05	0.43	0.04
Ile	3.85	10.8	10.2	3.19	23.9	39.5	3.31	4.92	3.74
Leu	12.8	18.1	16.9	4.72	40.0	60.7	4.88	10.0	7.26
Lys	2.85	9.98	11.9	5.13	32.6	52.9	3.61	3.64	2.33
Met	2.26	3.43	3.80	1.53	6.43	12.3	1.22	2.48	2.02
Phe	5.18	14.8	11.5	3.92	25.6	42.4	4.83	7.53	4.39
Pro	11.3	16.5	12.9	3.02	28.4	36.5	2.64	23.2	5.41
Ser	5.05	14.4	14.0	3.55	30.2	50.6	4.54	6.90	4.09
Thr	3.58	8.12	7.14	3.19	20.9	34.3	3.74	4.09	3.02
Trp	0.75	2.72	2.62	1.01	7.02	12.7	0.48	1.70	1.12
Tyr	4.80	11.0	5.32	2.34	18.4	30.0	2.99	3.86	2.37
Val	5.07	12.7	13.2	4.98	24.6	40.1	4.46	6.24	5.19
Total	106.7	292.9	231.1	90.8	520.6	838.7	73.9	156.9	84.9

[a]Values, expressed as mg/g of dry matter. The molecular weights of intact amino acids were used to calculate the amount of peptide (protein)-bound amino acids in the acid, alkaline, or enzymatic hydrolysates of foods. This calculation always overestimates the true content of protein and peptides in foods. Taken from Hou et al. (2019) and Li and Wu (2020b). Hyp, 4-hydroxyproline

content of Asp plus Asn is not particularly high in non-tuber foods, but Asn is the most abundant AA in potatoes and sweet potatoes. Of note, Asn represents about 21% and 16% of total AAs in potatoes and sweet potatoes, respectively. The content of Arg in total AAs is relatively high in peanuts (12.1%) and pistachio nuts (10.3%), intermediate in soybeans (7.2%), white rice (8.6%), and potatoes (5.7%), but is relatively low in corn grains (4.1%), sweet potatoes (4.2%), and wheat flour (4.6%). Note that Arg and L-citrulline are highly abundant in watermelon (e.g., 1150 and 2014 mg/L in watermelon pomace juice, respectively; Wu et al. 2007). Given the important physiological functions of Arg (Durante 2020; McNeal et al. 2018; Wu et al. 2021), pistachio nuts may be highly beneficial for cardiovascular and metabolic health (e.g., improving vasodilation and reducing obesity) in humans. The content of branched-chain AAs (BCAAs) in plant-sourced foods is relatively high, and Leu is the most abundant AA in corn grains. The content of BCAAs (Leu + Ile + Val) in the total AAs of the analyzed foods is: 20.4%, corn grains; 14.2%, peanuts; 17.4%, pistachio nuts; 14.2%, potatoes; 17.0%, soybeans; 17.1%, sweet potatoes; 13.5%, wheat flour; and 19.1%, white rice. Note that most plant-sourced foods contain low percentages of total AAs and, therefore, provide a relatively small amount of BCAAs (Wu 2021). Cys and Met are

Table 11.2 Content of total amino acids (peptide-bound plus free amino acids) in animal-sourced foods from humans[a]

AA	Beef (loin cuts) (mg/g dry matter)	Chicken egg[b] (mg/g dry matter)	Casein protein (% of protein)	Whey protein (% of protein)	Gelatin (% of protein)	Human whole milk (mg/L)	Cow's whole milk (mg/L)	Sow's whole milk (mg/L)
Ala	45.4	27.1	2.79	2.61	7.68	400	1078	1567
Arg	52.4	30.7	3.42	2.71	6.55	361	1144	1864
Asp	41.1	29.0	3.90	3.96	2.45	449	1219	2369
Asn	33.4	21.0	2.57	2.30	1.21	410	1130	2167
Cys[c]	11.2	10.7	0.43	2.52	0.04	102	153	406
Glu	75.1	41.5	9.43	13.7	4.48	1043	3708	4988
Gln	49.9	22.6	11.3	9.10	2.58	851	3244	4764
Gly	33.7	16.2	1.87	1.56	28.7	220	605	1479
His	31.7	12.0	2.80	2.40	0.63	230	807	766
Hyp	1.77	0.51	0.14	0.13	10.9	134	481	823
Ile	41.1	24.3	4.94	4.07	1.00	531	1587	1364
Leu	66.7	41.5	8.87	11.6	2.23	1041	3332	3817
Lys	72.0	33.4	7.53	6.26	3.20	710	2895	4927
Met	25.3	14.9	2.65	2.50	0.88	161	874	918
Phe	33.5	25.3	4.90	4.59	1.42	370	1685	1526
Pro	32.9	18.3	10.9	10.6	17.6	951	3362	5561
Ser	35.4	37.7	5.11	3.86	2.93	611	1881	1734
Thr	37.0	21.3	4.12	3.44	2.94	441	1418	1406
Trp	10.0	7.76	1.25	1.85	0.19	202	429	560
Tyr	30.1	19.8	5.09	4.80	0.79	460	1582	1939
Val	47.4	29.9	6.06	5.53	1.67	511	1746	1640
Taurine	2.92	0.36	–	–	–	40	7.1	190
Total	809.9	485.8	100	100	100	10,227	34,366	46,775

[a]Adapted from Li et al. (2011), Wu (2021), and Wu et al. (2016). The molecular weights of intact amino acids were used to calculate the amount of peptide (protein)-bound amino acids in the acid, alkaline, or enzymatic hydrolysates of foods. Sow's milk is included for comparison with human and cow's milk

[b]Containing 48.5% protein on the dry matter basis

[c]Total cysteine (cysteine + ½ cystine)

[d]Present mainly in the peptide- or protein-bound form, depending on the type of food

the second and third least abundant AAs, respectively, in corn grains, pistachio nuts, potatoes and white rice, but the order of their abundances was reversed in peanuts, soybeans, sweet potatoes, and wheat flour. Furthermore, the content of Lys and threonine is relatively low in most plant proteins. Thus, although grains and vegetables are important sources of nutrients in human diets, plant protein alone is not desirable for the optimum growth and maintenance of skeletal muscle. This tissue contains a large amount of not only Gly and Pro but also Lys and other AAs in its proteins (Li and Wu 2018).

Except for potatoes and sweet potatoes, most plant-sourced foods contain only a small amount of free AAs. For example, the content of free AAs in the total AAs (peptide-bound plus free AAs) of the analyzed foods are: 3.1%, corn grains; 1.4%, peanuts; 2.1%, pistachio nuts; 0.81%, soybeans; 0.82%, wheat flour; and 0.57%, white rice. However, free AAs represent 34.4% and 28.5% of total AAs in potatoes and

sweet potatoes, respectively. Note that Gly accounts for 26.2% and 21.5% of total free AAs in peanuts and pistachio nuts, respectively, whereas Asn represents 32.3% and 17.5% of total free AAs in potatoes and sweet potatoes, respectively. Gln and Glu are the second most abundant free AA in potatoes and sweet potatoes, respectively. Thus, these two foods may be beneficial for improving the integrity and function of the small intestine. The type and content of free AAs in food confer its taste (Wu 2020c).

All plant-sourced foods contain no taurine (an antioxidant sulfur-containing AA) and only a small amount of β-alanine and 4-hydroxyproline. Thus, these foods alone or in combination have a limited ability to increase the concentration of carnosine (an antioxidant dipeptide containing β-alanine and histidine) in skeletal muscle and, therefore, are not ideal for individuals performing moderate or intensive exercise (Posey et al. 2021). Likewise, dietary 4-hydroxyproline makes little contribution to Gly synthesis in those individuals consuming plants as the sole source of protein.

11.3.2 Animal-Sourced Foods for Humans

Animal agriculture provides sustainable high-quality protein for human consumption (Wu et al. 2014). On the dry matter (DM) basis, animal-sourced foods generally contain much more protein or AAs (particularly Cys, Gly, Lys, Met, Pro, Thr, and Trp) than plant-sourced foods. For example, protein content in beef cuts ranges from 63 to 68% on a DM basis, whereas most staple foods of plant origin (except for legumes) have a protein content of <12% (DM basis) and are deficient in most AA, including all the above-mentioned AAs. In addition, compared with plant protein, animal protein is more digestible and more balanced in the composition of all AAs, such that protein in animal products has a higher digestibility (~95-99%) than proteins isolated from plants (~85–97%) or proteins in whole plant foods (~65–85%) which generally contain anti-nutritional factors (Moughan 2003; Moughan and Rutherfurd 2012). Thus, nutritionally, 1 g plant protein is not equal to 1 g animal protein, although the food label indicates 1 g protein. Because meat provides a large amount of both Gly and Pro, there is no need for their energy-dependent biosynthetic processes in humans, thereby eliminating excessive production of ammonia from AA catabolism. A balanced and sufficient supply of all AAs stimulates protein synthesis in tissues (particularly skeletal muscle) to minimize AA oxidation and wasting (Wu 2021). Furthermore, as an abundant source of glycine, proline, and 4-hydroxyproline, gelatin and meat can accelerate wound healing and recovery from injury (e.g., burns and connective tissue damage).

Adequate intake of animal protein is crucial for the optimum growth of children (Dror and Allen 2011; Grillenberger et al. 2003), the maintenance of skeletal muscle mass and function in adults (Laymen et al. 2015; Leidy et al. 2015), as well as the health and well-being of all individuals (Wu 2021). Thus, a dietary deficiency of AAs results in not only stunting and impaired physical development but also cardiovascular dysfunction, impaired immunity, and high risk for infectious disease in humans (Wu 2016). Although results of some epidemiological studies suggest that consuming animal-sourced protein increases risk for obesity, diabetes, cardiovascular disorders, and cancer (Levine et al. 2014; Shang et al. 2016; van Nielen et al. 2014), there is no compelling experimental evidence to support this claim (Arends et al. 2017; Beresford et al. 2006; Lanza et al. 2001). By contrast, much evidence shows that adequate protein intake is important for preventing or alleviating protein loss in patients with type-I or type-II diabetes (Hebert and Nair 2010; Marković-Jovanovic 2013; Marliss and Réjeanne 2002; Pfeiffer et al. 2020; Prado et al. 2020), as well as cardiovascular dysfunction (Hu 2005; Wu and Meininger 2002).

Meat contains high amounts of taurine, β-alanine, 4-hydroxyproline, and creatine (Wu et al. 2016). Taurine plays crucial roles in physiology by serving as a: (1) nutrient to conjugate bile acids to form bile salts in the liver that facilitate intestinal absorption of dietary lipids (including lipid-soluble vitamins) and eliminate cholesterol

in bile via the fecal route; (2) major antioxidant, anti-inflammatory, and anti-apoptotic factor in the body; (3) physiological stabilizer of cell membranes; (4) regulator of Ca^{2+} signaling, fluid homeostasis in cells, and retinal photoreceptor activity; (5) contributor to osmoregulation; (6) key component of nerve and muscle conduction networks; (7) stimulator of neurological development; (8) inhibitory neurotransmitter in the central nervous system; and (9) conjugator with bile acid to form bile salts in the liver (Wu 2020a, b). Because humans (particularly infants) have a low ability to synthesize taurine, children and adults fed taurine-free diets are deficient in this AA (El Idrissi 2019; Laidlaw et al. 1988; Stapleton et al. 1997). Under stress or diseased conditions (e.g., heat stress, infection, obesity, diabetes, and cancer), taurine synthesis in the body is impaired due to hepatic dysfunction and the reduced availability of Met or Cys (Wu 2020a, b). Much evidence shows that taurine has anti-obesity, anti-diabetes, and anti-cancer effects (Schaffer et al. 2014). As a precursor of carnosine and carcinine, dietary β-alanine contributes to anti-oxidative reactions in tissues (particularly skeletal muscle and brain), as well as intracellular proton buffering, osmolality, and function (Boldyrev et al. 2013). As a precursor of glycine and a scavenger of free radicals, dietary 4-hydroxyproline confers anti-oxidative and anti-inflammatory effects to the small intestine and other tissues (Wu et al. 2019). Furthermore, creatine is crucial for energy metabolism in skeletal muscle and brain, as well as other tissues (e.g., those in the reproductive tract) and, therefore, the health and well-being of humans (Wu 2020b).

11.3.3 Optimum Ratios of Animal Protein to Plant Proteins in Human Diets

The whole food contains not only proteins but also other nutrients such as carbohydrates, lipids, vitamins and minerals. All of these nutrients are essential for human growth, development, health, and survival. It must be recognized that while dyslipidemia is a major risk for cardiovascular disease, dietary lipids are essential components of cells, essential bioactive molecules, and major sources of energy for vital organs (e.g., the liver, heart, kidneys, and skeletal muscle), and, therefore, are essential for human nutrition, metabolism, and health (Jobgen et al. 2006). In addition, animal products are abundant sources of functional fatty acids to improve human health. For example, 114 g of grass-fed beef can provide nutritionally significant amounts of oleic acid and conjugated fatty acids to increase high-density lipoproteins (HDL; "good cholesterol") and decrease low-density lipoproteins (LDL; "bad cholesterol") in the blood of men and women (Smith et al. 2020). Similarly, a large amount of ω3 polyunsaturated fatty acids in seafood is beneficial for elevating HDL and lowering LDL in individuals to reduce the risk of congestive heart failure, coronary heart disease, ischemic stroke, and sudden cardiac death (Rimm et al. 2018). Thus, an appropriate intake of lipids from animal products should not be regarded as an unhealthy feeding behavior.

The content of most nutrients differ greatly between plant- and animal-sourced foods (Wu 2018). For example, dietary fiber from plants is crucial for maintaining intestinal motility, providing energy for the large intestine, and preventing colon cancer in both men and women (Turner and Lupton 2011). However, animal-sourced foods lack dietary fiber. In addition, plants are abundant sources of many vitamins such as folate, vitamin C, and pantothenic acid that are essential for nutrient metabolism (Wu 2018). However, except for dairy products (e.g., milk and yogurt) and eggs, animal-sourced foods generally contain relatively small amounts of some water-soluble vitamins for humans, including folate, vitamin C, and pantothenic acid. By contrast, animal products provide vitamin B_{12} and thiamin (essential vitamins) but plant products lack vitamin B_{12} and polished rice grain (a staple food in many regions of the world) contains little thiamin. Furthermore, animal-soured foods contain much more biologically available minerals (e.g., iron, calcium, phosphorus, magnesium, selenium, and zinc) than plant products. As noted previously, animal-sourced proteins (e.g., meat, dairy

products, egg, poultry, seafood, and other products) contain higher quantities and more balanced proportions of amino acids relative to animal tissues than plant-sourced proteins (e.g., rice, wheat, corn, potato, vegetables, cereals, beans, peas, processed soy products, nuts, and seeds). Thus, a complementary mix of both animal and plant products is ideal for human diets to ensure optimum growth and health. Based on the finding that consuming <65% of total protein from animal-sourced foods results in the deficiency of at least one EAA in adult humans, leading to protein undernutrition (Dasgupta et al. 2005), an optimum ratio of animal protein to plant protein in the diets of children, adolescents, and adults is at least 0.65–0.35 and may be up to 0.70–0.30 or even 0.75–0.25. A combination of animal- and plant-sourced foods can meet the functional needs such as promoting lean tissue growth in children and mitigating sarcopenia in adults, while maintaining optimum health and well-being in all individuals.

11.4 Composition of AAs in Feedstuffs Consumed by Farm and Companion Animals

Principles of nutrition in farm and companion animals are the same as those in humans, but feeding practices are very different. In the production of animals (including swine, poultry, ruminants, fish, shrimp, and crabs), a great emphasis is placed on the cost of feeds, feed efficiency, weight gain, and profits although much attention is also paid to animal health and well-being (Wu 2018). The traditional term "NEAA" had underestimated the important functions of AASAs in animal nutrition, physiology, and health (Wu 2018). Thus, information about AASA composition in feed ingredients is missing from the current National Research Council (NRC 2012)-recommended nutrient requirements for animals. However, AASAs play versatile roles in regulating protein metabolism, gene expression, cell signaling, and whole-body homeostasis to favor lean tissue growth, as well as anti-oxidative, anti-inflammatory, anti-obesity, and anti-infectious responses in farm and companion animals (Che et al. 2021; He et al. 2021a,b; Herring et al. 2021; Hou et al. 2015; Zhang et al. 2021). Fortunately, information about the complete composition of AASAs (including Glu, Gln, Asp, and Asn) in plant- and animal-sourced feedstuffs is now available for animal producers, pet owners, and researchers (Tables 11.3 and 11.4). Most of plant-sourced feedstuffs for farm and companion animals contain small amounts of Cys, Gly, Lys, Met, Pro, Thr, and Trp. By contrast, these AAs are abundant in animal-sourced feedstuffs. Sufficient knowledge of AA composition in feedstuffs is essential for providing animals with the optimum amounts and optimum ratios of all proteinogenic AAs and, in the case of carnivores, also taurine.

11.4.1 Plant-Sourced Feedstuffs for Farm and Companion Animals

Among plant-sourced feedstuffs, Arg is the most abundant AA in peanut meal (5.7%) and cottonseed meal (4.5%), a predominant AA in soybean meal (4.6%), and low in corn and sorghum grains (0.38–0.41%). Histidine is also low in corn and sorghum grains (0.23%). Of note, most plant-sourced feedstuffs contain low percentages of Cys (<0.25%) but relatively high percentages of Glu plus Gln (15–20%) and BCAAs (15–25%). However, as noted previously for most plant-sourced foods consumed by humans, a majority of plant feedstuffs for animals contain low percentages of total AAs and, therefore, only a small amount of BCAAs. In addition, all plant-sourced feedstuffs lack taurine and creatine, and provide only a limited amount of β-alanine and 4-hydroxyproline. Due to the presence of protease inhibitors, fiber, and other anti-nutritional factors in plant-sourced feedstuffs, their proteins

Table 11.3 Content of dry matter and total amino acids (peptide-bound plus free amino acids) in feed ingredients for farm and companion animals

DM or AA	Blood meal	Cookie meal	Corn grain	CSM	Feather meal	Gelatin	MBM	Peanut meal	PBM	SBM	SBM (P)	SGH grain
DM	91.8	90.8	89.0	90.0	95.1	88.9	96.1	91.8	96.5	89.0	96.4	89.1
Ala	7.82	0.52	0.71	1.42	4.18	9.01	4.78	1.86	4.91	1.95	2.08	0.96
Arg	4.91	0.58	0.38	4.32	5.74	7.68	3.67	5.68	4.63	3.18	3.12	0.41
Asn	4.67	0.40	0.35	1.57	1.67	1.42	2.21	1.80	2.73	2.10	2.41	0.31
Asp	6.20	0.45	0.43	1.94	2.92	2.86	3.08	2.52	4.10	3.14	3.40	0.36
Cys	1.92	0.18	0.20	0.70	4.16	0.05	0.49	0.65	1.05	0.70	0.69	0.19
Gln	4.32	1.44	1.02	3.60	2.86	3.03	2.81	2.66	3.54	3.80	4.11	0.85
Glu	6.38	1.92	0.64	4.59	4.81	5.26	4.05	4.18	4.89	4.17	4.53	1.18
Gly	3.86	0.78	0.40	2.12	8.95	33.6	8.67	3.17	9.42	2.30	2.72	0.39
His	5.57	0.22	0.23	1.08	0.88	0.74	1.19	0.95	1.30	1.13	1.15	0.23
Hyp	0.20	0.00	0.00	0.05	4.95	12.8	2.88	0.07	3.31	0.09	0.07	0.00
Ile	2.54	0.51	0.34	1.19	3.79	1.17	1.92	1.41	2.32	2.03	2.10	0.38
Leu	11.4	0.88	1.13	2.26	6.75	2.61	3.56	2.48	4.21	3.44	3.70	1.21
Lys	8.25	0.41	0.25	1.66	2.16	3.75	3.13	1.37	3.44	2.80	2.87	0.21
Met	1.16	0.19	0.21	0.66	0.75	1.03	1.10	0.47	1.39	0.60	0.64	0.20
Phe	5.83	0.50	0.46	2.02	3.95	1.67	1.85	1.93	2.36	2.21	2.44	0.51
Pro	6.29	0.98	1.06	1.89	11.7	20.6	5.86	2.29	6.72	2.40	3.18	0.96
Ser	4.49	0.56	0.45	1.72	8.80	3.44	2.08	2.03	2.67	2.12	2.35	0.46
Trp	1.30	0.15	0.07	0.44	0.79	0.22	0.39	0.38	0.49	0.62	0.63	0.10
Thr	3.95	0.42	0.31	1.25	3.97	3.45	2.42	1.67	2.85	1.76	2.03	0.32
Tyr	2.86	0.55	0.43	1.10	2.04	0.93	1.45	1.39	1.84	1.66	1.72	0.45
Val	8.21	0.53	0.44	1.69	5.76	1.96	2.23	1.69	2.89	2.09	2.25	0.50
TAAs	102	12.2	9.51	37.3	91.6	117	59.8	40.7	71.1	44.3	48.2	10.2

Sources Li et al. (2011). Values are % of feedstuff (as-fed basis)
CSM cottonseed meal, *DM* dry matter, *MBM* meat and bone meal, *P* processed, *PBM* poultry byproduct meal, *SBM* soybean meal, *SGH* sorghum, *TAAs* total amino acids
[a]Molecular weights of intact amino acids (AAs) were used to calculate the content of AAs in feed ingredients

generally have lower digestibilities than animal proteins (Moughan 2003; Wu 2014). For the optimum growth and optimum health of animals, soybean meal or peanut meal should be used along with animal protein or supplemental key AAs. Soybean meal or peanut meal should not be the sole source of protein for carnivores, such as cats, dogs, and largemouth bass, simply because this feedstuff lacks taurine. Cats and dogs fed plant-based commercial diets containing inadequate taurine develop a fatal form of heart muscle failure called dilated cardiomyopathy (Che et al. 2021; Oberbauer and Larsen 2021).

11.4.2 Animal-Sourced Feedstuffs for Farm and Companion Animals

Quality assurance programs have been established to produce high-quality animal-source protein feedstuffs for farm and companion animals (Meeker and Hamilton 2006). Except for gelatin and feather meal, animal-sourced feedstuffs contain high percentages of Glu plus Gln, BCAAs, and Asp plus Asn. Except for blood meal, the content of Glu plus Gln is much higher than that of Asp plus Asn. Interestingly, Leu and

Table 11.4 Content of dry matter and total amino acids (peptide-bound plus free amino acids) in additional feed ingredients for farm and companion animals[a]

DM or AA	BSFM	CBPM	CVD	FM-M	FM-P	FM-SE	SDPM	PBM (PFG)	SDPP	SDEP	Algae SM	Bermuda grass hay	DDGS
DM	95.3	98.0	93.2	92.8	91.6	92.1	88.7	96.8	95.4	95.1	96.5	87.2	90.3
Ala	3.14	4.54	4.12	3.63	4.74	4.59	3.51	3.98	4.98	3.43	5.85	0.89	1.90
Arg	2.64	4.75	4.02	3.67	3.58	4.15	3.59	4.15	4.94	3.82	5.18	0.58	1.19
Asn	1.93	2.61	2.50	2.26	2.28	2.60	1.52	2.56	2.57	2.69	3.34	0.52	1.11
Asp	3.04	3.93	3.66	3.33	3.41	3.87	3.72	3.86	5.17	3.60	4.29	0.73	1.49
Cys	0.69	1.07	1.22	0.67	0.72	0.69	0.99	1.04	2.65	1.37	0.88	0.17	0.52
Gln	2.51	3.88	3.73	3.60	3.39	3.96	2.78	3.41	4.49	2.90	4.25	0.63	2.33
Glu	3.44	5.34	6.34	5.32	5.13	5.97	5.77	4.75	6.77	5.48	6.81	1.18	3.04
Gly	2.91	5.94	8.25	4.26	5.09	5.87	4.95	6.86	3.40	2.45	4.19	0.55	1.08
His	1.71	1.36	0.75	1.41	1.43	1.46	1.29	1.31	3.66	1.45	1.29	0.24	0.78
Hyp	1.27	1.85	1.73	1.57	1.82	1.98	0.79	2.25	0.021	0.023	0.014	0.010	0.035
Ile	2.02	2.72	3.84	2.42	2.75	2.79	2.44	2.38	2.77	2.97	4.09	0.50	1.01
Leu	3.25	5.28	6.10	4.50	5.27	4.59	4.30	4.33	7.56	4.61	6.32	0.92	3.14
Lys	3.21	4.52	5.02	4.46	5.25	4.57	4.07	3.54	6.85	4.09	4.18	0.52	0.92
Met	1.20	1.43	1.58	1.97	2.16	2.03	1.20	1.38	1.97	2.01	2.17	0.19	0.63
Phe	2.18	2.70	3.70	2.31	2.31	2.65	2.33	2.32	4.74	3.04	3.88	0.59	1.36
Pro	2.92	4.30	5.53	3.72	4.27	4.80	3.16	5.07	4.32	3.39	3.12	0.52	2.01
Ser	2.34	3.05	6.45	2.52	2.91	3.28	3.37	2.62	4.99	5.39	4.35	0.50	1.33
Thr	2.55	2.77	1.99	2.50	2.85	3.14	2.85	2.56	4.45	3.10	4.13	0.53	1.08
Trp	0.71	0.76	1.03	0.70	0.68	0.70	0.63	0.63	1.36	0.97	1.02	0.18	0.24
Tyr	3.85	2.29	2.56	1.89	2.01	2.36	2.24	1.88	3.51	2.61	3.79	0.38	0.95
Val	2.87	3.32	5.57	3.02	3.65	3.25	3.05	2.91	4.96	3.57	4.36	0.66	1.45
TAAs	50.4	68.4	79.7	59.7	65.7	69.3	58.6	63.8	86.1	63.0	77.5	11.0	27.6

Source: Li and Wu (2020a, b) and Gilbreath et al. (2020). Values are % of feedstuff (as-fed basis)
BSFM black soldier fly larvae meal, *CBPM* chicken by-product meal, *CVD* chicken visceral digest, *DDGS* dried distillers' grains with solubles, *DM*, dry matter, *FM-M*, fishmeal (United States Menhaden), *FM-P*, fishmeal (Peruvian anchovy), *FM-SE*, fishmeal (Southeast Asian miscellaneous marine fishes), *Hyp* 4-hydroxyproline, *PBM (PFG)*, poultry by-product meal (pet-food grade), *SDEP* spray-dried egg product, *SDPM* spray-dried peptone from enzymes-treated porcine mucosal tissues, *SDPP* spray-dried poultry plasma, *SM* spirulina meal, *TAAs* total amino acids
[a]Molecular weights of intact amino acids (AAs) were used to calculate the content of AAs in feed ingredients

Gln are most abundant in blood meal and casein (13% of total protein), respectively, whereas gelatin, feather meal, fish meal, meat and bone meal, and poultry byproduct have high percentages of Gly, Pro plus 4-hydroxyproline, and Arg, which are 10–35, 9.6–35, and 7.2–7.9% of total protein, respectively. The content of Gly is lower in blood meal (3.9%) and casein (1.9%), as compared with other animal-sourced feedstuffs. Furthermore, chicken by-product meal, poultry by-product meal, and spray-dried poultry plasma contain large amounts of carnosine and anserine (potent antioxidants), whereas balenine is enriched in porcine meat (Wu 2021). Carnosine is highly abundant in the skeletal muscles of farm mammals (e.g., 20–30 mM in pigs; and 28–48 mM in cattle) and the red blood cells of mammals and birds. Anserine is the major histidine-containing dipeptide in the skeletal muscles of birds, dog, cat, lion, rabbit, agouti, mouse, kangaroo, wallaby, opossum, cod, smelt, marlin, whiting, croaker, tuna, Japanese char, salmon, and trout (Boldyrev et al. 2013). Anserine is also abundant in the skeletal muscles

of cattle and pigs, as well as the red blood cells of nonprimate mammals and birds. Balenine is highly abundant in the skeletal muscles of whale (~45 mmol/kg wet weight), relatively low in swine (~0.7–1 mmol/kg wet weight or 5–8% of carnosine content), very low in cattle, chickens and sheep (~0.05–0.1 mmol/kg wet weight), and barely detectable in rat skeletal muscles. By contrast, meat and bone meal contains less cysteine and tryptophan than other animal-sourced feedstuffs. This is because meat and bone meal contains a large proportion of bone, cartilage, tendons, and other connective tissue in which collagen is the major protein. All types of collagen proteins contain no tryptophan and little cysteine (Wu 2021). Other characteristics of animal-sourced feedstuffs can be summarized as follows.

First, animal-sourced feedstuffs are generally palatable due to their high content of Glu and Gly, and can induce feed intake by animals (He and Wu 2020; Wu 2020c). Second, blood meal, feather meal, meat and bone meal, and poultry byproduct meal provide large amounts of AASAs. Unfortunately, this important nutritional aspect of these ingredients has long been ignored in animal nutrition. Third, Ile is not deficient in blood meal, and rather Ile is low relatively to Leu. Of particular note, blood meal is particularly rich in Asp, Asn, Cys (a major component of hemoglobin), Glu, Gln, His (another major component of hemoglobin), Leu, Lys, Met, Trp, and Val. The content of most of these AAs (namely, Asp, Asn, Glu, Gln, His, Leu, Lys, Trp, and Val) in blood meal is the highest among all animal-sourced feedstuffs. Likewise, Cys and His are highly abundant in spray-dried poultry plasma.

Fourth, feather meal contains the highest content of Arg, Cys, 4-hydroxyproline, Ile, Pro, and Ser among all common animal protein feedstuffs. Similarly, both Gly and Ser are highly abundant in chicken viscera digest. Ser is a major AA in one-carbon metabolism essential for DNA and protein synthesis (Bazer et al. 2021; Seo et al. 2021). Like Gly and 4-hydroxy-proline, Ser is highly abundant not only in feather meal but also in other animal-source feedstuffs, but Ser is relatively low in all plant-source feedstuffs. Specifically, Ser plus Gly plus 4-hydroxyproline accounts for ~25% of total protein in feather meal. Thus, this feedstuff is a unique source for these functional AAs to support one-carbon metabolism, DNA and protein syntheses. Without one-carbon metabolism, there would be no life or cell growth and development. Although little attention has been paid to serine nutrition in animals, much work should be directed to this new emerging research area to capitalize on the fundamental role of Ser in the metabolism, growth performance, feed efficiency, and health of farm and companion animals.

Fifth, both blood meal and feather meal provide the highest content of Thr among all common animal protein feedstuffs. Thus, feather meal is a highly abundant source of functional AAs for animal feeding, and may substitute certain crystalline AAs (e.g., Arg, Cys, Gly and Pro) for the diets of animals that have particularly high requirements for those AAs, such as piglets, gestating swine, lactating sows, ruminants, poultry, and fish. This indicates a unique aspect of feather meal in animal nutrition and such a low-cost feedstuff should be maximally utilized to sustain animal agriculture and aquaculture. Feather meal can be used alone or with another animal protein feedstuff to further bring in complementary effects.

Sixth, poultry by-product meal contains the highest content of Gly among all common animal protein feedstuffs and is also rich in Pro and 4-hydroxyproline. Likewise, meat and bone meal contains large amounts of Gly, Pro, and 4-hydroxyproline. Finally, we have recently identified Tyr as the most abundant AA in black soldier fly larvae meal (Li and Wu 2020a, b). Overall, animal-sourced feedstuffs contain greater quantities and more balanced proportions of AAs relative to animal requirements than plant-sourced proteins (e.g., rice, wheat, corn, potato, vegetables, cereals, beans, peas, processed soy products, nuts, and seeds). Thus, animal-sourced feedstuffs are excellent sources of all AAs, including nutritionally essential AAs (EAAs) and the traditionally classified nutritional nonessential AAs (AASAs). Blood meal, feather

meal, meat and bone meal, and poultry byproduct provide large amounts of AASAs, and this important nutritional aspect has long been ignored in animal nutrition and now must be recognized.

Although there is belief that blood meal has an undesirable AA balance for animal feeding (Heuzé and Tran 2016), this is not true when blood meal is used as a protein supplement in a compounded diet. The ratio of Ile:Leu in blood meal is 0.223:1, as compared to the optimum ratio of Ile:Leu (0.50:1) in the diets of growing pigs and the optimum ratio of 0.61:1 in the diets of broiler chickens (He et al. 2021a; Zhang et al. 2021; Wu 2014). Note that all plant-based diets contain a high content of Ile. Thus, blood meal can be used as a dietary protein supplement either alone or with another animal protein feedstuff to further bring in complementary effects.

There are very large variations (e.g., up to 100 and even 500 times) in the content of AAs in the same kind of feedstuffs reported by different laboratories (Li and Wu 2020a, b). Those values as the sum of AAs account for <1% or >200% of protein content, and therefore are clearly incorrect. This is possibly due to technical problems in chemical analyses and/or mis-calculations of experimental results. Likewise, some studies reported that Glu and Gln were absent from proteins in black soldier fly meal (e.g., Shumo et al. 2019), and such data are not consistent with our knowledge of protein biochemistry that Glu and Gln are abundant AAs in proteins (Wu 2021). Thus, care must be exercised in reading the scientific literature of animal nutrition, and incorrect data must not be used for dietary formulation.

11.4.3 Complementary Use of plant- and Animal-Sourced Feedstuffs for Farm and Companion Animals

An ideal diet for omnivores should consist of both plant- and animal-sourced proteins for both cost consideration and complementary effects in nutrition (Gatlin et al. 2007; Gaylord and Rawles 2007; Wu 2018). Inclusion of a small amount of animal-sourced food can substantially reduce the need for a large amount of plant-sourced food, while meeting the requirements of individuals for dietary AAs. Notably, dietary taurine is required by carnivores to maintain hepatic structure and function (Li et al. 2021a, b). By contrast, high intake of plant-sourced feedstuffs results in excess starch ingestion, possibly leading to excess glycogen deposition in the liver, hepatic dysfunction, and excess fat accumulation in farm and companion animals, particularly those (e.g., largemouth bass) that are sensitive to high starch intake (Li et al. 2020d; 2021a, b). Much evidence shows that animal-sourced feedstuffs contain: (1) proteins that help balance dietary AAs, (2) functional nutrients that activate cell signaling, and (3) bioactive molecules that improve intestinal function and immunity (Wu 2018). Among the functional AAs, Glu, Gln and Asp are the major metabolic fuels for the small intestine of mammals (Hou and Wu 2018; Zhang et al. 2021), fish (Li et al. 2020c), and crustaceans (Li et al. 2021b). Glu and Asp serve the same metabolic function in the avian gut (He et al. 2021b). Thus, adequate knowledge of principles of animal nutrition is crucial to maximize the benefits of animal-source feedstuffs in the production of livestock, poultry, fish, and crustaceans.

Growth of cells, tissues, and the whole body depends on protein gain. Deposition of 1 g proteins is associated with the retention of 3 g water (Wu 2018). Thus, increasing protein synthesis is the most efficient way to increase lean tissue gain and feed efficiency in all animals. By contrast, gaining fats (that are hydrophobic molecules) expels water in tissues. Studies with cattle and pigs have shown that the deposition of 1.4 g fat in the body is associated with the replacement of 0.4 g water, and the actual gain in the whole body is only 1 g (Wu 2018). Thus, to promote animal growth and feed efficiency, we must promote protein synthesis and protein accretion. Likewise, Arg is a key stimulator of fatty acid oxidation and mitochondrial biogenesis in white adipose tissue, while enhancing protein synthesis in skeletal muscle (Jobgen et al. 2006). For this

reason, diets must contain sufficient Arg to prevent liver dysfunction and obesity.

Appropriate combinations of corn and soybean meal in diets could provide adequate levels of most EAAs (with exception of lysine, threonine, and tryptophan) for the maximum growth and maximum feed efficiency of farm animals, including swine, poultry, and fish (He et al. 2021a; Li et al. 2020a, b; Zhang et al. 2021). Animal products must be fed to carnivores, such as tigers (Herring et al. 2021), cats (Che et al. 2021), and dogs (Oberbauer and Larsen 2021). Dependence on plant products as the sole source of protein to meet the dietary requirements for AAs results in the waste of a large amounts of AAs (Wu 2018). For example, a diet for post-weaning pigs containing a high level (e.g., 37%) of soybean meal supplies 40–60% greater amounts of most EAAs than needed as substrates for tissue protein accretion (Hou et al. 2016). Excess EAAs must be degraded via inter-organ cooperation by both the small intestine and extra-intestinal tissues because most of free AAs are not stored in the body. This constitutes a significant waste of dietary protein, the most expensive component of any diet for animals. Although NRC (2012) currently does not recommend dietary requirements of swine for most AASAs (except Arg and Gln), growing evidence shows that at least some of them are not adequately synthesized by animals (Hou and Wu 2018; Wu et al. 2018). For example, the typical corn- and soybean meal-based diet does not provide adequate amounts of Arg, Pro, Asp, Glu, Gln, or Gly for post-weaning pigs. Thus, these six AAs can be provided from black-soldier fly larvae meal, blood meal, chicken by-product meal, chicken visceral digest, feather meal, fishmeal, poultry by-product meal, ruminant meat and bone meal, spray-dried egg product, spray-dried porcine mucosal peptone, spray-dried poultry plasma, and other animal feedstuffs. Any of these feedstuffs can be used as a single supplement to plant-based diets for animals (including fish and shrimp) to balance their AA content and provide all the needed functional AAs.

Selection of animal-sourced protein feedstuffs for diet formulations will depend on animal species, production stage, region (e.g., tropical or subtropical), season, and feedstuff ingredient availability. Informed choice based on up-to-date science is key to success. For example, for feeding weanling, gestating, and lactating pigs, as well as all animals exposed to heat stress, single feedstuffs or their combinations providing high amounts of Arg, Glu, Gln, Gly, Pro, and 4-hydroxyproline, as well as polyamines, taurine and creatine can be used to: (1) improve the integrity and function of the small intestine, liver, reproductive tract, and mammary glands; (2) enhance blood flow to peripheral tissues so as to facilitate heat dissipation from animals; (3) protect animals from oxidative stress and reduce risk for bacterial, fungal, parasitic, and viral infections; and (4) promote feed intake by animals. In contrast to plant products, animal products contain taurine, which is a major antioxidant, a key regulator of cellular redox state, and a key osmolyte in the body, as noted previously. Thus, through complementary effects, an appropriate addition of a small amount of animal-sourced feedstuffs (e.g., blood meal, hydrolyzed feather meal, fish meal, meat and bone meal, and poultry by-product meal) to plant protein-based diets plays crucial roles in formulating AA-balanced diets for nursery, growing, gestating, and lactating livestock species. This will help substantially to improve their production performance, efficiency of nutrient utilization, and health.

It should be borne in mind that more does not necessarily mean better in animal nutrition, but an insufficiency of AAs will certainly impair animal growth, feed efficiency, and health. For example, piglets with a low birth weight can grow when fed a diet containing 20% protein but have a high rate of mortality when fed a diet containing 30% protein (Jamin et al. 2010). Thus, for an efficient use of animal- and plant-sourced feedstuffs to improve the growth, intestinal health, immunity and productivity of farm and companion animals, one must understand the underlying nutritional principles. First, the gut must be well-fed and healthy with adequate AASAs. Second, diets must contain sufficient Gly and taurine to form bile conjugates that

are essential for the digestion and absorption of lipids and lipid-soluble vitamins. Third, energy is essential for physiological processes (including biochemical reactions) and tissue integrity, and is, therefore, essential for animal growth, health and productivity. Fourth, Glu, Gln, and Asp must be sufficient in diets to support energy needs by the small intestine of all animals. Fifth, all nutrients in the diet must be sufficient and balanced. Some of the nutrients interact to influence (a) mineral and AA absorption by the small intestine, (b) nutrient metabolism, (c) antioxidative and anti-inflammatory responses, and (d) immune responses to kill pathogens. Sixth, an excess intake of dietary protein can cause ammonia toxicity environmental pollution due to nitrogen accumulation, and an excess intake of lipids and starch presents high risk for dyslipidemia, insulin resistance, and metabolic dysfunction in animals. Seventh, there is active interorgan metabolism of AAs in animals to transport nitrogen in a nontoxic form (e.g., Ala and Gln) and to synthesize important nitrogenous metabolites (e.g., creatine, heme, and glutathione) that are essential to normal physiological functions in animals.

To integrate the above nutritional principles with feeding practices, let's give the following examples involving the addition of single feedstuffs or their combinations to either a plant-based diet for omnivores or a meat-based diet for carnivores. (1) For feeding gestating sows, the content of dietary protein should not exceed 12.5% to prevent toxic effects of ammonia to embryos and fetuses, but such a diet does not provide sufficient Arg (Wu et al. 2018). Thus, because Arg is known to enhance milk production and fetal survival in swine and Arg is highly abundant in feather meal, this feedstuff may be effective to improve both lactation and reproduction in swine, particularly under the conditions of heat stress. (2) For feeding neonatal poultry, single feedstuffs or their combinations providing high amounts of Arg, Cys, Glu, Gln, Gly, Met, Pro, and Trp, as well as polyamines, taurine, and creatine can be used to promote intestinal maturation and growth, while reducing risk for infections by pathogens. (3) For feeding growing broilers in a thermo-neutral environment, single feedstuffs or their combinations providing high amounts of Cys, Gly, Leu, Lys, Met, Pro, Thr, and Trp can be used to support feather and whole-body growth. (4) For feeding laying hens in a thermo-neutral environment, single feedstuffs or their combinations providing high amounts of Cys, Gly, Leu, Lys, Met, Pro, Thr, and Trp, as well as calcium and phosphorus can be used to support egg production, while maintaining the health of hens. (5) For feeding animals of the Felidae family (e.g., cats and tigers), single feedstuffs or their combinations provide high amounts of BCAAs, Cys, Glu, Gly, and Met to support their production of special AAs such as felinine, isovalthine, and isobuteine (Che et al. 2021; Herring et al. 2021). (6) For feeding cats, single feedstuffs or their combinations providing high amounts of Arg, Tyr, and taurine can be used to prevent ammonia toxicity and maintain hair color, while maintaining the health of the eyes, heart, and skeletal muscle. (7) For feeding racing horses, single feedstuffs or their combinations providing high amounts of Arg, BCAAs, Gln, Gly, Met, and Pro, as well as creatine and carnosine can be used to enhance skeletal muscle mass and health, while maintaining the health of bone and joints. (8) For feeding shrimp, single feedstuffs or their combinations providing high amounts of Arg, Gly, His, and taurine can be used to produce phosphoarginine and maintain normal osmotic pressure in tissues. (9) For feeding ruminants, single feedstuffs or their combinations providing high amounts of rumen-undegradable protein and Glu can be used to support their growth, lactation, and pregnancy, particularly for lactating cows exposed to heat stress. Nearly all plant-source proteins are fermented in the rumen of the ruminants [e.g., cattle (lactating cows, beef cows, and steers), sheep, goats, and deer]. Interestingly, animal proteins, particularly when they are mixed with the blood, are resistant to attack by ruminal bacteria. Rumen-undegraded crude protein is 55 to 80% for animal-sourced protein feedstuffs (Wu 2018). Of note, we recently discovered that dietary Glu (a major metabolic fuel for the small intestine and an activator of the

secretion of digestive enzymes) is not degraded by ruminal bacteria due to its negligible uptake. Importantly, animal-sourced protein feedstuffs are abundant sources of Glu and all other proteinogenic AAs for ruminants. (10) Additional general examples for combinations of animal-sourced feedstuffs to feed all species of livestock, poultry fish, and crustaceans are summarized in Table 11.5.

As noted previously, only a relatively small amount of animal-sourced feedstuffs is added to animal diets to improve their composition and balance of AAs. As summarized in Table 11.6, inclusion rates of the ingredients in diets vary with ingredient and animal species, ranging from 1.5 to 2% feather meal and 5–10% meat and bone meal in the diets of broilers and turkey (Firman 2006) to 20–30% poultry by-product meal in the diets of cats and dogs (Aldrich 2006; Carciofi et al. 2009), as well as many species of fish (Bureau 2006) and 33–66% feather meal in the diets of shrimp (Yu 2006). Dietary supplementation with animal-sourced feedstuffs can cost-effectively enhance the growth performance and feed efficiency of young pigs and poultry (Hou et al. 2017). Furthermore, Appropriate amounts of blood meal, poultry by-product, and feather meal can cost-effectively replace up to 80% fishmeal in the diets of fish and shrimp (Bureau 2006; Yu 2006).

Table 11.5 General examples for combinations of animal-sourced feedstuffs to feed all species of livestock, poultry fish, and crustaceans

Feedstuff	Amount	Amino acids	Amount	Amino acids
Blood meal	High	Leu (11.4), Lys (8.25), His (5.57)	Low	Gly (3.86); Hyp (0.51); Ile (2.54), Ser (4.49)
Feather meal	Low	Leu (6.75), Lys (2.16), His (0.88)	High	Gly (8.95); Hyp (4.95); Ile (3.79), Ser (8.80)
Mix of 50% each	Desirable	Leu (9.08), Lys (5.21), His (3.23)	Desirable	Gly (6.41); Hyp (2.73); Ile (3.17), Ser (6.65)
Soybean meal	Reference	Leu (3.44), Lys (2.80), His (1.13)	Reference	Gly (2.30); Hyp (0.08); Ile (2.03), Ser (2.12)
Blood meal	High	Cys (1.92), Trp (1.30), His (5.57)	Low	Gly (3.86), Hyp (0.51)
Meat and Bone meal	Low	Cys (0.49), Trp (0.39), His (1.19)	High	Gly (8.67), Hyp (2.88)
Mix of 50% each	Desirable	Cys (1.21), Trp (0.85), His (3.38)	Desirable	Gly (6.27), Hyp (1.70)
Soybean meal	Reference	Cys (0.70), Trp (0.62), His (1.13)	Reference	Gly (2.30), Hyp (0.08)
Blood meal	High	His (5.57), Thr (3.95)	Low	Gly (3.86), Ile (2.54)
CVD	Low	His (0.75), Thr (1.99)	High	Gly (8.25), Ile (3.84)
Mix of 50% each	Desirable	His (3.16), Thr (2.97)	Desirable	Gly (6.06), Ile (3.19)
Soybean meal	Reference	His (1.13), Thr (1.76)	Reference	Gly (2.30), Ile (2.03)
SDPP	High	Lys (6.85), His (3.66), Trp (1.36)	Low	Gly (3.40), Hyp (0.02)
Feather meal	Low	Lys (2.16), His (0.88), Trp (0.80)	High	Gly (8.95); Hyp (4.95)
Mix of 50% each	Desirable	Lys (4.51), His (2.27), Trp (1.08)	Reference	Gly (6.18), Hyp (2.49)
Soybean meal	Reference	Lys (2.80), His (1.13), Trp (0.62)	Reference	Gly (2.30); Hyp (0.08)

CVD Chicken visceral digest, *Hyp* 4-hydroxyproline

[a]The number within the parentheses refers to the content of an amino acid, expressed as % of feedstuff (on the as-fed basis). The mix of two animal-sourced feedstuffs in the same ratio (50% each) results in nutritionally and economically more desirable content of amino acids than the reference value for soybean meal

Table 11.6 Inclusion levels of animal-sourced feedstuffs in the diets of livestock, poultry, companion animals, fish, and shrimp

Feedstuff	Diets of animals	Inclusion in diet (%)	Comment	References
Blood meal	Fish	8–20	Replace fishmeal	Bureau (2006)
Blood meal	Shrimp	3–5	Replace fishmeal and help balance dietary AAs	Yu (2006)
Blood meal, and mucosal protein hydrolysate	Weanling pigs	2–5	Help balance dietary AAs and provide anti-oxidative molecules	Cromwell (2006)
Blood meal, feather meal, fishmeal, meat and bone meal, and meat meal[a]	Lactating cows	3–5	Contains 55–80% rumen-undegraded crude protein	Jenkins (2006)
Dried porcine solubles (originating from porcine mucosal tissues)	Rice field eel	5	Replace fishmeal	Tang et al. (2021)
Feather meal	Salmon	15	Replace fishmeal	Bureau (2006)
Feather meal	Rainbow trout	20–25	Replace fishmeal	Bureau (2006)
Feather meal	Other fish	≥ 10	Replace fishmeal	Bureau (2006)
Feather meal	Shrimp	33[b]	Replace fishmeal	Yu (2006)
Feather meal	Shrimp	66[c]	Replace fishmeal	Yu (2006)
Feather meal	Poultry[d]	1.5–2	Help offset the costs of SAAs	Firman (2006)
Fishmeal	Fish	15–75	Provide AAs and minerals	Tacon and Metian (2008)
Fishmeal	Shrimp	15–20	Provide AAs and minerals	Li et al. (2021b)
Fishmeal	Weanling pigs	2–5	Help balance dietary AAs and confer palatability	Cromwell (2006)
Meat and bone meal	Yellowtail	10	Replace fishmeal	Bureau (2006)
Meat and bone meal	Fish[e]	10	Replace fishmeal	Bureau (2006)
Meat and bine meal	Shrimp	60	Replace fishmeal	Yu (2006)
Meat and bone meal, poultry by-product meal	Poultry[d]	5–10	Help balance dietary AAs and provide calcium and phosphorus	Firman (2006)
Meat and bone meal, poultry by-product meal	Various[f]	5–30	Help balance dietary AAs and provide calcium and phosphorus	Meeker and Hamilton (2006)
Meat meal, and meat and bone meal	Pigs	5–10	Help balance dietary AAs and provide anti-oxidative molecules	Cromwell (2006)
Meat and bone meal	Cats and dogs	10–15	Cost-effectively provide adequate AAs (including tauine)	Aldrich (2006), Carciofi et al. (2009)
Poultry by-product meal	Cats and dogs	20–30	Cost-effectively provide adequate AAs (including tauine)	Aldrich (2006), Carciofi et al. (2009)
Poultry by-product meal	Hybrid striped bass	61.2	Fully replace 56.6% fishmeal	Gaylord and Rawles (2007)

(continued)

Table 11.6 (continued)

Poultry by-product meal	Largemouth bass	22–35	Replace fishmeal	Li et al. (2021c)
Poultry by-product meal	Fish	20–30	Replace fishmeal	Bureau (2006)
Poultry by-product meal	Shrimp	30	Replace fishmeal	Yu (2006)
Spray-dried porcine plasma	Salmon	5–7.5	Replace fishmeal	Bureau (2006)
Spray-dried porcine plasma	Weanling pigs	5–7.5	Help balance dietary AAs and provide anti-oxidative molecules	Cromwell (2006)

AAs amino acids, *SAAs* crystalline sulfur-containing amino acids (cysteine and methionine)
[a]If phosphorus content is $\geq 4.0\%$, the product is designated as meat and bone meal. If phosphorus content is <4.0%, the product is designated as meat meal. Meat and bone meal of ruminant origin is generally the most cost-effective source of the same kind of feedstuff. According to the AAFCO's official definition, the calcium level should not be more than 2.2 times the phosphorus level
[b]Without lysine or methionine supplement
[c]With lysine and methionine supplement
[d]Broilers and turkey
[e]Rainbow trout, tilapia, and gilthead seabream
[f]Horses, rabbits, ferrets, rodents, and golden fish

11.5 Conclusion

Dietary AAs play important roles in the growth, development, and health of humans and other animals. Results of extensive research over the past three decades have shown that animals have dietary requirements for AASAs, including Gln, Glu, Gly, Asp, and Asn. Recent advances in analytical methods have allowed us to determine all AASAs and other proteinogenic AAs in staple foods consumed by humans, livestock, poultry, fish, and crustaceans. Knowledge of AA composition in foodstuffs is necessary to guide the practice of human and animal nutrition, and also helps to assess the rates of inter-organ AA synthesis and catabolism in the body. This will further aid in establishing a role of dietary AA intake in the maintenance of cell-, tissue-, and species-specific expression of genes and proteins, as well as cell-, tissue-, and species-specific patterns of nutrient and energy metabolism (e.g., the use of dietary Gln, Glu, and Asp as the major metabolic fuels in the mammalian small intestine). Proteins of both plant- and animal-sourced foods contain relatively high percentages of Glu, Gln, Asp, Asn, BCAAs, Phe, and Tyr. However, plant products generally have relatively small amounts of Cys, Gly, Lys, Met, Pro, Thr, and Trp. By contrast, these AAs are enriched in animal-sourced foods. Of particular note, taurine and creatine are absent from plants but are abundant in meat and present in all animal-sourced foods. A combination of plant- and animal products is desirable for the healthy diets of humans and omnivorous animals. In addition, animal-sourced feedstuffs can be used to cost-effectively replace a large proportion or all of fishmeal in the diets of fish and crustaceans. Furthermore, these ingredients can be used in the diets of carnivores, such as cats, dogs, mink, ferrets, and tigers, to reduce their dependence on edible foods for the growing human population.

Acknowledgements This work was supported by Texas A&M AgriLife Research (H-8200). We thank research associates and students in our laboratory for helpful discussions.

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12 Dietary Intakes of Amino Acids and Other Nutrients by Adult Humans

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Abstract

Measuring usual dietary intake in freely living humans is difficult to accomplish. As a part of our recent study, a food frequency questionnaire was completed by healthy adult men and women at days 0 and 90 of the study. Data from the food questionnaire were analyzed with a nutrient analysis program (www.Harvardsffq.date). Healthy men and women consumed protein as 19–20% and 17–19% of their total energy intakes, respectively, with animal protein representing about 75 and 70% of their total protein intakes, respectively. The intake of each nutritionally essential amino acid (EAA) by the persons exceeded that recommended for healthy adults with a minimal physical activity. In all individuals, the dietary intake of leucine was the highest, followed by lysine, valine, and isoleucine in descending order, and the ingestion of amino acids that are synthesizable de novo in animal cells (AASAs) was about 20% greater than that of total EAAs. The intake of each AASA met those recommended for healthy adults with a minimal physical activity. Intakes of some AASAs (alanine, arginine, aspartate, glutamate, and glycine) from a typical diet providing 90–110 g food protein/day does not meet the requirements of adults with an intensive physical activity. Within the male or female group, there were not significant differences in the dietary intakes of all amino acids between days 0 and 90 of the study, and this was also true for nearly all other essential nutrients. Our findings will help to improve amino acid nutrition and health in both the general population and exercising individuals.

Keywords

Amino acids · Diets · Humans · Intake · Health

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G. Wu (ed.), *Amino Acids in Nutrition and Health*, Advances in Experimental Medicine and Biology 1332,
https://doi.org/10.1007/978-3-030-74180-8_12

Abbreviations

AASAs	Amino acids that are synthesizable de novo in animal cells
BW	Body weight
EAA	Nutritionally essential amino acid
FAO	Food and Agriculture Organization of the United Nations
IOM	Institute of Medicine
NEAA	Nutritionally nonessential amino acid
NO	Nitric oxide

12.1 Introduction

Except for glycine, all proteinogenic amino acids (AAs) are L-isomers in mammals (Wu 2013). Humans have dietary requirements for AAs that are provided primarily in the form of proteins. Historically, based on nitrogen balance studies, AAs have been classified as nutritionally essential or nonessential in human nutrition (Reeds et al. 2000; Young 1987). Nutritionally essential AAs (EAAs) were considered to be histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Other proteinogenic AAs, alanine, arginine, aspartate, asparagine, cysteine, glutamate, glutamine, glycine, proline, serine, and tyrosine, were considered to be nutritionally nonessential. It had long been thought that humans have no dietary requirements for the so-called nutritionally nonessential AAs (NEAAs) (IOM 2005). However, based on functional needs (e.g., spermatogenesis in men, embryonic survival in gestating women, as well as immune and anti-oxidative responses, and recovery from wounds in all individuals), adults and infants clearly have dietary requirements for NEAAs, including arginine, taurine, and proline (see Hou et al. 2015 for review). In addition, although taurine (a nonproteinogenic AA) is synthesized by the liver of many mammals (including humans), the absence of this sulfur-containing β-AA from diets causes retinal damage in infants and children (Wright et al. 1986), indicating that taurine is nutritionally essential for these human populations. Furthermore, glycine and serine participate in one-carbon metabolism that is essential for the growth of normal cells and tumors (Bazer et al. 2015; Seo et al. 2021). Thus, the century-old term "NEAA" is now recognized as a misnomer in nutritional sciences and should be replaced with "AASAs," namely AAs that are synthesizable de novo in animal cells (Hou and Wu 2017).

Based on studies with animal models (e.g., pigs) and investigations of human AA metabolism, Wu (2016) recommended dietary requirements of young and adult humans for AASAs (Table 12.1). The content of AASAs in the bodies of mammals (including humans) is about 50% greater than that of EAAs (Wu 2013). This is consistent with the higher requirements for AASAs than for EAAs in both humans (Table 12.2) and other animals, such as pigs and chickens (He et al. 2021; Wu 2014). AAs are required by humans not only for the synthesis of proteins but also for the production of nitrogenous and sulfurous substances with enormous physiological significance. These metabolites include nitric oxide (NO, a major vasodilator), polyamines (essential for DNA and protein syntheses), creatine (crucial for energy metabolism in the brain and skeletal muscle), glutathione (the most abundant low-molecular-weight antioxidant in cells), and H_2S (a gaseous signaling molecule for neurological and vascular functions). Although there is information about dietary intakes of EAAs by freely living healthy adult humans (Chae et al. 2020; Ishikawa-Takata and Takimoto 2018), little is known about dietary intakes of AASAs by humans. The availability of such data will help to assess the adequacy of AASA intakes relative to the recommended AASA requirements and to provide a much needed database for future studies of human AA nutrition. Because the metabolism of AASAs is closely integrated with that of EAAs, lipids, carbohydrates, vitamins, and minerals, it is imperative to quantify dietary human intakes of these nutrients as well.

The Institute of Medicine (IOM 2005) recommended requirements of healthy adults with a normal body mass index (20–24.9 kg/m^2) for dietary protein at 0.8 g/kg of body weight (BW).

Table 12.1 Recommended requirements of healthy humans for amino acids that are formed de novo or from EAAs in humans

Age group	EAA[b]		Amino acids that are formed de novo or from EAAs in humans (AASAs)											
	Total	Lys	Total	Ala	Arg	Asn	Asp	Cys	Glu	Gln	Gly	Pro	Ser	Tyr
mg/kg of body weight/day														
Infants[c] (0.3 to 1 years)	402	71.3	750	69.2	71.3	48.6	69.2	21.6	121	108	76.7	82.1	42.2	39.9
Children[d] (1 to 3 years)	295	52.3	550	50.7	52.3	35.6	50.7	15.8	88.7	79.2	56.2	60.2	30.9	29.3
Adult men and women (>18 years)														
Minimal PA	268	47.5	500	46.1	47.5	32.4	46.1	14.4	80.6	72.0	51.1	54.7	28.1	26.6
Moderate PA	348	61.8	650	60.0	61.8	42.1	60.0	18.7	105	93.6	66.4	71.1	36.5	34.6
Intense PA	429	76.0	799	73.8	76.0	51.8	73.8	23.0	129	115	81.8	87.5	45.0	42.6
mg/kg of lean body mass/day														
Adult men (>18 years)[e]														
Minimal PA	315	55.9	588	54.2	55.9	38.1	54.2	16.9	94.8	84.7	60.1	64.4	33.1	31.3
Moderate PA	409	72.7	765	70.6	72.7	49.5	70.6	22.0	124	110	78.1	83.6	42.9	40.7
Intense PA	505	89.4	940	86.8	89.4	60.9	86.8	27.1	152	135	96.2	103	52.9	50.1
Adult women (>18 years)[f]														
Minimal PA	367	65.1	685	63.2	65.1	44.4	63.2	19.7	110	98.6	70.0	74.9	38.5	36.4
Moderate PA	477	84.7	890	82.2	84.7	57.7	82.2	25.6	144	128	91.0	97.4	50.0	47.4
Intense PA	588	104	1095	101	104	71.0	101	31.5	177	158	112	120	61.6	58.4

Source Adapted from Wu (2016). Except for glycine, all amino acids are L-isomers. Expressed as mg/kg of lean body mass/day, adult women have higher requirements for amino acids that are formed de novo or from EAAs than adult men. This is because adult women generally have a higher metabolic rate per kg of lean body mass than adult men
EAAs nutritionally essential amino acids, *PA* physical activity, *y* year(s)
[a]Values refer to true digestible amounts
[b]Values for adults with minimal physical activity are calculated as the IOM (2005) value × 1.25. Nutritionally essential amino acids (EAAs) include His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val, as defined by IOM
[c]Values are calculated as 1.5 × adult value (minimal PA)
[d]Values are calculated as 1.1 × adult value (minimal PA)
[e]Calculated on the basis of 85% lean body mass in adult men
[f]Calculated on the basis of 73% lean body mass in adult women

Given the current overweight or obesity pandemic worldwide (Nuttall 2015), the expression of nutrient requirements should be based on lean body mass (fat-free mass), which is defined as the mass of the body minus the mass of the body fat, rather than on BW. Lean body mass includes skeletal muscles, bones, blood, and the skin. In general, men have more lean body mass than women, and younger people have more lean body mass than older individuals (Lee et al. 2018). Normal ranges of lean body mass and body fat mass in healthy, non-obese, and non-overweight adult humans of different ages are summarized in Table 12.3.

12.2 General Methods for Estimating Dietary Intakes of AAs by Freely Living Adult Humans

Dietary intake of individuals refers to their daily eating patterns, such as the types and amounts of foods and calories consumed and also the relative

Table 12.2 Dietary requirements of healthy human adults for nutritionally essential amino acids (EAAs)

EAA	Estimates from nitrogen balance experiments[a]		MIT values[a] (tracer studies) (2000) IOM[b] (2005) FAO/WHO/UNU[c] (2007)		
	Men[d]	Women[e]			
mg/kg body weight/day					
His	–	–	–	14	10
Ile	10	9.17	23	19	20
Leu	15.7	12.1	40	42	39
Lys	11.4	9.07	30	38	30
Met	2.36	3.23	–	–	–
Met + Cys	15.7	11.7	13	19	15
Phe	4.29	4.30	–	–	–
Phe + Tyr	15.7	–	39	33	25
Thr	7.14	6.25	15	20	15
Trp	3.57	2.80	6	5	4
Val	11.4	10.4	20	24	26
Total	90.6	65.8	186	214	184
mg/kg lean body mass/day[f]					
His	–	–	–	16.5	11.8
Ile	11.8	12.6	27.1	22.4	23.5
Leu	18.5	16.6	47.1	49.4	45.9
Lys	13.4	12.4	35.3	44.7	35.3
Met	2.8	4.4	–	–	–
Met + Cys	18.5	16.0	15.3	22.4	17.6
Phe	5.0	5.9	–	–	–
Phe + Tyr	18.5	–	45.9	38.8	29.4
Thr	8.4	8.6	17.6	23.5	17.6
Trp	4.2	3.8	7.1	5.9	4.7
Val	13.4	14.2	23.5	28.2	30.6
Total	107	90.1	219	252	217

Adapted from Young and Borgonha (2000) for values expressed as mg/kg body weight/day. All amino acids are L-isomers

[a]Recommended values of Massachusetts Institute of Technology (MIT) for adult men and women

[b]Recommended dietary allowance (RDA) published by the Institute of Medicine (IOM 2005) for adult men and women

[c]FAO/WHO/UNU (World Health Organization/Food and Agriculture Organization/United Nations University, 2007) for adult men and women

[d]Body weight = 70 kg

[e]Body weight = 60 kg

[f]Calculated on the basis of 85% and 73% lean body mass in normal adult men and women, respectively, for nitrogen balances experiments, and on the basis of 85% lean body mass in normal adult men for the MIT, IOM, and FAO values

quantities of energy and nutrients. Daily dietary intake is of interest in epidemiologic studies because of its association with chronic diseases, including obesity, hypertension, metabolic syndrome, and cancer (Schulze 2018). A well-planned diet is desirable for good health and

Table 12.3 Normal means and ranges of lean body mass and body fat mass in healthy humans

Age groups (years)	Males		Females	
	Lean body mass	Body fat mass	Lean body mass	Body fat mass
14–17	90 (88–92)	10 (8–12)	82 (79–85)	18 (15–21)
18–19	89 (86–92)	11 (8–14)	77 (69–83)	23 (16–30)
20–39	86 (80–92)	14 (8–20)	73 (67–79)	27 (21–33)
40–59	83 (78–89)	17 (11–22)	71 (66–76)	29 (24–34)
60–79	81 (75–87)	19 (13–25)	69 (64–75)	31 (25–36)

Values are express as %, with the ranges being indicated in parentheses
Adapted from Abbate (2019)

can potentially reduce the risk of many chronic diseases (White and Frank 1994). A plethora of factors is responsible for dietary patterns in humans, such as physiology and nutritional status (e.g., hunger, appetite, endocrine status, developmental stage, physical activity or energy expenditure, a deficiency or excess of nutrients, mood, and stress); environmental conditions (e.g., hot or cold temperatures); food characteristics (e.g., the palatability, taste, smell, texture, and appearance of a food), cost, accessibility, and availability; educational levels and the knowledge of nutrients and health; social determinants (e.g., cultural influences and social context); and disease (e.g., diabetes, infections, and cancers) (Birch 1999; Jabs and Devine 2006; Rose et al. 2010; Sanjur 1982). Food intake is regulated by the brain and its associated networks involving AAs (e.g., glutamate, aspartate, and glycine) and their metabolites (e.g., NO, γ-aminobutyrate, and serotonin) (He and Wu 2020).

We conducted a randomized, placebo-controlled, double-blinded trial regarding the physiological effects and safety of oral arginine administration in adult humans (McNeal et al. 2018). From this study, dietary intakes of energy, protein, AAs, and other nutrients by male and female participants not receiving arginine supplementation were used for calculation in the present article, and the physical characteristics of those individuals are presented in Table 12.4. The study participants were overweight or obese but otherwise healthy. There are reports that some obese persons may be classified as metabolically healthy (Muñoz-Garach et al. 2017; Stefan et al. 2013). To date, there is no universally accepted definition of metabolically healthy obesity, and most studies have defined this condition as a body mass index (BMI) of $\geq$ 30 kg/m^2 without the presence of metabolic diseases, such as type 2 diabetes, dyslipidemia, or hypertension (Muñoz-Garach et al. 2017; Stefan et al. 2013; Zheng et al. 2016). To be included in our study, individuals could not be taking any chronic medications, except for oral contraceptives. Exclusion criteria were: (1) chronic or acute infections at the time of enrollment or during the study (if they required antibiotic therapy); (2) active inflammatory or autoimmune disorders; (3) active malignancy, tumors, or pathological angiogenesis; (4) any gastrointestinal disease (with the exception of occasional, intermittent problems, such as gastroesophageal reflux not requiring chronic treatment); (5) any tobacco use (cigarettes, cigars, pipes, or use of smokeless tobacco or snuff) over the past year; (6) clinical evidence of hypertension, hypotension, or atherosclerosis documented in the medical record; (7) previous medical history resulting in hospitalization and/ or use of medications for >28 consecutive days in the past five years (e.g., cardiovascular, renal, hepatic, gastrointestinal, neuropsychiatric, allergic or endocrine disease); (8) use of any chronic medications (other than oral contraceptives); and (9) women who were pregnant or nursing or women who were planning conception during the course of the study.

The study participants were instructed to eat healthy diets and maintain exercise habits throughout the study. Measurement of food intake is difficult in free-living humans; in our study, the

Table 12.4 Physical characteristics of human individuals

Variable	Day of study	Males	Females
Age, years (minimum, maximum)	0 and 90	35 ± 2.5 (23, 50)	40 ± 2.6 (29, 53)
Height (cm)	0 and 90	176.4 ± 1.1	163.6 ± 1.4
Waist circumference (cm)	0	117.6 ± 7.1	109.5 ± 3.3
	90	119.6 ± 8.1	108.7 ± 3.3
Body weight (kg)	0	116.3 ± 8.5	100.1 ± 4.2
	90	117.1 ± 9.5	99.4 ± 4.1
Body mass index (kg/m^2)	0	36.1 ± 2.5	36.6 ± 1.8
	90	36.6 ± 2.9	36.3 ± 1.8
Lean body mass (kg)	0	68.0 ± 2.9	49.2 ± 1.5
	90	67.3 ± 3.2	47.9 ± 1.3*
Body fat mass (kg)	0	37.4 ± 6.1	42.2 ± 3.5
	90	38.8 ± 6.5	42.6 ± 3.5
Blood pressure systolic (mmHg)	0	130.8 ± 2.5	123.0 ± 2.8
	90	134.1 ± 3.0	125.2 ± 2.6
Diastolic (mmHg)	0	77.1 ± 1.9	73.4 ± 2.2
	90	80.8 ± 2.4	74.8 ± 2.5

Adapted from McNeal et al. (2018). Values are means ± SEM, $n = 13$
*$P < 0.01$ versus the value for day 0 (the beginning of the trial)

individuals completed a standardized food frequency questionnaire (Block et al. 1992) at days 0 and 90 of the study. Data from the food questionnaire were analyzed with a nutrient analysis program designed by the Channing Laboratory, Brigham and Women's Hospital and Harvard Medical School (www.Harvardsffq.date). The analysis included dietary intakes of energy, protein, AAs, lipids, carbohydrates, vitamins, and minerals. Data were exported into an Excel spreadsheet. The paired *t*-test was used to determine differences in nutrient or energy intake between days 0 and 90 of the study for both male and female groups (McNeal et al. 2018).

12.3 Estimation of Dietary Intakes of EAAs by Freely Living Adult Humans

Male and female participants in our study consumed protein as 19–20% and 17–19% of their total energy intakes, respectively (Table 12.5). Within the male or female group, there were not significant differences ($P > 0.05$) in the dietary intakes of total energy, total protein (Table 12.6), each EAA (Table 12.6), the contribution of protein to dietary energy intake, or BW between days 0 and 90 of the study. Expressed per kg of BW or kg of lean body mass, the intake of each EAA in our study participants (males or females) exceeded that recommended by IOM (2005) and Food and Agriculture Organization of the United Nations (FAO 2007) for healthy adults with a minimal physical activity. In both males and females, the intake of leucine was the highest, followed by lysine, valine, and isoleucine in descending order. This is consistent with the composition and amounts of EAAs in animal protein (Wu et al. 2016) and our finding that animal protein represented about 75 and 70% of total protein intake in the men and women of our study, respectively (Table 12.6). The intakes of EAAs by our study participants were generally similar to those reported for the Japanese adults of similar ages (Ishikawa-Takata and Takimoto 2018) but were about 50–100% greater than those reported for Korea adults of similar ages

Table 12.5 Daily dietary intakes of energy per kg of body weight (BW) or lean body mass (LMB) on days 0 and 90 in adult males and females

Variable	Day of study	kcal/person/day		kcal/kg BW/day		kcal/kg LBM/day	
		Males	Females	Males	Females	Males	Females
Total energy	0	2443 ± 295	2020 ± 168	21.0	20.2	35.9	41.1
	90	1866 ± 218	1660 ± 292	15.9	16.7	27.7	34.7
Energy from protein	0	465 ± 78	342 ± 30	4.00	3.42	6.84	6.96
	90	376 ± 66	320 ± 70	3.21	3.22	5.59	6.68

Values are means ± SEM, n = 13

Chae et al. 2020). Dietary intakes of all EAAs meet the requirements of adults with a minimal or moderate physical activity, but not adults with an intense physical activity. For example, cysteine intake from a typical diet providing 90–110 g food protein/day does not meet the requirements of adults with strenuous training.

Sulfur AAs (including methionine, cysteine and taurine) play important roles in human nutrition and health, such as DNA and protein methylation, glutathione synthesis, and H_2S production (Wu 2013). Humans cannot synthesize de novo the carbon skeleton of methionine but can convert methionine into cysteine in the liver. Neither the IOM (2005) nor the FAO (2007) provided values for dietary methionine or cysteine requirements for humans. Rather, these two sulfur AAs were considered as one entity due to the lack of sufficient experimental data. Based on nitrogen balance studies, Young and Borgonha (2000) recommended that dietary requirements for cysteine and methionine be 13.34 and 2.36 mg/kg BW/day in adult men, respectively, and 8.47 and 3.23 mg/kg BW/day in adult women, respectively, with the ratios of cysteine to methionine (g/g) in dietary requirements being 5.65 and 2.62 for adult men and adult women, respectively. These recommended ratios of cysteine to methionine in diets are very different from those (0.59 and 0.57 for men and women, respectively) that we found in our study (Table 12.5). The ratio of cysteine to methionine in the diet of men (0.59) is only about 10% of that recommended by Young and Borgonha (2000) for dietary requirements of adult men for these two AAs. Common food sources of these EAAs include dairy products, eggs, fish, and some meats. The content of methionine in the whole body of humans is 20 mg/g protein, which is similar to that for pigs (18.7 mg/g protein; Wu 2013). Although there is no published data on the content of cysteine in the whole body of humans, the values are 13.2, 14.5, and 14.6 mg/g protein in pigs, rats, and sheep, respectively (Wu 2013). Because the body composition of AAs is generally similar among mammals, we expect the ratio of cysteine to methionine (g/g) in the whole body of humans is similar to that in pigs (namely, 0.71), which is not substantially different from the values in human diets (namely 0.59 and 0.57 for men and women, respectively). Thus, it is possible that the previously recommended ratios of dietary requirements for cysteine and methionine by adult humans are grossly incorrect and should be reconsidered.

12.4 Estimation of Dietary Intakes of AASAs by Freely Living Adult Humans

To our knowledge, this is the first report of dietary intakes of all AASAs (including glutamine, glutamate, glycine, asparagine, and aspartate) by humans (Table 12.5). Based on the consumption of staple foods by the US population (USDA 2018) and the composition of AAs in those foods (Hou et al. 2019; Wu et al. 2016), we estimated the intakes of glutamine, glutamate, asparagine, and aspartate by US adults (Table 12.7). Note that potatoes and wheat flour are highly abundant in glutamine (Hou et al.

Table 12.6 Daily dietary intakes of energy, protein, amino acids, and aspartame on days 0 and 90 in adult males and females

Variable	Day of study	g/person/day		mg/kg BW/day		mg/kg LBM/day	
		Males	Females	Males	Females	Males	Females
Protein[a]							
Plant protein	0	30.46 ± 3.21	26.34 ± 1.98	262	263	448	535
	90	21.18 ± 2.39*	22.29 ± 4.05	181	224	315	465
Animal protein	0	85.84 ± 17.07	59.12 ± 6.25	738	591	1262	1203
	90	72.84 ± 15.99	57.72 ± 13.65	622	581	1082	1206
Dairy protein (part of animal protein)	0	20.04 ± 3.86	13.58 ± 1.42	172	136	294	277
	90	16.75 ± 3.88	10.32 ± 1.46	143	104	249	216
Total protein	0	116.30 ± 19.55	85.46 ± 7.44	1000	854	1710	1738
	90	94.02 ± 16.58	80.00 ± 17.58	803	805	1397	1670
Amino acids that are not synthesized de novo in animal cells (EAAs)							
Cysteine	0	1.60 ± 0.29	1.16 ± 0.10	13.8	11.6	23.6	23.6
	90	1.35 ± 0.27	1.07 ± 0.21	11.5	10.8	20.0	22.4
Histidine	0	3.29 ± 0.56	2.46 ± 0.23	28.3	24.6	48.4	50.1
	90	2.69 ± 0.49	2.29 ± 0.51	23.0	23.0	40.0	47.7
Isoleucine	0	5.37 ± 0.96	3.91 ± 0.34	46.2	39.1	79.0	79.6
	90	4.42 ± 0.86	3.66 ± 0.79	37.8	36.8	65.8	76.4
Leucine	0	9.22 ± 1.62	6.78 ± 0.59	79.3	67.7	136	138
	90	7.55 ± 1.38	6.31 ± 1.33	64.5	63.5	112	132
Lysine	0	8.37 ± 1.50	6.17 ± 0.59	72.0	61.6	123	125
	90	7.01 ± 1.37	5.84 ± 1.33	59.9	58.8	104	122
Methionine	0	2.75 ± 0.51	1.97 ± 0.19	23.7	19.7	40.5	40.1
	90	2.28 ± 0.46	1.88 ± 0.42	19.5	18.9	33.9	39.2
Phenylalanine	0	5.14 ± 0.90	3.80 ± 0.31	44.2	38.0	75.6	77.3
	90	4.17 ± 0.75	3.50 ± 0.72	35.6	35.2	61.9	73.0
Threonine	0	4.47 ± 0.81	3.30 ± 0.31	38.4	33.0	65.7	67.2
	90	3.64 ± 0.69	3.11 ± 0.69	31.1	31.3	54.1	65.0
Tryptophan	0	1.26 ± 0.23	0.898 ± 0.079	10.8	8.97	18.5	18.3
	90	1.01 ± 0.19	0.833 ± 0.170	8.63	8.38	15.0	17.4
Tyrosine	0	4.08 ± 0.73	2.97 ± 0.25	35.1	29.7	60.0	60.4
	90	3.34 ± 0.63	2.77 ± 0.58	28.5	27.9	49.6	57.9
Valine	0	6.13 ± 1.11	4.47 ± 0.38	52.7	44.7	90.1	91.0
	90	4.99 ± 0.94	4.14 ± 0.86	42.6	41.7	74.1	86.5
Amino acids that are synthesizable de novo in animal cells (AASAs)							
Alanine	0	5.85 ± 1.04	4.30 ± 0.41	50.3	43.0	86.0	87.5
	90	4.79 ± 0.92	4.15 ± 0.98	40.9	41.8	71.2	86.7
Arginine	0	6.77 ± 1.17	5.01 ± 0.46	58.2	50.1	99.5	102
	90	5.39 ± 1.00	4.72 ± 1.09	46.0	47.5	80.0	98.6

(continued)

Table 12.6 (continued)

Variable	Day of study	g/person/day		mg/kg BW/day		mg/kg LBM/day	
		Males	Females	Males	Females	Males	Females
Aspartate + Asparagine	0	10.84 ± 1.92	8.03 ± 0.73	93.2	80.2	137	163
	90	8.84 ± 1.59	7.60 ± 1.73	75.5	76.5	131	159
Aspartate[b]	0	5.60 ± 0.99	4.15 ± 0.38	48.2	41.5	70.8	84.2
	90	4.57 ± 0.82	3.93 ± 0.89	39.0	39.5	67.7	82.2
Asparagine[b]	0	5.24 ± 0.93	3.88 ± 0.35	45.0	38.8	66.2	78.8
	90	4.27 ± 0.77	3.67 ± 0.84	36.5	37.0	63.3	76.8
Glutamate + Glutamine	0	21.22 ± 3.25	15.95 ± 1.27	183	159	313	324
	90	16.98 ± 2.73	14.50 ± 2.95	145	146	252	303
Glutamate[c]	0	7.98 ± 1.22	6.00 ± 0.48	68.8	59.8	118	122
	90	6.38 ± 1.03	5.45 ± 1.11	54.5	54.9	94.7	114
Glutamine[c]	0	13.24 ± 2.03	9.95 ± 0.79	114	99.2	195	202
	90	10.60 ± 1.70	9.05 ± 1.84	90.5	91.1	157	189
Glycine	0	5.13 ± 0.86	3.85 ± 0.37	44.1	38.5	75.4	78.3
	90	4.11 ± 0.73	3.78 ± 0.95	35.1	38.0	61.1	78.9
Proline	0	6.99 ± 1.07	5.18 ± 0.39	60.1	51.8	103	105
	90	5.58 ± 0.91	4.69 ± 0.90	47.7	47.2	83.0	97.9
Serine	0	5.40 ± 1.00	3.93 ± 0.32	46.4	39.3	79.3	80.0
	90	4.39 ± 0.83	3.60 ± 0.73	37.5	36.2	65.3	75.1
Added sweeter							
Aspartame	0	0.077 ± 0.027	0.037 ± 0.012	0.662	0.370	1.13	0.753
	90	0.030 ± 0.012	0.032 ± 0.014	0.256	0.322	0.445	0.668

Values are means ± SEM, n = 13. Except for glycine, all amino acids are L-isomers
LBM, lean body mass
[a]Crude protein (food protein), $N \times 6.25$
[b]Estimated on the basis of the ratio of total asparagine to total aspartate (0.935:1.000) in the staple foods consumed by the US population
[c]Estimated on the basis of the ratio of total glutamine to total glutamate (1.66:1.00) in the staple foods consumed by the US population

2019). The estimated intake of glutamine plus glutamate (20.8 g/person/day; Table 12.7) by healthy adults is remarkably similar to that from our food survey (21.2 g/person/day; Table 12.6) for adults with a similar protein intake. Likewise, the estimated intake of asparagine plus aspartate (8.7 g/person/day; Table 12.7) is not substantially different from that (10.8 g/person/day; Table 12.6) for adults with a similar protein intake based on our food questionnaire (McNeal et al. 2018). In both males and females, the intake of glutamine was the highest, followed by glutamate, proline, and arginine in descending order. This is consistent with the pattern of AAs in wheat flour-, potato-, and animal protein-based foods (Hou et al. 2019; Wu et al. 2016) and the fact that these are the staple ingredients of diets consumed by the US population (USDA 2018). Such a high intake of dietary glutamine may not apply to regions of the world where four and potato are rarely consumed as foods.

The ingestion of total AASAs was about 20% greater than that of total EAAs in both adult men and women. Although AASAs can be synthesized from EAAs, these biochemical processes are limited by both substrates and enzymes, are

not 100% efficient, and can generate a large amount of ammonia (Hou et al. 2016). Elevated concentrations of ammonia in plasma are highly toxic to humans, particularly during pregnancy (Herring et al. 2018). Thus, it is imperative to provide AASAs in diets for humans to ensure their optimum health. Although dietary AASAs had long been thought not to be required by humans (IOM 2005), the term "NEAA" is now recognized to be a misnomer in nutritional sciences (Hou and Wu 2017). Within the male or female group, there were not significant differences ($P > 0.05$) in the dietary intakes of AASAs between days 0 and 90 of the study. Expressed per kg of lean body mass, the intake of each AASA in our study participants (males or females) met those recommended for healthy adults with minimal physical activity (Wu 2016). Except for glycine, the intake of all AASAs in our study participants (males or females) met those recommended for healthy adults with moderate physical activity (Wu 2016). Dietary glycine is sufficient for adult women with moderate physical activity. Plant foods are deficient in glycine relative to animal foods (e.g., meat) (Hou et al 2019; Wu et al. 2016). Intakes of some AASAs (alanine, arginine, aspartate, glutamate, and glycine) from a typical diet providing 90–110 g food protein/day do not meet the requirements of adults with an intensive physical activity. These AAs can be supplemented in the form of intact proteins (e.g., casein and whey) or free AAs. Thus, our findings will help to improve AA nutrition in both the general population and exercising individuals.

12.5 Estimation of Dietary Intakes of Energy, Lipids, Carbohydrates, Vitamins, and Minerals

Effects of dietary AAs on health and exercise performance can be influenced by dietary supplementation with other nutrients, such as digestible carbohydrate, dietary fiber, fats, vitamins, and nutritionally essential minerals, as well as energy (Jequier 1994; Kaur et al. 2014; Lissner et al. 1987). Expressed per kg of BW, adult men and women have a similar intake of dietary energy (Table 12.5). However, expressed per kg of LMB, adult women have a 14.5% to 25% higher intake of dietary energy than adult men. Based on our current knowledge of nutritional biochemistry, digestible carbohydrate (e.g., sugar) provides energy and NADPH (required for anti-oxidative reactions and NO synthesis; Wu and Meininger 2009), and dietary fiber improves gut health. Of note, fats supply additional energy to the body and inhibit the oxidation of AAs to CO_2 (thereby sparing AAs for protein synthesis and other biosynthetic pathways). Furthermore, minerals serve as coenzymes for cellular ATP production, as well as ATP-dependent and independent reactions, including nutrient absorption and transport, protein synthesis, gluconeogenesis, and regulation of acid–base balance (Bagchi et al. 2018; Kerksick et al. 2018; Wu et al. 2004; Wu 2018). Except for myristic acid and gamma-tocopherol, dietary intakes of fatty acids (Table 12.8), sugars (Table 12.9), minerals (Table 12.10), and vitamins (Table 12.11) did not differ between days 0 and 90 of the study. Fatty acids include saturated, monounsaturated, polyunsaturated, and *trans*-fatty acids. In all subjects, the dietary intake of oleic acid (C18:1, ω9) was the highest, followed by palmitic acid (C16:0), linoleic acid (C18:2, ω6), and stearic acid (C18:0) in descending order, and the total ω3 polyunsaturated fatty acids in the diets were only about 2.9–4.3% (men) and 1.6–2% (women) of the total ω6 polyunsaturated fatty acids. This likely reflected a low intake of marine seafood. In men and women, the consumption of myristic acid was reduced ($P < 0.05$) by 20% and 26%, respectively, at day 90 as compared with day 0. Furthermore, the ingestion of gamma-tocopherol by men was 29% lower ($P < 0.05$) at day 90 than at day 0, but no difference was detected for women. These results indicate that men and women may not constantly consume the same foods with exactly the same nutrient composition during a three-month period. Such moderate changes in

Table 12.7 Intakes of dietary glutamine, glutamate, asparagine, and aspartate from foods by US adults

Food	Food intake (g/person/day)[a]		Dietary intake of amino acids (mg/person/day)[b]			
	As-consumed food	Dry matter food	Glutamine	Glutamate	Asparagine	Aspartate
Corn grain	43.8	8.02	94.6	57.1	31.1	38.0
Peanut	8.22	7.96	231	228	157	113
Pistachio nut	6.03	5.80	156	107	59.2	57.4
Potato	54.8	11.5	187	11.6	221	46.6
Soybean	1.26	1.21	52.1	63.8	29.8	45.2
Sweet potato	9.86	2.26	4.97	12.3	27.1	7.54
Wheat flour	164.4	156.3	7112	438	699	560
White rice	27.4	24.9	198	184	115	106
Meat[c]	230	69.0	3443	5182	2305	2836
Chicken egg	39	10.3	233	427	216	299
Dairy product	320	64.0	1184	1107	275	426
Total[d]	315.8	217.9	12,896	7817	4133	4534

[a]Mean values of food consumption by US adults in 2016 (USDA 2018)
[b]Calculated on the basis of the content of amino acids in foods (Hou et al. 2019; Wu et al. 2016; Wu 2021). All the amino acids presented in this table are L-isomers
[c]Loin cuts of beef
[d]The ratio of total glutamine to total glutamate in the combined foods is 1.66:1.00. The ratio of total asparagine to total aspartate in the combined foods is 0.935:1.000

the dietary intakes of a few nutrients did not appear to affect the health of the individuals (McNeal et al. 2018).

12.6 Conclusion

Healthy men and women consumed protein as 19–20% and 17–19% of their total energy intakes, respectively, with animal protein representing about 75 and 70% of total protein intake, respectively. Expressed per kg of lean body mass, the intake of each EAA by the persons exceeded that recommended by the IOM (2005) and the FAO (2007) for healthy adults with a minimal physical activity. The staple foods consumed by the US population contained more AASAs than EAAs. In both males and females, the dietary intake of leucine was the highest, followed by lysine, valine, and isoleucine in descending order. In all the individuals, the ingestion of total AASAs was about 20% greater than that of total EAAs in both adult men and women. Expressed per kg of lean body mass, the intake of each AASA met those recommended for healthy adults with a minimal physical activity. Intakes of some AASAs (alanine, arginine, aspartate, glutamate, and glycine) from a typical diet providing 90–110 g food protein/day did not meet the requirements of adults with an intensive physical activity. Within the male or female group, there were not significant differences in the dietary intakes of all AAs between days 0 and 90 of the study, and this was also true for nearly all the other nutrients such as ω3 and ω6 polyunsaturated fatty acids, sugars and fiber, as well as nutritionally essential vitamins and minerals. We believe that the consistency in nutrient intakes between days 0 and 90 add further validity to our data. To our knowledge, this is the first report of dietary AASA intakes by humans. Our findings will help to improve AA nutrition and health in both the general population and exercising individuals.

Table 12.8 Daily dietary intakes of fatty acids on days 0 and 90 in adult males and females

Variable	Day of study	Males	Females
Saturated fatty acids			
Total saturated fatty	0	28.99 ± 3.93	22.91 ± 2.28
acids	90	23.82 ± 3.38	18.40 ± 3.35
Butyric acid	0	0.555 ± 0.086	0.378 ± 0.045
(C4:0)	90	0.434 ± 0.064	0.244 ± 0.028
Caproic acid	0	0.291 ± 0.058	0.182 ± 0.023
(C6:0)	90	0.212 ± 0.035	0.126 ± 0.019
Caprylic acid	0	0.215 ± 0.046	0.156 ± 0.018
(C8:0)	90	0.153 ± 0.017	0.112 ± 0.021
Capric acid	0	0.423 ± 0.070	0.313 ± 0.032
(C10:0)	90	0.326 ± 0.043	0.213 ± 0.028
Lauric acid	0	0.557 ± 0.087	0.619 ± 0.106
(C12:0)	90	0.502 ± 0.068	0.469 ± 0.136
Myristic acid	0	2.22 ± 0.32	1.68 ± 0.16
(C14:0)	90	1.77 ± 0.22*	1.24 ± 0.19*
Palmitic acid	0	16.56 ± 2.27	12.75 ± 1.22
(C16:0)	90	13.66 ± 2.00	10.66 ± 2.08
Stearic acid	0	7.12 ± 0.94	5.99 ± 0.76
(C18:0)	90	5.97 ± 0.87	4.59 ± 0.88
Monounsaturated fatty acids (cis)			
Total monounsaturated	0	33.92 ± 4.30	27.03 ± 2.47
fatty acids	90	28.03 ± 4.11	23.32 ± 4.61
Palmitoleic acid	0	1.60 ± 0.24	1.17 ± 0.12
(C16:1, ω7)	90	1.45 ± 0.28	1.11 ± 0.28
Oleic acid	0	31.58 ± 4.00	25.25 ± 2.31
(C18:1, ω9)	90	25.93 ± 3.77	21.68 ± 4.20
Eicosenoic fatty acid	0	0.316 ± 0.041	0.260 ± 0.034
(C20:1, ω11)	90	0.283 ± 0.047	0.203 ± 0.048
trans-Fatty acids			
trans-Palmitoleic acid	0	0.115 ± 0.015	0.084 ± 0.009
(7-trans-C16:1, ω7)	90	0.092 ± 0.012	0.072 ± 0.018
trans-Oleic acid	0	2.08 ± 0.24	1.85 ± 0.22
(9-trans-C18:1, ω9)[a]	90	1.83 ± 0.26	1.50 ± 0.29
11-*trans*-Eicosenoic	0	0.001 ± 0.001	0.001 ± 0.001
Acid (11-trans-C20:1, ω11)	90	0.001 ± 0.001	0.001 ± 0.001
Cis-9, *trans*-11	0	0.122 ± 0.020	0.084 ± 0.008
conjugated linoleic acid	90	0.096 ± 0.013	0.075 ± 0.018
Polyunsaturated fatty acids			
Total polyunsaturated	0	18.36 ± 2.33	14.28 ± 1.33

(continued)

Table 12.8 (continued)

Variable	Day of study	Males	Females
fatty acids	90	13.87 ± 2.10	13.22 ± 2.63
Linoleic acid	0	15.37 ± 1.92	12.21 ± 1.16
(C18:2, ω6)	90	11.46 ± 1.78	11.32 ± 2.36
Arachidonic acid	0	0.322 ± 0.079	0.197 ± 0.026
(C20:4, ω6)	90	0.298 ± 0.095	0.193 ± 0.051
Eicosapentaenoic acid	0	0.182 ± 0.044	0.101 ± 0.030
(EPA; C20:5, ω3)	90	0.215 ± 0.064	0.070 ± 0.030
Docosapentaenoic acid	0	0.039 ± 0.007	0.023 ± 0.004
(DPA; C22:5, ω3)	90	0.041 ± 0.012	0.024 ± 0.006
Docosahexaenoic acid	0	0.334 ± 0.099	0.177 ± 0.042
(DHA; C22:6, ω3)	90	0.350 ± 0.113	0.142 ± 0.029
Total ω3 polyunsaturated	0	0.515 ± 0.135	0.278 ± 0.069
fatty acids	90	0.566 ± 0.171	0.212 ± 0.054
Total lipids (triacylglycerols plus free fatty acids)			
Plant lipids	0	43.90 ± 5.02	39.32 ± 4.32
	90	34.71 ± 5.80	33.03 ± 5.61
Animal lipids	0	46.32 ± 8.46	31.95 ± 3.30
	90	38.14 ± 6.68	28.19 ± 6.68
Dairy lipids (part of animal lipids)	0	16.76 ± 2.95	10.91 ± 1.24
	90	13.04 ± 1.88	7.32 ± 0.95
Total lipids	0	90.22 ± 11.59	71.27 ± 6.48
	90	72.85 ± 10.35	61.21 ± 11.87

Values, expressed as g/person/day, are means ± SEM, $n = 13$

*$P < 0.05$ versus the value for day 0 (the beginning of the trial)

[a]The 9-trans-isomer of oleic acid (also known as elaidic acid or 9-octadecenoic acid)

Acknowledgements The portion of this work on the food intake questionnaire of study participants was funded by International Council of Amino Acid Science (Brussels, Belgium). Tapasree R. Sarkar was supported by a training grant from the National Cancer Institute (T32-CA00301). Bani K. Mallick was supported, in part, by grants from the National Cancer Institute (R01CA194391) and the National Science Foundation (CCF 1934904). Raymond Carroll was supported by a grant from the National Cancer Institute (U01-CA057030). Guoyao Wu was supported by Texas A&M AgriLife Research (H-8200).

Table 12.9 Daily dietary intakes of sugars on days 0 and 90 in adult males and females

Variable	Day of study	Males	Females
Natural sugars in foods	0	62.00 ± 12.24	46.86 ± 5.35
	90	43.13 ± 6.28	38.35 ± 5.18
Sucrose	0	50.94 ± 8.80	50.80 ± 6.25
	90	31.63 ± 5.38	34.89 ± 4.55
Fructose	0	33.70 ± 6.16	39.87 ± 10.66
	90	24.29 ± 7.09	20.81 ± 3.07
Lactose	0	17.79 ± 4.46	12.82 ± 1.86
	90	14.18 ± 3.30	10.38 ± 1.99
Maltose	0	2.82 ± 0.37	2.39 ± 0.33
	90	2.07 ± 0.27	1.67 ± 0.22
Added sugars	0	75.66 ± 17.93	93.36 ± 21.65
	90	50.10 ± 17.77	48.39 ± 7.77
Total sugars	0	137.66 ± 22.32	140.20 ± 23.28
	90	93.23 ± 16.38	86.73 ± 10.83

Values, expressed as g/person/day, are means ± SEM, $n = 13$

Table 12.10 Daily dietary intakes of minerals on days 0 and 90 in adult males and females

Variable	Day of study	Males	Females
Sodium	0	2795 ± 282	2285 ± 175
	90	2247 ± 212	1960 ± 400
Potassium	0	3911 ± 542	2978 ± 245
	90	3029 ± 375	2720 ± 530
Calcium	0	1158 ± 187	1011 ± 114
	90	876 ± 127	737 ± 106
Phosphorous	0	1828 ± 282	1364 ± 103
	90	1439 ± 207	1194 ± 215
Magnesium	0	454 ± 73	346 ± 37
	90	346 ± 54	312 ± 55
Zinc	0	28.3 ± 7.66	14.1 ± 1.48
	90	19.0 ± 5.36	20.7 ± 6.49
Copper	0	2.23 ± 0.50	1.64 ± 0.14
	90	1.64 ± 0.23	1.78 ± 0.32
Iron	0	15.9 ± 2.05	14.2 ± 1.27
	90	11.8 ± 1.18	13.8 ± 2.82

Values, expressed as mg/person/day, are means ± SEM, $n = 13$

Table 12.11 Daily dietary intakes of vitamins on days 0 and 90 in adult males and females

Variable	Day of study	Males	Females
Vitamin A[a]	0	1.4 ± 0.36	1.0 ± 0.14
	90	1.3 ± 0.19	1.2 ± 0.23
Vitamin D[a] (IU/person/day)	0	428 ± 123	303 ± 71
	90	411 ± 71	342 ± 69
Vitamin C[a]	0	280 ± 84	193 ± 55
	90	199 ± 96	223 ± 77
Thiamin (vitamin B_1)	0	8.79 ± 4.68	1.79 ± 0.20
	90	6.02 ± 4.18	1.91 ± 0.39
Riboflavin (vitamin B_2)	0	9.72 ± 4.93	2.20 ± 0.20
	90	6.85 ± 4.38	2.40 ± 0.43
Niacin (vitamin B_3)	0	51.3 ± 13.5	42.0 ± 14.8
	90	36.4 ± 6.6	30.6 ± 6.8
Pantothenic acid (vitamin B_5)	0	16.5 ± 6.0	6.98 ± 0.91
	90	13.9 ± 5.0	8.19 ± 1.8
Pyridoxine (vitamin B_6)	0	1.20 ± 0.11	1.27 ± 0.17
	90	1.33 ± 0.19	1.26 ± 0.19
Food folate	0	0.399 ± 0.065	0.287 ± 0.024
	90	0.282 ± 0.035	0.260 ± 0.050
Total folate	0	0.667 ± 0.116	0.510 ± 0.054
	90	0.538 ± 0.059	0.567 ± 0.111
Alpha-tocotrienol	0	0.871 ± 0.181	0.779 ± 0.083
	90	0.770 ± 0.154	0.704 ± 0.164
Beta-tocotrienol	0	0.710 ± 0.107	0.575 ± 0.060
	90	0.445 ± 0.048	0.613 ± 0.178
Gamma-tocotrienol	0	0.446 ± 0.051	0.405 ± 0.039
	90	0.404 ± 0.078	0.399 ± 0.107
Delta-tocotrienol	0	0.033 ± 0.004	0.038 ± 0.004
	90	0.031 ± 0.008	0.041 ± 0.014
Beta-tocopherol	0	0.297 ± 0.029	0.254 ± 0.020
	90	0.213 ± 0.023	0.260 ± 0.065
Gamma-tocopherol	0	11.06 ± 1.24	9.43 ± 0.94
	90	7.81 ± 1.26*	8.56 ± 1.60
Delta-tocopherol	0	2.56 ± 0.28	2.30 ± 0.23
	90	1.97 ± 0.34	2.07 ± 0.45

Data are means ± SEM, $n = 13$. Except for vitamin D, all values are expressed as mg/person/day

*$P < 0.05$ versus the value for day 0 (the beginning of the trial)

[a]Taken from McNeal et al. (2018)

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