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Amino Acids Sensing *in vivo*, a Significant Research Area on Amino Acid Nutrition

[1]Yongfei Wang, [2]Zemeng Feng, [2]Yuzhe Zhang and [3]Fang Jun
[1]Research and Development Center, Twins Group Co., Ltd., Nanchang, 330096 Jiangxi, China
[2]Key Laboratory Agroecol Process Subtropical Region, Chinese Academy of Science,
Institute Subtropical Agriculture, Research Center of Healthy Breeding Livestock and Poultry,
Hunan Engineering and Research Center Animal and Poultry Science,
Scientific Oberving and Experimetal Station of Animal Nutrition and Feed Science in South-Central,
Ministry of Agriculture, Furong Road #644, Changsha City,
410125 Hunan, Peoples Republic of China
[3]College of Bioscience and Biotechnology, Hunan Agricultural University,
410126 Hunan Changsha, China

Abstract: Amino acids are important nutrients that function as both tissue building blocks and metabolic regulators. How amino acid balance is vital for the development and metabolism. Amino acid sensors, serials of particular functional molecules, possess the function of sensing amino acids has been reported in recent years. Here, both intracellular and extracellular amino acid sensors were expounded including their composition, amino acid sensing mechanism and their applications.

Key words: Amino acid-sensing, SPS-sensing pathway, mammalian Target of Rapamycin (mTOR), class C G-protein coupled receptors, nutrition

INTRODUCTION

As one of the three important macronutrients (protein, fat and carbohydrate) in diet, protein is the main nitrogen source and is essential for the development and metabolism. In the small intestine, dietary protein is digested into Amino Acids (AA) and small peptides (finally discomposed into AA in cell) and function as the building blocks for new proteins and metabolic precursors (Adibi and Mercer, 1973). Besides, AA also regulate nutrient metabolism and impacts both protein synthesis regulation and proteolysis (Paddon-Jones *et al*., 2004; Nakashima *et al*., 2005) thus contributing to the regulation of a number of intracellular enzymes. Thus, AA play fundamental roles by linking the deconstruction of protein in the diet and construction of protein or peptides in tissues.

Amino acid balance, especially Indispensable Amino Acids (IAA) is vital for normal metabolism. After billions of years of evolution, several discrete mechanisms were developed to sense AA concentrations *in vivo* and to keep the AA balance by regulating various pathways. Biological sensing of AA *in vivo* play key roles in coupling changes in whole body protein and AA metabolism to appropriate physiological responses.

Amino acid sensors are particular functional molecules sensing AA which can detect and transmit AA stimulito following cascaded signal pathways inducing genes expression or secretion of hormones that controlling AAs metabolism. Beyond AA transporting, AA transporters also play in the sensing of AA levels both directly as initiators of nutrient signal and indirectly as regulators of external AA access to intracellular receptor/signaling mechanisms (Hyde *et al*., 2003). Now, studies of AA sensing mainly focus on two key areas: The discovery and identification of AA receptors function as sensors and the identification of AA transporters possess AA sensing function. Amino acid sensing containing two parts, one is extracellular AA sensing to ascertain the AA concentration in the cell surrounding environments and to regulate the AA transporting by controlling AA transporters, the other

Corresponding Author: Zemeng Feng, Key Laboratory Agroecol Process Subtropical Region, Chinese Academy of Science, Institute Subtropical Agriculture, Research Center of Healthy Breeding Livestock and Poultry, Hunan Engineering and Research Center Animal and Poultry Science, Scientific Oberving and Experimetal Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Furong Road #644, Changsha City, 410125 Hunan, Peoples Republic of China

is intracellular AA sensing to ascertain the AA concentration in the inside of cell and to regulate protein breakdown, AA synthesis and uptake and to coordinate Central Nervous System (CNS) feeding responses.

EXTRACELLULAR AA SENSING

Extracellular AA receptors have been identified in numerous cells and include for example, the neuronal receptors for glutamate (Matthews and Anderson, 2002) and glycine (Betz *et al*., 1999). In the CNS, AA sensitivities should be significantly higher because extracellular AA levels are substantially lower in the cerebrospinal fluid than in systemic plasma (Brown *et al*., 2004). Outside the nervous system, extracellular AA sensing systems seem to operate primarily to coordinate hormonal, digestive and trans-epithelial transport responses following protein ingestion. In gastrointestinal tract there lie the first AA sensing mechanisms. These AA sensors are responsible for sensing the AA contained in consumed food and control the digestion and absorption either via the release of gut hormones or via direct effects on epithelial transport (Strunz *et al*., 1978; Csendes and Grossman, 1972). Several different extracellular AA sensing systems have been formed in evolution in various species.

Taz: Taz is a typical chimeric receptor consisting of part of the aspartate receptor (Tar) of *Escherichia coli* fused to the C-terminal part of the *E. coli* osmosensor EnvZ (Utsumi *et al*., 1989). All 20 natural AA have interaction with Taz. Leu, Met, Val and Ser can reduce Taz expression which in the case of Leu is likely attributed to a direct effect on the receptor. Taz exhibits a higher sensitivity to Leu compared with Asp. Taz has exquisite sensitivity to structure conformations and small conformational changes can induce the change of the way it responds to different AA (Michalodimitrakis *et al*., 2005).

Ssy1-Ptr3-Ssy5 (SPS) AA sensing pathway: Yeast can sense the presence of low levels of extracellular AA with activating respective transporters to facilitate nutrient import (Forsberg and Ljungdahl, 2001a, b). After years of research, the SPS AA sensing pathway responsive to extracellular AA was identified using *saccharomyces cerevisiae* as a model organism (Didion *et al*., 1998; Iraqui *et al*., 1999; Klasson *et al*., 1999). The SPS complex includes Ssy1and two downstream factors, Ptr3 and Ssy5 (Klasson *et al*., 1999; Forsberg *et al*., 2001a, b). The three components are reported to interact physically, Ssy1 to Ptr3 (Uetz *et al*., 2000) and Ptr3 to itself and Ssy5 (Bernard and Andre, 2001a) and they are involved in the same cellular functions for Ssy1, Ptr3 and Ssy5 mutations have identical pleiotropic phenotypes (Jorgensen *et al*., 1998; Klasson *et al*., 1999; Bernard and Andre, 2001a; Forsberg and Ljungdahl, 2001a, b).

Ssy1 is a plasma membrane protein evolutionarily related to the AA permeases but unable to transport AA and was first functionally identified in a screen for mutants with decreased uptake of the branched chain AA (Jorgensen *et al*., 1997; Nelissen *et al*., 1997; Didion *et al*., 1998; Jorgensen *et al*., 1998; Iraqui *et al*., 1999; Klasson *et al*., 1999). Amino acid transport is not required for signaling and support the notion that sensing by Ssy1 occurs via its direct interaction with extracellular AA (Gaber *et al*., 2003). The amino terminus of Ssy1 plays an important role in an intracellular aspect of this signaling (Bernard and Andre, 2001b; Forsberg and Ljungdahl, 2001a, b). A key function of the SPS sensor is to activate the transcription factors Stp1 (Jorgensen *et al*., 1997) and Stp2 which bind to the promoters and induce increased transcription of the responsive AA permease genes (Nielsen *et al*., 2001). When extracellular AA bind to Ssy1, Stp1 and Stp2 are activated through Ptr3, Ssy5 (Forsberg and Ljungdahl, 2001a, b; Klasson *et al*., 1999) and Grr1, a component of the SCFGrr1 ubiquitin ligase (Abdel-Sater *et al*., 2004a, b; Andreasson and Ljungdahl, 2002). Though Stp1 and Stp2 are two homologous transcription factors both ubiquitin-dependent degradation of Stp1 and Stp2 and their intracellular localization are differentially regulated. The E2 ubiquitin-conjugating enzyme Cdc34 is required for degradation of both full-length and processed Stp1 but not Stp2. The full-length Stp1 is localized both in the cytoplasm and at the cell periphery, whereas full-length Stp2 is localized only diffusely in the cytoplasm. The N-terminal domain of Stp1 is required for localization of full length Stp1 to the cell periphery. As the primary factor, Stp2 is involved in basal activation of target gene expression (Tumusiime *et al*., 2011). Grr1, the F-box component of the SCFGrr1 E3 ubiquitin ligase is the primary factor in degradation of full-length Stp1 whereas both Grr1 and Cdc4 are required for degradation of processed Stp1. The signal is transmitted to the Stp transcription factors and then released from their cytosolic location to the nucleus (Andreasson and Ljungdahl, 2002; Eckert-Boulet *et al*., 2004). The activation of Ssy5, a novel protease leads to endoproteolytic processing and activation of Stp1 and Stp2 (Abdel-Sater *et al*., 2004a, b; Andreasson *et al*., 2006; Poulsen *et al*., 2006). The Ssy5-dependent processing of Stp1 and Stp2 is a key activation step in the

regulation of the SPS AA sensing pathway. Both Ssy5 and Ptr3 remain essential for AA signaling (Gaber *et al*., 2003) which results in cleavage of the transcription factors Stp1 and Stp2, removing 10 kDa of the N terminus of each of them (Poulsen *et al*., 2005). This confers the transcription factors with the ability to gain access to the nucleus and activate transcription of AA permease genes. Research have found that Ssy1 was required for normal AA-induced transcription of several AA permease genes (i.e., AGP1, BAP3, GNP1, BAP2, VAP1 and TAT2), the peptide transporter gene PTR2 and the arginase gene CAR1 (Didion *et al*., 1998; Jorgensen *et al*., 1998; Iraqui *et al*., 1999; Klasson *et al*., 1999). Besides AA permease genes, Ssy1 also regulate the transcription of nitrogen catabolite repression sensitive genes and methionine-biosynthesizing genes (Kodama *et al*., 2002). Although, protein phosphatase 2A negatively regulates SPS signaling (Eckert-Boulet *et al*., 2006), two isoforms of the yeast casein kinase I proteins, Yck1 and Yck2, function as positive regulators of this pathway (Abdel-Sater *et al*., 2004a; Liu *et al*., 2008; Spielewoy *et al*., 2004).

Class III G-protein-coupled receptor family: A group of extracellular AA sensors has recently been identified within class III of the G-protein-coupled receptor superfamily. These sensors control G-protein regulated signaling pathways and are widely distributed. They seem to be designed for the detection of subgroups of AA or even specific AA including L-glutamate (the metabotropic Glutamate (mGlu) receptors). They also include several broad-spectrum AA sensors, the extracellular Ca^{2+}-sensing Receptor (CaR) which responds to aromatic, aliphatic and polar AA but not to positively charged or branched chain amino acids (Conigrave *et al*., 2000), a heterodimeric taste receptor composed of the T1R1 and T1R3 receptors (T1R1/T1R3) which responds to aliphatic, polar, charged and branched chain AA but not to aromatic AA (Nelson *et al*., 2002) and the goldfish 5.24 receptor (Kuang *et al*., 2003; Speca *et al*., 1999) and its mammalian ortholog, G-protein-coupled receptor family C member 6A (GPRC6A) (Wellendorph *et al*., 2005; Kuang *et al*., 2005; Wellendorph and Brauner-Osborne, 2004) which respond to basic, aliphatic and polar amino acids.

Ca^{2+}-sensing receptor: Ca^{2+}sensing receptor is widely expressed in mammalian tissues and cells (Ruat *et al*., 1995; Chattopadhyay *et al*., 1997, 1998; Hofer and Brown, 2003) and is activated by a variety of divalent and polyvalent cations (Hofer and Brown, 2003). With various signals to process and numerous signaling pathways to access, CaR possesses pluripotency, providing ligand selective control of physiological responses. Along with other class III GPCRs, CaR is involved in AA sensing (Conigrave *et al*., 2000; Conigrave and Brown, 2006). Ca^{2+}-sensing receptor is a broad-spectrum a AA sensor but not a universal AA sensor. Ca^{2+}-sensing receptor is the only member of this receptor family o select for aromatic L-AAs (Conigrave *et al*., 2000, 2004) and less sensitive to the acidic AA (aspartate and glutamate) and is essentially unresponsive to basic and branched chain AA. These effects are stereo-selective. Ca^{2+} and AA sensing function of CaR can be dissociated (Mun *et al*., 2005). The CaR's response to AA is dependent not only on variations in AA concentration to which the receptor is exposed but also on the prevailing Ca^{2+} concentration with different AA exhibiting differential Ca^{2+} concentration thresholds for receptor activation. However when the plasma concentrations of different AA are taken into consideration (aliphatic and polar AA>aromatic AA). Thus, it is significantly means that universal AA sensing would require co-expression of the CaR with one or more additional AA sensors such as GPRC6A or T1R1/T1R3.

In the brain, the CaR is expressed in the ionic strength-sensing subfornical organ that provides inputs to hypothalamic centres that control antidiuretic hormone secretion, various other organs including the hippocampus and more diffusely on myelin-producing oligodendrocytes. In the GI tract, the CaR is expressed at high levels on the basolateral membranes of gastric parietal cells (Cheng *et al*., 1999) and may be involved in Ca^{2+}-dependent and AA-dependent activation of acid secretion (Geibel *et al*., 2001; Busque *et al*., 2005). The CaR's selectivity for aromatic AA provides an explanation for the recognized selectivity of gastric acid secretion for aromatic AA (Strunz *et al*., 1978). Ca^{2+}-sensing receptor mediates the known effects of aromatic AA on cholecystokinin release, bile flow and pancreatic enzyme secretion (Conigrave and Brown, 2006). It seems likely that AA dependent activation of CaR also modulates the secretion of hormones involved in the control of calcium metabolism, growth, nutrient disposition and the intestinal digestion and absorption of nutrients. Ca^{2+}-sensing receptor can integrate signals arising from distinct classes of nutrients: mineral ions and AA.

G-protein-coupled receptor family C member 6A: G-protein-coupled receptor family C member 6A was classified as an orphan class III GPCR until the recent demonstration that both the mouse and human orthologs are activated by AA (Wellendorph *et al*., 2005; Kuang *et al*., 2005). The goldfish 5.24 chemosensory receptor is expressed in the olfactory epithelium and is

thought to have a role in feeding-related navigation by sensing gradients of AA in water. In mammals, GPRC6A which is widely expressed in mammalian tissues including brain, skeletal muscle, heart, lung, spleen, kidney and liver (Kuang *et al*., 2005) shows the highest AA sequence similarity to the goldfish 5.24 chemosensory receptor (41%) and responds sensitively to polar and aliphatic with an apparent preference for the basic AA (Arg and Lys) (Speca *et al*., 1999) but not other AA (Wellendorph *et al*., 2005; Kuang *et al*., 2005). The 5.24 receptor is more tolerant of variations in the size, shape and even charge of the AA side chain than is GPRC6A. Three alternatively spliced forms of GPRC6A are present in both the mouse and the human, two of these are short forms that have inframe deletions of all or parts of exons 3 and 4 which code for sequences in the Venus Fly Trapdomain. Surprisingly, whereas the mouse long protein isoform is readily expressed on the cell surface of transfected HEK293 cells, the corresponding human isoform is expressed at much lower levels (Wellendorph *et al*., 2005; Kuang *et al*., 2005).

T1R1/T1R3: Though much less is known about the expression and function in enteroendocrine cells of heterodimers of T1R1/T1R3 this G-protein-coupled taste receptor heterodimeris believed to be sensitive to a broad range of AA and act broadly tuned AA sensors, although the precise ligand specificity appears to be species-dependent (Nelson *et al*., 2002; Wellendorph *et al*., 2009).

These receptors including CaR, GPRC6A andT1R1/T1R3 provide mechanisms by which changes in the intestinal and plasma levels of AA regulate digestion, absorption, growth and metabolism and by which oral AA mediate the sensation of umami taste. Amino acid binding depends on highly conserved residues in the N-terminal Venus Flytrap (VFT) domains that bind the a-amino and a-carboxylate functional groups. The VFT domains mediate AA binding in the class III GPCR. These bilobed structural motifs have an ancient lineage providing the basis of nutrient sensing by the periplasmic binding proteins of Gram-negative bacteria (Felder *et al*., 1999). The CaR VFT domain but none of its other domains is required for AA sensing (Mun *et al*., 2004). Mutational analysis of conserved binding residues in the CaR VFT domain has led to the identification of two mutants, T145A and S170T which selectively impair AA sensing, leaving extracellular Ca^{2+} sensing intact. Furthermore, the double mutant T145A/S170T selectively disables AA sensing (Mun *et al*., 2005).

Amino acid transporters function as AA sensors: Besides surface membrane receptors, AA transporters are also involved in sensing extracellular AA levels (Hyde *et al*., 2003). In *S. cerevisiae*, CaGappermeases, at least three of the six studied genes (CaGAP1, CaGAP2 and CaGAP6), possess the ability to participate in AA sensing (Kraidlova *et al*., 2011). Gap1 acts as an AA sensor for rapid activation of the fermentable growth medium. Na^{+}-coupled AA Transporters (SNAT) which belong to the *SLC38* gene family also possess the function as AA sensors. In these transporters, SNAT2 is widely distributed and has considerable preference for Pro (Mackenzie and Erickson, 2004). In enteroendocrine cells located in the distal region of the small bowel and in the colon, SNAT2 is involved in the sensing for sudden surges in the change of luminal or local neutral AA concentration (Young *et al*., 2010). The SNAT2-mediated plasma membrane transport of metabolizable AA can have an impact on cellular metabolism.

INTRACELLULAR AA SENSING

The intracellular concentration of any given AA is governed by the rates of AA influx and efflux, protein synthesis, protein degradation and amino acyl-tRNA production, plus AA catabolism and biosynthesis. All these processes should be accurately regulated and the first step is to sense the intracellular AA concentration which are well done by AA sensors located intracellular.

Amino acyl-tRNA synthetases: Most of the AAs transported into cell are first used for the biosynthesis of proteins or/and peptides in ribosome. In this process, AAs are sensed based on amino Acyl-free tRNA Synthetases (ARSs) (Carter, 1993; Hao *et al*., 2005; Gietzen *et al*., 2007; Tan *et al*., 2009). In genetic studies on yeast, eukaryotic Initiation Factor 2α (eIF2α) kinase is identified function as the primary sensor of AA starvation and is involved in the regulation of AA homeostasis (Hinnebusch, 2005). This pathway has been shown to be conserved from yeast to mammals (Harding *et al*., 2000; Chaveroux *et al*., 2010). Following prolonged AA deprivation, uncharged tRNA molecules including General Control Nonderepressible-2 (GCN2) which is homologous to histidyl-tRNA synthetases, mediated phosphorylation to form the phospho-protein eIF2α (Wek *et al*., 1995) are accumulated and may also be sensed within the cell. The 6-Phosphofructo-1-kinaseis inhibited by uncharged tRNA *in vitro* (Rabinovitz, 1992) potentially leading to a reduction in intracellular fructose-1, 6-bisphosphate an activator of the translation factor eukaryotic initiation factor 2β (Singh and Wahba, 1995; Kimball and Jefferson, 1995). A mammalian homologue of GCN2 has been cloned and it functions in response to AA starvation (Zhang *et al*., 2002), however, it is presently uncertain

whether mammalian GCN2 is activated by uncharged tRNA. In particular, GCN2 activation initiates various physiological responses such as food intake inhibition (Hao *et al*., 2005; Maurin *et al*., 2005) or regulation of hepatic lipid metabolism (Guo and Cavener, 2007) during nutritional deprivation of one essential AA. Unbalanced AA supplies induce accumulation of uncharged transfer RNA which induces phosphorylation of eIF2α via the GCN2 kinase. Through eIF2α phosphorylation, GCN2 regulates both protein synthesis rate and stress-induced gene expression at transcriptional level. tRNA/GCN2/phospho-proteineIF2α system signals IAA deficiency in the output neurons of the brain area essential for the adaptive rejection of an IAA-deficient diet within a 20-min time frame of the behavioral response (Koehnle *et al*., 2003; Hao *et al*., 2005).

mTOR: An intimate connection exists between the AA-sensing pathway and the rapamycin-sensitive Target Of Rapamycin (TOR) signaling pathway (Shin *et al*., 2009). When observing rapamycin exhibits immunosuppressive effects in yeast (Heitman *et al*., 1991) first identified a conserved member of the PI3K superfamily, TOR which regulates cell growth and metabolism in response to environmental cues. There exist at least two isoform of TOR, TOR complex 1 (TORC1) and 2 (TORC2). TORC1 and TORC2 are two distinct complexes that act as the target of rapamycin (Bhaskar and Hay, 2007) that appear to act downstream and upstream of Akt, respectively. TORC1 is inhibited by rapamycin with the strongest effect while TORC2 was reported just to be inhibited in some cell types (Sarbassov *et al*., 2004, 2006). The key components of the TOR signaling pathway are structurally conserved between yeast and mammals, despite divergence of sequence to a degree that thwarts detection through simple homology searches (Kogan *et al*., 2010). mTOR is the mammalian version of TOR.

TORC1 mediates anabolic processes that promote growth by directly phosphorylating p70S6K (p70S6 kinase) and eukaryotic initiation factor 4E-Binding Protein 1 (4E-BP1) to modulates cell growth in response to local AA levels by relocalization (Christie *et al*., 2002; Findlay *et al*., 2007; Sancak *et al*., 2008). The low concentrations of extracellular nutrients and reduced TORC1 signaling lead to enhanced autophagy (Pattingre *et al*., 2008; Cecconi and Levine, 2008) where organelles are destroyed and their component macromolecules recycled through autophagolysosomes. mTORC1 is placed in a central position in cell growth regulation and dysregulation of mTOR signaling pathway and has been implicated in many serious human diseases including cancer, diabetes and tissue hypertrophy. mTORC1 can be activated by intracellular AA, particularly Leu (Beugnet *et al*., 2003; Lynch, 2001; Avruch *et al*., 2009). Though some model were proposed (Kim, 2009), the mechanism of how AA activate mTOR signaling pathway is largely uncharted for the diversiform characters of AA including structure and metabolism. The Proton-Assisted AA Transporter (PAT/SLC36) family was identified as uniquely potent regulators of TORC1-mediated growth in Drosophila (Goberdhan *et al*., 2005). Members of PAT/SLC36 family have been identified as critical components of the AA-sensing system that regulates mTORC1 present in endosomal and lysosomal membranes (Heublein *et al*., 2010). In human skeletal muscle, mTORC1 activity increases the expression of several AA transporters which may be important adaptive responses to sensitize muscle to a subsequent increase in AA availability (Dickinson and Rasmussen, 2011).

APPLICATION

Traditionally, ninhydrin is used when classical AA analyzers are employed whereas fluorescence labeling is used for both classical AA analyzers and HPLC Methods. The shortcoming of these methods is large volume of organic solvents requirement and the long processing time (Cohen and Michaud, 1993). Research on biosensor is emerging in recent years and various biosensors were designed based on DNA, protein, cell and tissue levels. As many AA sensors have been identified in life, researchers can design biosensors to detect or monitor AA in the environment and in body fluid *in vitro* or *in vivo*. As early as 1991, Pseudomonas L-Phe oxidase was immobilized on a nylon membrane to act as an AA sensor for routine analyses of the total aromatic AA in fermentation media (Nakajima *et al*., 1991). The fundamental conditions for reaction and detection in AA sensing by ARS can also be applied to biosensor technology. A rapid and easy method was described by Kugimiya *et al*. (2008) which could be realized without any AA labeling process without a large volume of organic solvents and within a reasonable measurement time. Nanotechnology is now concentrating on *in vivo* sensors (nano-sized devices) so that on being injected it could act as reporters of *in vivo* concentrations of chief analytes (Lavan *et al*., 2003).

CONCLUSION

A mathematic model has been built by the research group to explore the relationship between the expression levels of AA sensing genes belong to Class III G-protein-coupled receptor family in liver and AA concentration in

plasma. Wire-less biosensor can be designed to sense AA (particularly IAA) concentration in plasma in real time and can be used for the diagnosis of disease and for accurately determining the condition of diseases in clinical settings (Noguchi *et al*., 2006).

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