[Frontiers in Bioscience 17, 2725-2739, June 1, 2012]

Nutritional and regulatory role of branched-chain amino acids in lactation

Jian Lei[1], Dingyuan Feng[1], Yongliang Zhang[1], Feng-Qi Zhao[2], Zhenlong Wu[3], Ana San Gabriel[4], Yoshiyuki Fujishima[4], Hisayuki Uneyama[4], Guoyao Wu[3,5]

[1]College of Animal Science and Technology, South China Agricultural University, Guangzhou, P. R. China 510642, [2]Department of Animal Science, University of Vermont, Burlington, VT, USA 05405,[3]State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing, China 100193,[4]Institute for Innovation, Ajinomoto Inc., 15-1, Kyobashi 1-chome, Chuo-ku, Tokyo, Japan 104-8315,[5]Department of Animal Science and Faculty of Nutrition, Texas A&M University, College Station, TX, USA 77843-2471

TABLE OF CONTENTS

1. ABSTRACT

Optimal growth and health of suckling neonates critically depend on milk production by their mothers. In both humans and animals, branched-chain amino acids (BCAA) are not only the major components of milk proteins but are also nitrogenous precursors for the synthesis of glutamate, glutamine, alanine, and aspartate in the mammary gland. These synthetic pathways, which are initiated by BCAA transaminase, contribute to the high abundance of free and peptide-bound glutamate, glutamine, aspartate and asparagine in milk. In mammary epithelial cells, the carbon skeletons of BCAA can be partially oxidized via branched-chain α-ketoacid dehydrogenase to provide energy for highly active metabolic processes, including nutrient transport, protein turnover, as well as lipid and lactose syntheses. In addition, results of recent studies indicate that BCAA play regulatory roles in mammary metabolism. For example, leucine can activate the mammalian target of rapamycin cell signaling pathway to enhance protein synthesis in mammary epithelial cells. Dietary supplementation with BCAA may have great potential to enhance milk synthesis by the lactating mammary gland, thereby improving neonatal survival, growth and development.

2. INTRODUCTION

Based on nitrogen balance, amino acids have been traditionally classified as nutritionally essential or nonessential (1). For nonruminants, essential amino acids are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. The carbon skeletons of these amino acids are not synthesized by animal cells and, therefore, these amino acids must be provided in diets. For some mammals, including cats and ferrets, dietary arginine is essential for maintenance, growth and lactation. Nonessential amino acids are alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, proline, serine, and tyrosine. Historically, the so-called "nonessential amino acids" have not received much attention from nutritionists because of the assumption that these nutrients are synthesized adequately by the body. However, growing evidence shows that this assumption is not valid for at least some amino acids (2). For example, Haynes et al. (3) found that glutamine in sow's milk is inadequate for maximal growth of suckling piglets although concentrations of this amino acid (free and peptide-bound) are particularly high (Figures 1 and 2). Additionally, Mateo *et al.* (4) reported that endogenous synthesis of arginine (a nutritionally essential amino acid for neonates) cannot meet the need of maximal milk production by lactating sows.

Table 1. Concentration-dependent increases in leucine degradation by mouse and bovine mammary epithelial cells

	Mouse mammary epithelial cells			Bovine mammary epithelial cells		
Leucine concentration	0.25 mM	0.5 mM	2 mM	0.25 mM	0.5 mM	2 mM
CO_2 from all carbons (A)	1.31±0.11^{c}	2.13±0.15^{b}	4.60±0.23^{a}	0.18±0.02^{c*}	0.44±0.03^{b*}	1.60±0.09^{a*}
CO_2 from carbon 1 (B)	0.50±0.02^{c}	0.79±0.04^{b}	1.63±0.09^{a}	0.15±0.02^{c*}	0.34±0.04^{b*}	0.71±0.09^{a*}
CO_2 from carbons 2 to 6 (C)	0.81±0.10^{c}	1.34±0.13^{b}	2.97±0.17^{a}	0.03±0.00^{c*}	0.11±0.02^{b*}	0.89±0.10^{a*}
Net release of KIC (D)	0.51±0.04^{c}	0.72±0.02^{b}	1.41±0.14^{a}	0.79±0.06^{c*}	1.28±0.19^{b*}	2.44±0.13^{a*}
Net transamination (E=B+D)	1.01±0.05^{c}	1.51±0.05^{c}	3.04±0.15^{a}	0.96±0.18^{c}	1.62±0.22^{b}	3.15±0.16^{a}
Transaminated leucine released as KIC (D/E), %	50.4±1.28	48.0±1.38	46.1±2.29	80.0±2.77*	78.7±1.75*	77.5±2.31*
Decarboxylated leucine oxidized as CO_2 [(A-B)/(5×B)], %	32.4±3.34	33.9±2.49	36.6±2.19	3.56±0.38^{b*}	6.80±2.15^{b*}	27.8±5.45^{a*}

HC11 cells (mouse mammary epithelial cells) and Mac-T cells (bovine mammary epithelial cells) were obtained from American Type Culture Collection (Manassa, VA). Approximately 1×10^{6} viable cells (HC11 cells or Mac-T cells) were incubated in 1 mL of Krebs buffer containing 20 mmol/L HEPES, 5 mmol/L D-glucose, 0.3 mmol/L NH_4Cl, 5 μL of 20 U/mL insulin, and 0, 0.5 or 2 L-leucine plus L-[1-^{14}C]- or L-[U-^{14}C]-labeled leucine, and other amino acids at physiological concentrations found in plasma (36). Values, expressed as nmol/10^{6} cells per 2h unless indicated otherwise, are means ± SEM, n=6. Within each cell type, data were analyzed by one-way ANOVA and the Student-Newman-Keuls multiple comparison. Comparison between mouse and bovine mammary epithelial cells was made by the t-test. * $P < 0.05$ versus the corresponding value for mouse mammary epithelial cells. Within each cell type, means within a row sharing different superscript letters differ ($P < 0.05$).

The milk synthetic capacity of the lactating mammary gland is determined by many factors, including the number and activity of functional mammary epithelial cells, stimulation of mammary cell differentiation, degree of premature involution of the mammary gland, precursor availability, and concentrations of lactogenic hormones (5-8). Over the past decades, studies have revealed that lactogenic hormones (prolactin, growth hormone, insulin, and glucocorticoids) are associated with proliferation, functional differentiation and involution of mammary epithelial cells, as well as transcriptional and translational regulation of gene expression in mammary epithelial cells (5, 6, 9). Recent studies also led to the recognition that amino acids, particularly branched-chain amino acids (BCAA), are capable of regulating cell cycle progression, cell differentiation (9-11), cell apoptosis (12), as well as milk protein synthesis (13, 14). Under the present feeding regimen, lactating dams (e.g., sows and cows) are not able to produce sufficient milk to sustain the maximal growth of progeny (8, 15). This provides an impetus to better understand the mechanisms responsible for regulation of lactogenesis in mammals. The major objective of this article is to highlight recent developments in mammary metabolism and nutrition of BCAA as well as their regulatory roles related to milk synthesis and lactation.

3. BCAA CATABOLISM IN MAMMARY EPITHELIAL CELLS

3.1. BCAA degradation

BCAA catabolism by the mammary gland (16) and mammary epithelial cells (Table 1) is initiated by BCAA transaminase (BCAT) in the presence of α-ketoglutarate to form branched-chain α-ketoacids (BCKA) and glutamate. Using leucine as a prototype BCAA, we found both mouse and bovine mammary epithelial cells rapidly take up and degrade leucine (Table 1). Interestingly, rates of leucine transport and transamination between mouse and bovine mammary epithelial cells are identical (Figure 3 and Table 1). In both cell lines (HC11 and Mac-T cells), increasing extracellular concentration of leucine from 0.25 to 2 mmol/L dose-dependently increased its uptake (Figure 3) as well as rates of leucine transamination, production of α-ketoisocaproate (KIC), leucine oxidative decarboxylation, and oxidation of decarboxylated leucine carbons 2 to 6 (Table 1). Intriguingly, the rates of leucine oxidative decarboxylation and oxidation of carbons 2-6 in mouse mammary epithelial cells is greater than those in bovine mammary epithelial cells, resulting in a lower rate of KIC production by mouse mammary epithelial cells.

In bovine mammary epithelial cells, nearly 80% of the transaminated leucine is released as KIC, and only 5% of the decarboxylated leucine was oxidized to CO_2 in the presence of 0.25 or 0.5 mmol/L leucine (Table 1). However, 28% of the decarboxylated leucine is oxidized to CO_2 by these cells in the presence of 2 mmol/L leucine. In contrast, in mouse mammary epithelial cells, approximately 50% of transaminated leucine is released from mouse mammary epithelial cells as KIC and approximately 35% of the decarboxylated leucine is oxidized to CO_2, regardless of extracellular leucine concentration (Table 1). These differences in leucine oxidation between mouse and bovine mammary cells, despite similar rates of leucine transamination, may be explained by differences in the activity of branched-chain α-ketoacid dehydrogenase (BCKAD). Thus, concentrations of BCKA in milk may be relatively high, and milk-born BCKA may serve as energy substrates for the small intestine of suckling neonates. In support of this view, there is evidence that substantial amounts of BCKA are utilized in the first pass by enterocytes of the small intestine (1) and the splanchnic bed of animals (17, 18).

BCAA transaminase exists in two isoforms (19): mitochondrial BCAA transaminase (BCATm) and cytosolic BCAA transaminase (BCATc). Both BCATm and BCATc are present in mammary epithelial cells (Figure 4). This enzyme is activated by octanoate, a medium-chain amino acid. The BCAA-derived BCKA undergo oxidative decarboxylation by the mitochondrial BCKA dehydrogenase complex (BCKAD). This enzyme complex consists of BCKA decarboxylase (E_1 which requires thiamine pyrophosphate as a cofactor), dihydrolipoamide acyltransferase (E_2 which requires lipoate and coenzyme A as cofactors), and dihydrolipoamide dehydrogenase (E_3

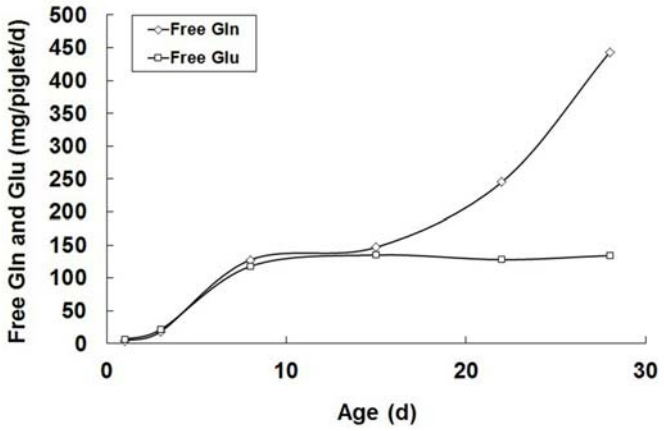

Figure 1. Intake of free glutamine and glutamate from sow's milk by normal-birth-weight piglets during lactation. Values were calculated based on concentrations of free glutamine or glutamate in sow's milk (22) multiplied by the volume of milk consumption by piglets (3). Total Gln or Glu represents free plus protein-bound glutamine or glutamate in sow's milk.

Figure 2. Intake of total glutamine and glutamate from sow's milk by normal-birth-weight piglets during lactation. Values were calculated based on concentrations of total glutamine or glutamate (free plus protein-bound) in sow's milk (22) multiplied by the volume of milk consumption by piglets (3).

which requires FAD and NAD as cofactors) (20). The BCKAD E1 is composed of two subunits: E1a and E1b. BCKAD is regulated by phosphorylation and dephosphorylation in cells. Of particular interest, protein levels for BCAT and BCKAD E1α, as well as de-phosphorylation of BCKAD E1α are enhanced by leucine (Figure 5). For example, leucine at 2 mmol/L increased the protein levels for mitochondrial BCAT (Figure 4) and total BCKAD E1α (Figure 5) by 39% and 42%, respectively, while decreasing the protein levels of phosporyalated BCKAD E1α by 33% (Figure 5). As a result, the ratio of phosporyalated BCKAD E1α to total BCKAD E1α (P-E1α/total E1α value) is reduced by 51%. Lactation increases mammary tissue BCAT activity by a factor of ten and promotes the BCKAD complex in the fully activated state (19). For comparison, only 20% of BCKAD exists in an active state in the mammary tissue of non-lactating cows (20). Thus, the mammary gland may become a significant site for BCAA catabolism at the whole body level during lactation (21).

BCAT catalyzes the transamination of BCAA with α-ketoglutarate to form glutamate and BCKA in mammary epithelial cells. The BCAA-derived glutamate is either amidated to generate glutamine or transaminated with pyruvate (or oxaloacetate) to produce alanine (or aspartate) (16). Rates of synthesis of alanine, aspartate, asparagine, glutamate, and glutamine from BCAA increase with increasing leucine concentration from 0 to 5 mmol/L (Table 2), with the value being the highest for glutamine, followed by glutamate, aspartate, alanine, and asparagine in the descending order (Table 2). Glutamine synthetase (GS) and phosphate-dependent glutaminase (glutaminase hereafter) are the two key enzymes involved in glutamine synthesis and degradation, respectively (22). Interestingly, glutaminase activity is absent from lactating mammary

Table 2. Concentration-dependent increase in net synthesis of amino acids from leucine in bovine mammary epithelial cells

Amino acid	Leucine concentration in incubation medium (mmol/L)			
Amino acid	0	0.25	1	5
Alanine	0.22±0.02^{d}	0.32±0.01^{c}	0.40±0.01^{b}	0.56±0.04^{a}
Aspartate	0.23±0.02^{d}	0.37±0.02^{c}	0.54±0.06^{b}	0.94±0.10^{a}
Asparagine	0.13±0.02^{d}	0.22±0.01^{c}	0.34±0.03^{b}	0.54±0.02^{a}
Glutamate	0.42±0.03^{d}	0.56±0.04^{c}	0.74±0.06^{b}	1.38±0.10^{a}
Glutamine	1.27±0.10^{d}	1.54±0.05^{c}	1.78±0.05^{b}	2.50±0.15^{a}

Mac-T cells (bovine mammary epithelial cells) were obtained from American Type Culture Collection (Manassa, VA). Approximately 1×10^{6} of viable bovine mammary epithelial cells (Mac-T cells) were incubated in 1 mL Krebs buffer containing 20 mmol/L HEPES, 5 mmol/L D-glucose, 0.3 mmol/L NH_4Cl, 5 μL of 20 U/mL insulin, and 0, 0.25, 0.5 or 2 mmol/L L-leucine, and other amino acids (except for alanine, aspartate, asparagine, glutamate, and glutamine) at physiological concentrations found in plasma (36). Data were analyzed by one-way ANOVA and the Student-Newman-Keuls multiple comparison. Values, expressed as nmol/10^{6} cells per 2h, are means ± SEM, n=6. a-d: Means within a row sharing different superscript letters differ ($P < 0.05$).

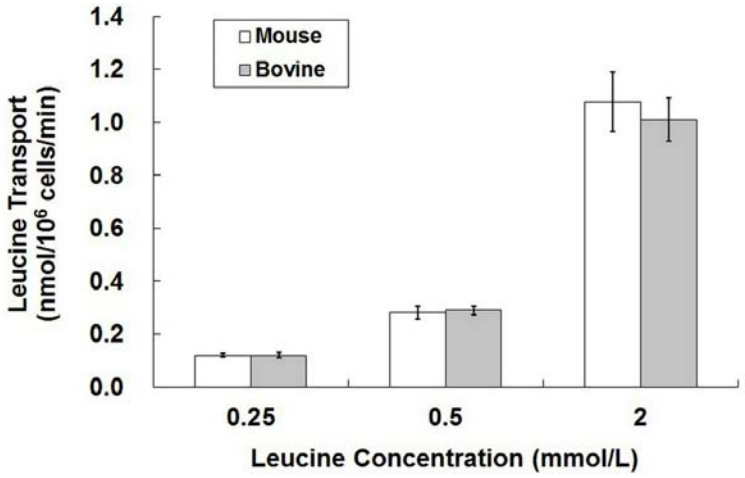

Figure 3. Concentration-dependent increases in leucine transport by mouse and bovine mammary epithelial cells. HC11 cells (mouse mammary epithelial cells) and Mac-T cells (bovine mammary epithelial cells) were obtained from American Type Culture Collection (Manassa, VA). Approximately 1×10^{6} viable cells (HC11 cells or Mac-T cells) were added to 0.2 mL of oxygenated Krebs-Henseleit bicarbonate buffer consisting 0.25 or 0.5 or 2 mmol/L of leucine, 0.05 μCi of L-[U-^{14}C]leucine, 0.05 μCi [^{3}H]inulin (an extracellular marker that does not enter cells) (16), and physiological concentrations of other amino acids found in plasma (36). After 5-min incubation at 37^{o}C, the solution was immediately transferred to a micro-centrifuge tube containing an oil mixture which was overlaid on 0.2 ml of 1.5 M $HClO_4$ solution. The tube was centrifuged, and the upper layer was thoroughly rinsed with saline to remove [^{3}H]inulin (16). The bottom solution was analyzed for ^{14}C and ^{3}H radioactivities using a dual-channel counting program (36). Values are means ±SEM, n=6.

tissue (16), and, therefore, its epithelial cells maximizes glutamine production. The *de novo* synthesis of glutamate and glutamine helps explains the high abundance of these two amino acids in both the free and peptide-bound forms in milk (23, 24). Thus, BCAA are expected to play an important role in milk synthesis by lactating mammary epithelial cells. Consistent with this view, the mammary glands of lactating sows take up large amounts of BCAA (76 g/d) but secret only 46 g/d BCAA in milk (almost exclusively in the peptide-bound form), while the uptake of glutamine (16 g/d) by the lactating glands is considerably lesser than its output (36 g/d) in milk (Figure 2). Approximately 30 g/d BCAA must be degraded to form 20 g/d glutamine in the mammary glands of lactating sows.

3.2. Role of milk-born glutamine, glutamate and aspartate for suckling neonates

Since Windmueller and Spaeth reported in 1974 that glutamine is extensively utilized by the rat small intestine (25), much attention has been paid to the nutritional and physiological roles of glutamine in neonates (26-29). Additionally, *in vivo* studies have shown that luminal glutamate and aspartate are actively oxidized to CO_2 via the Krebs cycle in the intestinal mucosa (26, 27). Based on these findings, dietary glutamine, glutamate and aspartate are now regarded to be essential for the maintenance and function of the small intestine (30). Dietary glutamate is a preferential substrate for glutathione synthesis in the gut mucosa (31), and glutamine serves as the nitrogen moiety for the syntheses of purines, pyrimidines, and amino sugars (e.g., N-acetylglycosamine and N-acetylglactosamine) (22). Purines and pyrimidines are obligatory molecules for the synthesis of DNA, whereas amino sugars are critical for the production of mucosal mucin and, therefore, the maintenance of intestinal-mucosal integrity (32, 33). Given the high abundance of free glutamate and glutamine in the milk of both livestock species (7) and women (34), it is surprising that, except for

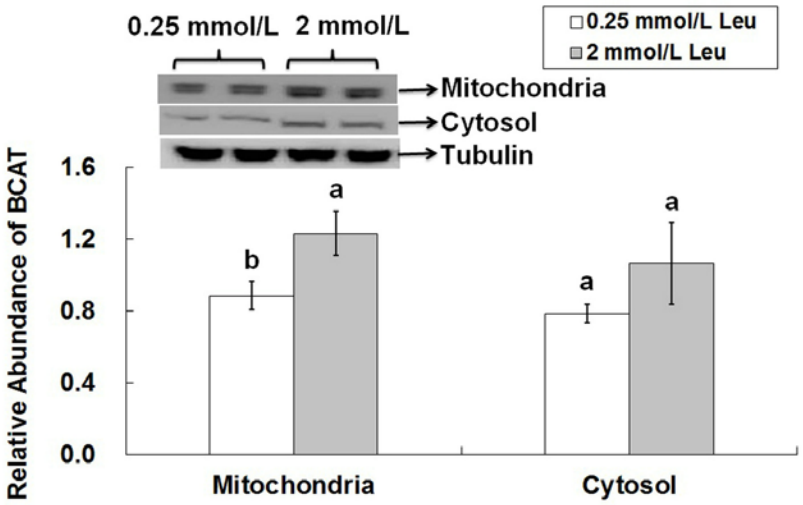

Figure 4. Relative protein levels for cytosolic and mitochondrial BCAT. Bovine mammary epithelial cells (Mac-T cells) were obtained from American Type Culture Collection (Manassa, VA). Cells were incubated for 2-h in the medium containing 20 mmol/L HEPES, 5 mmol/L D-glucose, 5 μL of 20 U/mL insulin, 0.25 or 2 mmol/L leucine, and other amino acids at physiological concentrations found in plasma (36). After 2-h culture, cells were collected for analysis of cytosolic and mitochondrial BCAT using the western blotting technique (16). Values are means ± SEM, n=6. a-b: Within each protein, means with different letters differ ($P < 0.05$).

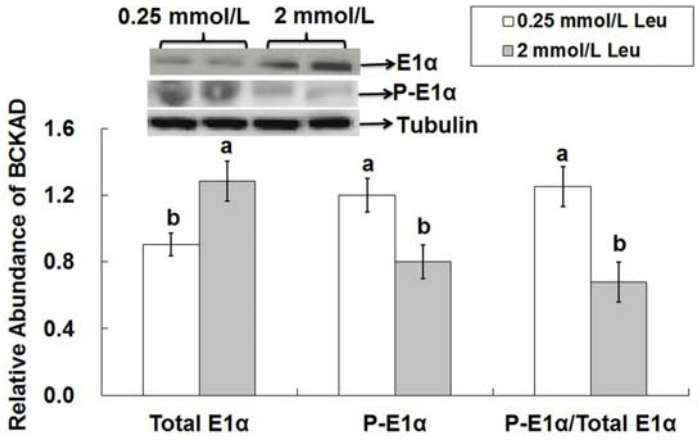

Figure 5. Relative protein levels for total and phosphorylated E1α of the mitochondrial BCKAD complex. Bovine mammary epithelial cells (Mac-T cells) were obtained from American Type Culture Collection (Manassa, VA). Cells were incubated for 2-h in the medium containing 20 mmol/L HEPES, 5 mmol/L D-glucose, 5 μL of 20 U/mL insulin, 0.25 or 2 mmol/L leucine, and other amino acids at physiological concentrations found in plasma (36). After 2-h culture, cells were collected for analysis of total and phosphorylated E1α of the mitochondrial BCKAD complex using the western blotting technique (16). Values are means ± SEM, n=6. a-b: Within each protein, means with different letters differ ($P < 0.05$).

protein-hydrolysate formulas, current infant formulas contain virtually no free glutamate and glutamine (34).

The catabolism of glutamine and glutamate in enterocytes is crucial for the synthesis of ornithine, citrulline, and arginine in most of mammalian neonates, including human infants and young pigs (35, 36). The endogenous synthesis of arginine compensates for a marked deficiency of arginine in the milk of most species, including humans, cows, sheep, and pigs (37-39). Among its versatile functions, arginine stimulates muscle protein synthesis, maintains the hepatic urea cycle in the active state for ammonia detoxification, and regulates the uptake and utilization of nutrients by tissues (40).

Physiological levels of glutamine upregulate intestinal expression of genes that are related to: a) anti-oxidative responses (41); b) proliferation and survival of mucosal cells (42); c) tight-junction stabilization (28); and d) anti-inflammation and anti-apoptosis (28). Glutamine

may also modulate the transport of nutrients (including amino acids, small peptides, fatty acids, glucose, vitamins and minerals) by enterocytes at transcriptional and translational levels (1). Moreover, through activation of the mammalian target of rapamycin (mTOR) signaling pathway, glutamine stimulates protein synthesis and inhibits protein degradation in enterocytes, thereby promoting cell growth (29) and migration (28).

Studies with the swine model demonstrate that milk-borne glutamine is inadequate for maximal growth of neonates (3). Sow's milk contains 4.3-10.8 g/L glutamine, depending on lactation stage (Figure 1). Based on milk consumption, a piglet with normal birth-weight obtains 2.9-3.5 g/day glutamine from milk during the suckling period (Figure 1). Owing to extensive catabolism of glutamine in the small intestine (43), less than 36% of milk-derived glutamine (i.e., 1.14 g) is accreted in the body of the piglet (2). As a consequence, the rate of endogenous synthesis of glutamine in the suckling piglet is relatively high (≥ 0.88 g/kg body weight per day) to sustain maximum growth (2). Another milk-derived amino acid closely related to glutamine is glutamate, which can spare glutamine oxidation by the intestine (44). A suckling piglet can obtain 3.3-3.8 g/L glutamate from milk (Figure 1), but more than 95% of this amount is removed by the portal-drained viscera (26). Thus, glutamate in milk is virtually not available for protein synthesis in extra-intestinal tissues. Although a young pig can obtain daily 6-7 g of glutamine plus glutamate from milk (Figure 1), oral administration of glutamine (1 g/kg body weight per day) can enhance daily weight gains and anti-inflammatory responses in the animal (3, 22). Thus, it is crucial to provide lactating mothers with an abundant supply of BCAA for the synthesis of glutamine and glutamate in mammary tissue. These amino acids promote the growth, development, health, and well-being of neonates.

4. ROLE OF BCAA IN CELL SIGNALING TO SUPPORT LACTOGENESIS

4.1. The biochemical machinery of milk production

The mammary gland is composed of epithelial cells, the surrounding stroma, and adipose tissue. The luminal epithelial cell is regarded as the milk-synthetic and secretory cell in the mammary gland (45). The newly synthesized milk is stored in the lactiferous duct tree and, thereafter, secreted to the neonates upon the initiation of oxytocin surge stimulated by suckling activity (5). However, before the luminal epithelial cells of the mammary gland are capable of lactogenesis, they must undergo functional differentiation and acquire the biochemical machinery for syntheses of lipids, proteins, and lactose (46). Thus, the number of functional mammary epithelial cells is crucial for the gland's milk synthetic capacity (47).

Mammary epithelial cells undergo proliferation during both the prepartum and postpartum periods. For example, DNA concentration in the sow's mammary tissue begins to increase by Day 30 of pregnancy, and the increase is dramatically accelerated between Days 75 and 90 and continues until parturition (45). This finding indicates that the interval between Days 75 and 90 is a critical period in the rapid proliferation of mammary epithelial cells. Between Day 90 and parturition, the mammary gland also undergoes remarkable changes in mammary structure, size and function (46, 47). One characteristic change is that alveoli are functionally differentiated from luminal epithelial cells and expanded greatly at the end of each branch, when the main lactiferous duct tree is almost completely formed at birth. Thus, there is a "window of opportunity" (i.e., between Days 75 and 114 of gestation in swine) to cost-effectively use nutritional and hormonal means during gestation to enhance mammary growth and milk production during lactation. Mammary hypertrophy also takes place during lactation (48). Measurement of mammary tissue composition reveals that tissue weight, mammary cross-sectional area, fat-free tissue content, tissue protein and amino acid content, as well as DNA content increase as lactation progresses (8, 46). However, rates of tissue protein and DNA syntheses increase from Day 0 to Day 14 and decrease thereafter. These results indicate that mammary epithelial cells undergo a series of dynamic changes of proliferation, differentiation, and involution as lactation advances (46). In the following sections, we discuss the underlying mechanisms by which BCAA regulate these physiological events.

4.2. BCAA stimulate growth and proliferation of mammary epithelial cells

A strong body of evidence indicates that BCAA have the ability to modulate cell metabolism through the synthesis of macromolecules (protein and DNA), cell growth, and cell proliferation in a variety of cell types, including pancreatic cells (9), hepatocytes (10), fibroblasts (11), embryonic kidney cells (49), as well as mammary epithelial cells (50). This suggests that besides serving as the building blocks of proteins, BCAA are also used as nutrient signals that regulate the cellular proliferation machinery, including the cell cycle, DNA synthesis, and protein synthesis.

The cell cycle consists of the interphase and mitosis. These series of ordered events, which are rapid and highly complex, lead to cell growth and division into two daughter cells. During the interphase of the cell cycle, the synthesis of cellular macromolecules (particularly proteins) is the primary event to restart the cell cycle (51, 52). Based on the stage of cell division, the interphase is divided into three sequential phases [Gap 1 (G_1), DNA synthesis (S), and Gap 2 (G_2)] and is the longer period of the cell cycle compared with the mitotic (M) phase. During the three phases of interphase, the cell grows by producing protein and cytoplasmic organelles and continues to grow by duplicating its chromosomes during S and G_2 phases, followed by cell mitosis (53). At the M stage of the cell cycle, nuclear and cytoplasmic division occurs. Thus, an increase in cell size through synthesis of macromolecules (particularly protein) is the prerequisite process before cell division progresses. At the end of the cell cycle, two daughter cells are generated by mitosis. The process of cell differentiation, which is critically affected by intracellular

levels of polyamines, requires activation of the necessary biochemical machinery (54, 55).

Constitutive proteins in mammary tissue are synthesized extensively during lactation, accounting for 40-70% of total mammary protein synthesis (tissue constitutive protein plus milk protein) (56). In lactating sows, the synthetic rate of mammary constitutive proteins increases to a peak level on Day 14 of lactation (7.8 g/kg wet tissue/day), and then decreases as lactation advances to 2.7 g/kg wet tissue/day on Day 21 and to 0.5 g/kg wet tissue/day on Day 28 (57). Consistent with protein synthesis, the rate of net DNA synthesis shows an identical trend (46). Notably, peak milk yield occurs around Day 14 of lactation, as estimated by weigh-suckle-weigh and deuterium oxide dilution method (57-59). This implicates that the synthesis of constitutive proteins in mammary tissue is proportional to the number of its functional mammary epithelial cells, which is a consequence of cell proliferation. As noted above, this physiological process is up-regulated by BCAA (50) although the underlying mechanisms remain unknown.

4.3. BCAA and the mTOR signaling pathway

The mechanisms whereby BCAA stimulate protein synthesis in mammary epithelial cells may involve activation of mRNA translation initiation by mTOR. Specifically, leucine stimulates the phosphorylation of mTOR, thereby promoting translation initiation and polypeptide formation (10, 60, 61). This, in turn, results in growth and proliferation of mammary epithelial cells (50, 62, 64). The mTOR signaling pathway consists of mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (65). mTORC1 is composed of mTOR, regulated associated protein (Raptor), mammalian LST8/G-protein β-subunit like protein (mLST8/GβL) and the partners PRAS40 and DEPTOR; this complex is sensitive to rapamycin and integrates signals induced by multiple extracellular stimuli such as hormones and nutrients to regulate mRNA translation initiation, cell growth and proliferation. mTORC2 is composed of mTOR, GβL, rapamycin-insensitive companion of mTOR (Rictor), and mammalian stress-activated protein kinase interacting protein 1 (mSIN1); this complex is non-rapamycin sensitive but is an important regulator of cytoskeleton development (65). At present, it is not known how leucine regulates the mTOR signaling pathway in animal cells.

Activation of mTORC1 directly phosphorylates ribosomal protein S6 kinase 1 (S6K1) and eukaryotic factor eIF4E binding protein-1 (4E-BP1), thereby regulating the translational machinery that controls cell growth and G1- to S-phase transition in the cell cycle progression (50). Phoshporylation of 4E-BP1 by mTOR leads to dissociation of the 4E-BP1/eIF4E complex, increasing the availability of eIF4E, and subsequently initiating the translation of a small number of cap-dependent mRNAs encoding Id protein, Cyclin D1/3, *ODC1*, all of which are associated with cell cycle progression and mitogenic stimulation (49, 50, 63). The helix-loop-helix protein Id1, which has a profound impact on proliferation of mammary epithelial cells, is a typical example. Id1 protein is required for mTOR to induce proliferation of mammary epithelial cells, and their mRNA levels are high in the cells under proliferative conditions (growing) but are low in confluent cultures (functionally competent cells). Overexpression of Id1 contributes to faster cell growth and completely resists to the inhibitory effect of rapamycin on cell growth (63). Additionally, mTOR-mediated phosphorylation of p70 S6K accelerates the translation of mRNAs with a 5'-TOP (terminal oligpyrimidine tract) encoding molecules associated with translation machinery, such as ribosomal proteins and elongation factors, thus promoting cell growth and division (9).

S6K1 and 4E-BP1 are two markedly different proteins but can act in concert to regulate cell growth and proliferation. Rapamycin inhibits effectively the phosphoryaltion of 4E-BP1 and S6K1 in mammary epithelial cells, contributing to the inhibition of cell proliferation and the arrest of the cell cycle (63). Notably, overexpression of S6K1 or eIF4E can partially block the rapamycin-induced reduction of cell size across all phases of the cell cycle. When coexpressed, eIF4E and S6K1 cooperate downstream of the mTOR protein to increase cell size further (51). Reduction of the mRNA level for S6K1 or overexpression of the 4E-BP1 gene suppresses G_1-phase progression in cells. Conversely, overexpression of the S6K1 or eIF4E gene partially rescues rapamycin-inhibited G1-phase progression (52). Emerging evidence shows that isoleucine or leucine increases S6K1 phosphorylation in Mac-T cells and bovine mammary tissue slices (64).

4.4. BCAA enhance the functional differentiation of mammary epithelial cells

As noted previously, mature secretory cells should have the biochemical machinery for milk synthesis and secretion (7, 8). Such a mechanism may develop during cell differentiation and has important implications for epigenetic regulation of lactogenesis (66). At present, little is known about the epigenome in the lactating gland. Results of recent studies demonstrate that the mTOR signaling cascade, which is well conserved in animals during evolution, plays an important role in regulating cell differentiation in response to nutrients, such as BCAA (9, 63). Branched-chain amino acids, especially leucine and its metabolites, are secretagogues for insulin secretion by β-cells (67) partly through phosphorylation of the mTOR downstream molecules p70 S6K and 4E-BP1, indicating the stimulatory role of those amino acids in cell differentiation. Both *in vitro* and *in vivo* evidence show that unlike the differentiating mammary epithelial cells, the undifferentiated and predifferentiated cells have low levels of mTOR phosphorylation; moreover, rapamycin abrogates the formation of mammary alveoli structure, β-casein secretion and the volume of the gland (63). Subsequent in-depth studies have concluded that mTOR-mediated expression of the Id2 protein (inhibitor of DNA-binding protein) is prerequisite for cell differentiation (63). These results indicate that mTOR is required for functional differentiation of mammary secretory cell and development of the mammary gland. Such a mechanism explains the stimulating effect of BCAA on protein synthesis and secretion by mammary epithelial cells.

4.5. BCAA increase the longevity of mammary epithelial cells

Mammary involution is a dramatic remodeling process associated with cell aging (64). An increase in mitochondrial biosynthesis takes place in response to the onset of lactation to meet the need of ATP for biosynthetic pathways. For example, in lactating mice, the density of mitochondrial inner membranes in mammary epithelial cells increases 7-fold and the activity of mammary cytochrome C oxidase increases 4- to 5-fold (68, 69). These results indicate increases in the number and activity of mitochondria in lactating mammary tissue. However, mitochondrial respiration is a double-edged sword for the mammary gland in that ATP production is required to support high rates of milk production yet endogenous free-radicals generated from this process concomitantly accumulate to negatively affect cell function. Specifically, accumulation of free-radicals results in progressive oxidative damage on cellular lipids, proteins and DNA, leading to cell aging and impaired synthetic capacity of the mammary epithelial cells (64). Thus, controlling mitochondrial respiration and optimizing the defense system against reactive oxygen species (ROS) are crucial for prolonging mammary cell survival and milk production.

Through activating the mTOR signaling pathway to promote the synthesis of anti-oxidative proteins, BCAA may beneficially increase the life span of mammary epithelial cells. In support of this proposition, emerging evidence shows that the anti-aging role of BCAA is associated with the enhanced growth of new mitochondria (70). Interestingly, leucine, isoleucine and valine can extend the chronological longevity of *Saccharomyces cerevisiae*, implicating that these nutrients are capable of promoting eukaryotic cell survival (70). Another study has also demonstrated that treatment of cardiac and skeletal myocytes with BCAA increases mitochondrial DNA concentration and expression of major genes related to activation of mitochondrial biogenesis and reduction of ROS production. These genes include peroxisome proliferator-activated receptor γ coactivator-1α and sirtuin 1 (12). The outcome is to prevent oxidative damage (and possibly apoptosis) in metabolically active cells and prolong their survival in spite of enhanced mitochondrial respiration by BCAA.

5. BCAA REGULATE MAMMARY METABOLIC CAPACITY FOR MILK SYNTHESIS

5.1. Intracellular protein synthesis

Production of milk proteins depends on the balance between rates of intracellular protein synthesis and proteolysis (7). The mammary gland has a high capacity for protein synthesis during lactation. Large amounts of milk protein and a variety of constitutive proteins (e.g., structural proteins and enzymes) are endogenously synthesized by the lactating gland (57). It can be estimated that the mammary glands of sows and cows produce daily 420 and 950 g milk protein, respectively, at peak lactation. However, the efficiency of converting dietary protein into milk protein is less than 30% (57, 71, 72). Major limiting factors include: a) entry of dietary amino acids into the portal circulation; b) mammary uptake of amino acids and peptides; c) concentrations of lactogenic hormones and nutrients that can regulate mammary metabolism of amino acids and peptides; and d) activation of signaling pathways that regulate syntheses of proteins, lipids and lactose. Because the mammary metabolic capacity for milk protein synthesis is greater than that expressed under the current feeding system and management conditions (5, 71), there is great potential for enhancing milk production in animals and humans.

Although regulatory roles for hormones in controlling the synthesis of milk proteins are well documented (6, 72), the functions of essential amino acids in this regard, besides their nutritional roles as building blocks of polypeptides, are poorly understood. Previous studies showed that BCAA could promote milk protein production (73), but it is obscure whether those nutrients merely serve as building blocks of protein or whether they act as signaling molecules in the control of metabolic pathways related to lactogenesis. There is also evidence that dairy cows receiving intragastric infusion of casein (a BCAA-rich protein) as a source of amino acids exhibited 10% greater production of milk protein (71). Interestingly, this effect of casein was further augmented (namely a 28% increase in the production of milk protein in comparison with the control group) when intragastric administration of casein was combined with intravenous infusion of insulin, indicating a synergistic action of amino acids and insulin on milk protein synthesis (71). Intravenous infusion of either total essential amino acids or leucine can activate the mTOR signaling pathway but infusion of methionine plus lysine or histidine has no effect (13). In contrast, either the lack of all amino acids or selective deprivation of leucine inhibits phosphorylation of the mTOR substrates, 4E-BP1 and p70 S6K1, thus decreasing the synthesis of total proteins and specific proteins (e.g., β-lactoglobulins) in bovine and mouse mammary cell lines (14). Addition of either all amino acids or leucine to the amino acid-lacking basal medium restored protein synthesis in the cells but such effects were blocked by rapamycin or LY294002 (a phosphatidylinositol 3-kinase inhibitor) in a dose-dependent manner (14). Taken together, these results suggest that leucine plays a signaling role in the translational machinery controlling milk protein synthesis, in addition to serving as a substrate for polypeptide formation. This is consistent with the findings of both *in vitro* and *in vivo* studies that BCAA (particularly leucine) have anabolic effects on protein synthesis in skeletal muscle and other tissues (71-76).

5.2. Intracellular protein degradation

Control of intracellular protein degradation in the lactating gland is crucial for maximizing the production of milk proteins. However, at present, little is known about the rates or regulation of protein degradation in mammary epithelial cells. An inhibitory effect of BCAA on proteolysis had been reported for skeletal muscle and the whole body under catabolic conditions (66, 74, 77, 78). Interestingly, α-ketoisocaproate (a product of leucine transamination) and 4-methyl-2-oxopentanoate (a metabolite of α-ketoisocaproate) can also suppress

proteolysis in incubated muscle (74, 76). However, the underlying mechanisms remain unknown. High concentrations of these leucine metabolites (> 0.25 mmol/L) required to inhibit muscle protein breakdown (74, 76), raising a question of whether they mediate the effect of physiological levels of leucine in myocytes.

Three proteolytic mechanisms are responsible for the catabolism of proteins, including the calcium-activated pathway, the ATP- and ubiquitin-dependent proteasome pathway, and the lysosomal pathway (79). The calcium-activated pathway plays a major role in degrading myofibrillar protein. In contrast, most of the intracellular proteins are hydrolyzed by the proteasome pathway, which is activated by a wide array of catabolic conditions, including trauma, sepsis, cancer, and starvation. The lysosomal system is mainly involved in the degradation of engulfed particles that enter lysosomes via a process known as autophagy. Busquets *et al.* (80) reported that the short-term effect of BCAA on proteolysis was associated with decreased activities of lysosomal proteases and the long-term effect of these amino acids might be mediated by the proteasome pathway. Increasing evidence shows that activation of the mTOR signaling pathway results in inhibition of intracellular proteolysis in enterocytes (81), but such an effect has not been reported for mammary epithelial cells. We suggest that BCAA, particularly leucine, can inhibit protein degradation in mammary epithelial cells via activation of the mTOR signaling pathway. Experimental evidence is required to support this hypothesis.

6. DIETARY SUPPLEMENTATION WITH BCAA TO LACTATING MAMMALS IMPROVES MILK PRODUCTION AND NEONATAL GROWTH

6.1. Low availability of dietary glutamine for mammary tissue in lactating dams

Although regular diets for animals contain high levels of glutamine and glutamate (82-84), dietary supplementation with 1% glutamine to lactating sows can enhance milk production (22). The underlying mechanisms may be complex and involve the actions of multiple organs, including the small intestine, skeletal muscle and lactating gland. At present, little is known about efficacy of dietary supplementation with glutamine versus BCAA to sustain high levels of glutamate and glutamine in milk. Studies with rats have shown that glutamine catabolism in the small intestine is increased at the onset of lactation, compared with virgin and non-lactating animals (85). Similarly, Okine *et al.* (86) reported that enterocytes from cows at the early stage of lactation oxidized more glutamine than cells from cows at either mid- or late-stage of lactation. Accordingly, the small intestine of lactating dams degrades approximately 70% of dietary glutamine (22). Thus, only a small percentage of dietary glutamine reaches the lactating gland (87). This explains the need for alternative means to increase glutamine and glutamate availability in milk. Supplementing BCAA to the diet of lactating dams may offer an attractive strategy (16).

6.2. Dietary BCAA supplementation enhances milk production in animals

The mammary gland of lactating sows produces 125% more glutamine in milk than its uptake from arterial plasma (16), whereas the uptake of arterial glutamate by the mammary gland is fairly matched by the amount of glutamate in milk (88). It is unlikely that the extra glutamine is derived from proline because porcine mammary tissue lacks the proline oxidase enzyme that would be needed for this activity (89). The synthesis of aspartate and asparagine is also of nutritional importance because the uptake of these two amino acids by the mammary gland of lactating sows accounts for only 50% of their output in milk (15). Aspartate plus asparagine are the third most abundant nonessential amino acids in porcine milk protein (23, 24). Thus, BCAA support protein synthesis in lactating mammary tissue partly by stimulating the synthesis of "nutritionally nonessential" amino acids. The modulation of BCAA catabolism for the synthesis of glutamate, glutamine, aspartate and asparagine may play a hitherto unrecognized important role in regulating milk synthesis.

Several lines of evidence support the notion that supplementing BCAA to the diets of lactating sows enhances milk yield and neonatal growth. First, Richert *et al.* (90) found that litter weaning weight and litter weight gain in high-producing lactating sows increased as dietary valine increased from 0.85 to 1.15%. Subsequently, these authors reported that increasing dietary valine (0.64-1.44%) to the lactating sow nursing 10 or more piglets increased litter weaning weights and litter weight gain (91). This effect of dietary valine on lactation was confirmed by Moser *et al.* (92). Furthermore, Paulicks *et al.* (93) observed that dietary supplementation of valine (0.85-1.45%) to the lactating sow increased milk production and milk protein content, in comparison with the control group (0.55% valine). Similarly, increasing dietary intake of valine, isoleucine, or total branched-chain amino acids by lactating sows resulted in an improvement of milk synthesis and litter weight gain (73). Consistent with the findings from swine, duodenal infusion of leucine to lactating cows increased concentrations of casein, whey proteins, and total proteins in milk (94). We expect that these results from animal studies have important implications for enhancing milk production in women, who have impaired lactogenesis under various stressful conditions (e.g., premature birth, maternal complications, and high or low ambient temperatures) and in response to low intake of dietary protein (95).

7. CONCLUSION AND PERSPECTIVES

Mammary synthetic capacity depends on the number and efficiency of functional mammary epithelial cells and determines the growth of suckling neonates. In the lactating mammary gland, BCAA are extensively transaminated with α-ketoglutarate to produce glutamate and BCKA, with glutamate being an essential substrate for synthesis of glutamine, alanine, aspartate and asparagine. Additionally, some of the BCAA carbons are either oxidized to provide ATP in mitochondria or utilized for

lipid synthesis in cytoplasm. Furthermore, BCAA enhance secretion of insulin by pancreatic β-cells (67) and activate the mTOR signaling pathway to initiate polypeptide formation and possibly inhibit protein degradation (60). At present, little is known about hormonal or nutritional regulation of BCAA catabolism in mammary tissue.

Emerging evidence from animal studies indicates that dietary supplementation with valine or a mixture of BCAA stimulates milk synthesis and lactation in sows (90-93). Because dietary supplementation with valine increases milk protein synthesis (90-93) but this amino acid has no effect on the mTOR signaling pathway (60), increases in substrate provision may contribute to enhanced lactation performance in sows. Interestingly, in contrast to enteral provision (94), intravenous administration of BCAA has been reported not to affect milk protein synthesis or milk production by lactating cows. Thus, the route of supplementation with BCAA may be a critical factor influencing physiological responses. It is possible that dietary supplementation with BCAA inhibits catabolism of amino acids by both mucosal cells and luminal microorganisms of the small intestine (97-100), thereby increasing the entry of dietary amino acids to the portal circulation for utilization by extra-intestinal cells and tissues. One of the resulting effects may be to enhance the availability of arginine for synthesis of nitric oxide (101), a major vasodilator (102), thereby augmenting the uptake of nutrients by the lactating mammary gland. Collectively, the findings on BCAA metabolism and nutrition in mammary tissue not only advance our knowledge about the biology of lactation but also have practical implications for improving milk production by female animals and women in both developed and developing countries. Much work involving advanced "-omics" technologies (103-106) and traditional research methods (107-110) is needed to define optimal requirements of BCAA by lactating mammals.

8. ACKNOWLEDGMENTS

Jian Lei was supported by a Postgraduate Scholarship from South China Agricultural University. Work in the authors' laboratories was supported by National Natural Science Foundation of China grants no. 31172217, the Thousand-People Talent program at China Agricultural University, National Research Initiative Competitive Grants no. 2008-35206-18764 and 2008-35203-19120 from the USDA National Institute of Food and Agriculture, Texas AgriLife Research Hatch Project no. H-8200, and American Heart Association. We thank Dr. Susan Hutson and Dr. Christopher Lynch for the kind provision of BCAT and BCKAD E1α antibodies, respectively.

9. REFERENCES

1. Guoyao Wu: Amino acids: metabolism, functions, and nutrition. *Amino Acids* 37, 1-17 (2009)

2. Guoyao Wu: Functional amino acids in growth, reproduction, and health. *Adv Nutr* 1, 31-37 (2010)

3. Tony E Haynes, Peng Li, Xilong Li, Kazuhiro Shimotori, Hiroyuki Sato, Nick E Flynn, Junjun Wang, Darrell A Knabe and Guoyao Wu: L-Glutamine or L-alanyl-L-glutamine prevents oxidant- or endotoxin-induced death of neonatal enterocytes. *Amino Acids* 37, 131-142 (2009)

4. RD Mateo, G Wu, HK Moon, JA Carroll and SW Kim: Effects of dietary arginine supplementation during gestation and lactation on the performance of lactating primiparous sows and nursing piglets. *J Anim Sci* 86, 827-835 (2008)

5. Dean Boyd, Ronald Kensinger, Robert Harrell and Dale Bauman: Nutrient uptake and endocrine regulation of milk synthesis by mammary tissue of lacating sows. *J Anim Sci* 73 (Suppl. 2), 36-56 (1995)

6. Robert Rhoads and Ewa Grudzien-Nogalska: Translational regulation of milk protein synthesis at secretory activation. *J Mammary Gland Biol Neoplasia* 12, 283-292 (2007)

7. Sung Woo Kim and Guoyao Wu: Regulatory role for amino acids in mammary gland growth and milk synthesis. *Amino Acids* 37, 89-95 (2009)

8. SW Kim, WL Hurley, Guoyao Wu and Fei Ji: Ideal amino acid balance for sows during gestation and lactation. *J Anim Sci* 87, E123-132 (2009)

9. Gang Xu, Guim Kwon, Connie Maeshall, Tai-An Lin, John Lawrence and Michael McDaniel: Branched-chain amino acids are essential in the regulation of PHAS-I and p70 S6 kinase by pancreatic ß-cells: A possible role in protein translation and mitogen signaling. *J Biol Chem* 273, 28178-28184 (1998)

10. Mitsutoshi Kimura and Masahiko Ogihara: Effects of branched-chain amino acids on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. *Europ J Pharmacol* 510, 167-180 (2005)

11. Estela Maria Gonçalves and Maria Cristina Gomes-Marcondes: Leucine affects the fibroblastic Vero cells stimulating the cell proliferation and modulating the proteolysis process. *Amino Acids* 38, 145-153 (2010)

12. Giuseppe D'Anona, Maurizio Ragni, Annalisa Cardile, Laura Tedesco, Marta Dossena, Flavia Bruttini, Francesca Caliaro, Giovanni Corsetti, Roberto Bottinelli, Michele Carruba, Alessandra Valerio, and Enzo Nisoli: Branched-chain amino acid supplementation promotes survival and supports cardiac and skeletal muscle mitochondrial biogenesis in middle-aged mice. *Cell Metab* 12 (4), 362-372 (2010)

13. Chanelle Toerien, Donald Trout, and John Cant: Nutritional stimulation of milk protein yield of cows is associated with changes in phosphorylation of mammary eukaryotic initiation factor 2 and ribosomal S6 kinase 1. *J Nutr* 140, 285-292 (2010)

14. Yana Moshel, Robert Rhoads, and Itamar Barash: Role of amino acids in translational mechanisms governing milk protein synthesis in murine and ruminant mammary epithelial cells. *J Cell Biochem* 98, 685-700 (2006)

15. Trottier NL, Shipley CF and Easter RA: Plasma amino acid uptake by the mammary gland of the lactating sow. *J Anim Sci* 75, 1266-1278 (1997)

16. Peng Li, Darrell Knabe, Sung Woo Kim, Christopher Lynch, Susan Hutson, Guoyao Wu: Lactating porcine mammary tissue catabolized branched-chain amino acids for glutamine and asparate synthesis. *J Nutr* 139, 1502-1509 (2009)

17. Mackenzie Walser: Role of branched-chain ketoacids in protein metabolism. *Kidney Int* 38, 595-604 (1990)

18. Guoyao Wu: Intestinal mucosal amino acid catabolism. *J Nutr* 128, 1249-1252 (1998)

19. Soledad Desantiago, Nimbe Torres, Agus Suryawan, Armando Tovar and Susan Hutson: Regulation of branched-chain amino acid metabolism in the lactating rat. *J Nutr* 128, 1165-1171 (1998)

20. John Brosnan and Margret Brosnan: Branched-chain amino acids: enzymes and substrate regulation. *J Nutr* 136, 207S-211S (2006)

21. Juan Viña and Dermot Williamson: Effects of lactation on L-leucine metabolism in the rat. *Biochem J* 194, 941-947 (1981)

22. Guoyao Wu, Fuller W. Bazer, Gregory A. Johnson, Darrell A. Knabe, Robert C. Burghardt, Thomas E. Spencer, Xilong Li, and Junjun Wang: Important roles for L-glutamine in swine nutrition and production. *J Anim Sci* 89: 2017-2030 (2011)

23. Guoyao Wu and Darrell Knabe: Free and protein-bound amino acids in sow's colostrum and milk. *J Nutr* 124, 415-424 (1994)

24. Teresa Davis, Hanh Nguyen, Roselina Garcia-Bravo, Marta Fiorotto, Evelyn Jackson, Douglas Lewis, Rick Lee and Peter Reeds: Amino acid composition in human milk. *J Nutr* 124, 1126-1132 (1994)

25. Herbert Windmueller, Albert Spaeth: Uptake and metabolism of plasma glutamine by the small intestine. *J Biol Chem* 249, 5070-5079 (1974)

26. Barbara Stoll, Douglas Burrin, Joseph Hebry, Hung Yu, Farook Jahoor, and Peter Reeds: Substrate oxidation by the portal drained viscera of fed piglets. *Am J Physiol Endocrinol Metab* 227, 168-175 (1999)

27. Guoyao Wu, Darrell Knabe, Yan W, and Nick Flynn: Glutamine and glucose metabolism in enterocytes of the neonatal pig. *Am J Physiol Regulatory Integrative Comp Physiol* 268, 334-342 (1995)

28. Marc Rhoads and Guoyao Wu: Glutamine, arginine, and leucine singaling in the intestine. *Amino Acids* 37, 111-122 (2009)

29. Pengbin Xi, Zongyong Jiang, Chuntian Zheng, Yingcai Lin and Guoyao Wu: Regulation of protein metabolism by glutamine: implications for nutrition and health. *Front Biosci* 16, 578-597 (2011)

30. Yulong Yin, Kang Yao, Zhaojin Liu, Min Gong, Zheng Ruan, Dun Deng, Bie Tan, Zhiqiang Liu and Guoyao Wu: Supplementing L-leucine to a low-protein diet increases tissue protein synthesis in weanling pigs. *Amino Acids* 39, 1477-1486 (2010)

31. Peter Reeds, Douglas Burrin, Barbara Stoll, Farook Jahoor, Linda Wykes, Henry Joseph, and Margaret Frazer: Enteral glutamate is the preferential source for mucosal glutathione synthesis in fed piglets. *Am J Physiol Endocrinol Metab* 273 E408-E415 (1997)

32. Peter Reeds and Douglas Burrin: Glutamate and the bowel. *J Nutr* 131, 2505S-2508S (2001)

33. Herbert Windmueller, Albert Spaeth: Respiratory fuels and notrogen metabolism *in vivo* in small intestine of fed rats. *J Biol Chem* 255, 107-112 (1980)

34. Carlo Agostoni, Brunella Carratu, Concetta Boniglia, Enrica Riva and Elisabetta Sanzini. Free amino acid content in standard infant formulas: comparison with human milk. *J Am Coll Nutr* 19, 434-438 (2000)

35. Xin Wu, Zheng Ruan, Yuling Gao, Yulong Yin, Xihong Zhou, Lei Wang, Meimei Geng, Yongqing Hou and Guoyao Wu: Dietary supplementation with L-arginine or N-carbamylglutamate enhances intestinal growth and heat shock protein-70 expression in weanling pigs fed a corn- and soybean meal-based diet. *Amino Acids* 39, 831-839 (2010)

36. Bie Tan, Yulong Yin, Xiangfeng Kong, Peng Li, Xilong Li, Haijun Gao, Xinguo Li, Ruiling Huang and Guoyao Wu: L-Arginine stimulates proliferation and prevents endotoxin-induced death of intestinal cells. *Amino Acids* 38, 1227-1235 (2010)

37. Guoyao Wu, Darrell Knabe and Sung Woo Kim: Arginine nutrition in neonatal pigs. *J Nutr* 134, 2783S-2390S (2004)

38. Sung Woo Kim and Guoyao Wu: Dietary arginine supplementation enhances the growth of milk-fed young pigs. *J Nutr* 134, 625-630 (2004)

39. Meimei Geng, Taijun Li, Xiangfeng Kong, Xiaoyan Song, Wuying Chu, Ruilin Huang, Yulong Yin and Guoyao Wu: Reduced expression of intestinal N-acetylglutamate synthase in suckling piglets: a novel molecular mechanism for arginine as a nutritionally essential amino acid for neonates. *Amino Acids* 40, 1513-1522 (2011)

40. Guoyao Wu, Fuller Bazer, Teresa Davis, Sung Woo Kim, Peng Li, Marc Rhoads, Carey Satterfield, Smith

Smith, Thomas Spencer and Yulong Yin: Arginine metabolism and nutrition in growth, health and disease. *Amino Acids* 37, 153-168 (2009)

41. Junjun Wang, Lixiang Chen, Peng Li, Xilong Li, Huaijun Zhou, Fenglai Wang, Defa Li, Yulong Yin and Guoyao Wu: Gene expression is altered in piglet small intestine by weaning and dietary glutamine supplementation. *J Nutr* 138, 1025-1032 (2008)

42. Michael Weiss, Vincent Demarco, Daniel Strauss, Donald Samuelson, Meredith Lane and Josef Nue: Glutamine synthetase: a key enzyme for intestinal epithelial differentiation? *J Parenter Enteral Nutr* 23, 140-146 (1999)

43. Yulong Yin, Ruilin Huang, Taijun Li, Zheng Ruan, Mingyong Xie, Zeyun Deng, Yongqing Hou and Guoyao Wu: Amino acid metabolism in the portal-drained viscera of young pigs: effects of dietary supplementation with chitosan and pea hull. *Amino Acids* 39, 1581-1587 (2010)

44. Guoyao Wu, Darrell Knabe, Wene Yan and Nick Flynn. Glutamine and glucose metabolism in enterocytes of the neonatal pig. *Am J Physiol Regulatory Integrative Comp Physiol* 268, R334-R342 (1995)

45. RS Kensinger, RJ Collier, FW Bazer, CA Ducsay and HN Becker: Nucleic acid, metabolic and histological changes in gilt mammary tissue during pregnancy and lactogenesis. *J Anim Sci* 54, 1297-1308 (1982)

46. SW Kim, WL Hurley, IK Han and RA Easter: Changes in tissue composition associated with mammary gland growth during lactation in sows. *J Anim Sci* 77, 2510-2516 (1999)

47. Sung Woo Kim and Guoyao Wu: Regulatory role for amino acids in mammary gland growth and milk synthesis. *Amino Acids* 37, 89-95 (2009)

48. Sung Woo Kim, Guoyao Wu and David Baker: Ideal protein and amino acid requirements by gestating and lactating sows. *Pig News Inform* 26, 89N-99N (2005)

49. Do-Hyung Kim, Dos Sarbassov, Siraj Ali, Jessie King, Robert Latek, Hediye Erdjument-Bromage, Paul Tempst, and David Sabatini: mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 110, 163-175 (2002)

50. So Hee Kim, Kim Zukowski and Raymond Novak: Rapamycin effects on mTOR signaling in benign, premalignant and malignant human breast epithelial cells. *Antican Res* 29, 1143-1150 (2009)

51. Diane Fingar, Sofie Salama, Christina Tsou, Ed Harlow, and John Blenis: Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. *Genes Dev* 16, 1472-1487 (2002)

52. Diane Fingar, Celeste Richardson, Andrew Tee, Lynn Cheatham, Christina Tsou, and John Blenis: mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4EBP1/eukaryotic translation initiation factor 4E. *Mol Cell Biol* 24 (1), 200-216 (2004)

53. Julian Blow and Tomoyuki Tanaka: The chromosome cycle: Coordinating replication and segregation. *EMBO Reports* 6, 1028-1034 (2005)

54. Guoyao Wu, Fuller Bazer, Robert Burghardt, Gregory Johnson, Sung Woo Kim, Darrell Knabe, Peng Li, Xilong Li, Jason McKnight, Carey Satterfield and Thomas Spencer: Proline and hydroxyproline metabolism: implications for animal and human nutrition. *Amino Acids* 40, 1053-1063 (2011)

55. G Wu, FW Bazer, RC Burghardt, GA Johnson, SW Kim, XL Li, MC Satterfield and TE Spencer: mpacts of amino acid nutrition on pregnancy outcome in pigs: mechanisms and implications for swine production. *J Anim Sci* 88, E195-E204 (2010)

56. BJ Bequette, FRC Backwell and LA Crompton: Current concepts of amino acid and protein metabolism in the mammary gland of the lactating ruminant. *J Dairy Sci* 81, 2540-2559 (1998)

57. Reza Rezaei, Darrell A. Knabe, Xilong Li, Shuo Feng and Guoyao Wu: Enhanced efficiency of milk utilization for growth in surviving low-birth-weight piglets. *J Anim Sci Biotech* 2, 73-83 (2011)

58. Karen Thodberg and Martin Sørensen: Mammary development and milk production in the sow: Effects of udder massage, genotype and feeding in late gestation. *Lives Sci* 101, 116-125 (2006)

59. King RH, Mullan BP, Dunshea FR and Dove H: The influence of piglet body on milk production of sows. *Lives Prod Sci* 47,169-174 (1997)

60. Agu Suryawan, and Teresa Davis. Regulation of protein synthesis by amino acids in muscle of neonates. *Front Biosci* 16, 1445-60 (2011)

61. Fengna Li, Yulong Yin, Bie Tan, Xiangfeng Kong and Guoyao Wu: Leucine nutrition in animals and humans: mTOR signaling and beyond. *Amino Acids* 41, 1185-1193 (2011)

62. Guoyao Wu: Recent advances in swine amino acid nutrition. *J Anim Sci Biotech* 1, 49-61 (2010)

63. Marcin Jankiewicz, Brernd Groner, and Sylvane Desrivières: Mammalian target of rapamycin regulates the growth of mammary epithelial cells through the inhibitor of deoxyribonucleic acid binding Id1 and their functional differentiation through Id2. *Mol Endocrinol* 20 (10) 2369-2381 (2006)

64. JAD Ranga Niroshan Appuhamy, Nicole A Knoebel, WA Deepthi Nayananjalie Jeffery Escobar, Mark Hanigan: Isoleucine and leucine independently regulate mTOR signaling and protein synthesis in MAC-T cells and bovine mammary tissue slices. J Nutr. doi:10.3945/jn.111.152595 (2012)

65. Estela Jacinto, Robbie Loewith, Anja Schmidt, Shuo Lin, Markus Rüegg, Alan Hall and Michael Hall: Mammalian TOR complex 2 controls the atin cytoskeleton and is rapamycin insensitive. *Nature Cell Biol* 6, 1122-1131 (2004)

66. Junjun Wang, Zhenlong Wu, Defa Li, Ning Li, Scott Dindot, Carey Satterfield, Fuller Bazer and Guoyao Wu. Nutrition, epigenetics, and metabolic syndrome. *Antioxid Redox Signal*. doi: 10.1089/ars.2011.4381 (2012)

67. Peng Li, Yulong Yin, Defa Li, Sung Woo Kim and Guoyao Wu: Amino acids and immune function. *Br J Nutr* 98, 237-252 (2007)

68. Thomas Rosano and David Jones. Developmental changes in mitochadria during the transition into lactation in the mouse mammary gland. I. Behavior on isopycnic gradient centrifugation. *J Cell Biol* 69, 573-580 (1976)

69. Thomas Rosano, SK Lee and David Jones: Developmental changes in mitochadria during the transition into lactation in the mouse mammary gland. □. Membrane marker enzymes and membrane ultrasructure. *J Cell Biol* 69, 581-588 (1976)

70. Ashley Alvers, Laura Fishwick, Michael Wood, Doreen Hu, Hye Chung, William Dunn Jr, and John Aris: Autophage and amino acid homeostasis are required for chronological longevity in Saccharomyces cerevisiae. *Aging Cell* 8, 353-369 (2009)

71. Grinari JM, McGuire MA, Dwyer DA, Bauman DE, Barbano DM, and House WA. The role of insulin in the regulation of milk protein synthesis in dairy cows. *J Dairy Sci* 80, 2361-2371 (1997)

72. Alan Bell and Dale Bauman: Regulation of amino acid metabolism in dairy and beef cattle. *21st Annual Southwest Nutrition & Management Conference*, Arizona, 34-44 (2006)

73. Richert BT, Goodband RD, Tokach MD and Nelssen JL: Increasing valine, isoluecine, and total brancded-chain amino acids for lactating sows. *J Anim Sci*, 75, 2117-2128 (1997)

74. Mark Tischler, M Desautels and Alfred Goldberg. Does leucine, leucyl-tRNA, or some metabolite of leucine regulate protein synthesis and degradation in skeletal and cardiac muscle? *J Biol Chem*, 257, 1613-1621 (1982)

75. Peter Garlick and Ian Grant: Amino acid infusion increases the sensitivity of muscle protein synthesis *in vivo* to insulin. Effect of branched-chain amino acid. *Biochem J* 254, 579-584 (1988)

76. William Mitch and Andrew Clark. Specificity of the effects of leucine and its metabolites on protein degradation in skeletal muscle. *Biochem J* 222, 579-586 (1984)

77. John Tayek, Bruce Bistrian, Dermot Hehir, Ramon Martin, Lyle Moldawer and George Blackburn: Improved protein kinetics and albumin synthesis by branched-chain amino acid-enriched total parenteral nutrition in cancer cachexia. *Cancer* 58, 147-157 (1986)

78. Balvin Chua, Daniel Siehl and Howard Morgan: Effect of leucine and metabolites of branched chain amino acids on protein turnover in heart. *J Biol Chem* 254, 8358-8362 (1979)

79. Aaron Ciechanover: Intracellular protein degradation: From a vague idea through the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. *Biochim Biophys Acta* 1824, 3-13 (2012)

80. Sílvia Busquets, Belén Alvarez, Marta Llovera, Neus Agell N, Francisco López-Soriano and Josep Argilés: Branched-chain amino acids inhibit proteolysis in rat skeletal muscle: mechanisms involved. *J Cell Physiol* 184, 380-384 (2000)

81. Kang Yao, Yulong Yin, Xilong Li, Pengbin Xi, Junjun Wang, Jian Lei, Yongqing Hou and Guoyao Wu: Alpha-ketoglutarate inhibits glutamine degradation and enhances protein synthesis in intestinal porcine epithelial cells. *Amino Acids*. doi: 10.1007/s00726-011-1060-6 (2011)

82. Xilong Li, Reza Rezaei, Peng Li and Guoyao Wu: Composition of amino acids in feed ingredients for animal diets. *Amino Acids* 40, 1159-1168 (2011)

83. Yongqing Hou, Lei Wang, Binying Ding, Yulan Liu, Huiling Zhu, Jian Liu, Yongtang Li, Xin Wu, Yulong Yin and Guoyao Wu: Dietary α-ketoglutarate supplementation ameliorates intestinal injury in lipopolysaccharide-challenged piglets. *Amino Acids* 39, 555-564 (2010)

84. WZ Wu, XQ Wang, GY Wu, SW Kim, F Chen and JJ Wang: Differential composition of proteomes in sow colostrum and milk from anterior and posterior mammary glands. *J Anim Sci* 88, 2657-2664 (2010)

85. M Salleh Ardawi and May F Majzoub: Glutamine and ketone-body metabolism in the small intestine of starved peak-lactating rats. *Biochimeie* 70, 749-755 (1988)

86. Okine EK, Glimm OR, Thompson JR, Kennelly JJ: Influence of stage of lactation on glucose and glutamine metabolism in isolated enterocytes from dairy cattle. *Metabolism* 44, 325-331 (1995)

87. L Doepel, GE Lobley, JF Bernier, P Dubreuil and H Lapierre: Effect of glutamine supplementation on

splanchnic metabolism in lactating dairy cows. *J Dairy Sci* 90, 4325-4333 (2007)

88. Shennan D and Peaker M: Transport of milk constituents by the mammary gland. *Physiol Rev* 80, 925-951 (2000)

89. O'Quinn PR, Darrell Knabe, Guoyao Wu: Arginine catabolism in lactating porcine mammary tissue. *J Anim Sci* 80, 467-474 (2002)

90. BT Richert, MD Tokach, RD Goodband, JL Nelssen, RG Campbell and Kershaw S: The effect of dietary lysine and valine fed during lactation on sow and litter performance. *J Anim Sci* 75, 1853-1860 (1997)

91. BT Richert, MD Tokach, RD Goodband, JL Nelssen, JE Pettigrew, RD Walker, LJ Johnston: Valine requirement of the high-producing lactating sow. *J Anim Sci* 74, 1307-1313 (1997)

92. SA Moser, MD Tokach, SS Dritz, RD Goodband, JL Nelssen and Loughmiller JA: The effects of branched-chain amino acids on sow and litter performance. *J Anim Sci* 78, 658-667 (2000)

93. BR Paulicks, H Ott and DA Roth-Maier: Performance of lactating sows in response to the dietary valine supply. *J Anim Physiol Anim Nutr* 87, 389-396 (2003)

94. Henri Rulquin and Pawel Pisulewski: Effects of graded levels of duodenal infusions of leucine on mammary uptake and output in lactating dairy cows. *J Dairy Res* 73, 328-339 (2006)

95. Dewey KG: Maternal and fetal stress are associated with impaired lactogenesis in humans. *J Nutr* 131, 3012S-3015S (2001)

96. JA Appuhamy, JR Knapp, O Becvar, J Escobar and MD Hanigan: Effects of jugular-infused lysine, methionine, and branched-chain amino acids on milk protein synthesis in high-producing dairy cows. *J Dairy Sci* 94, 1952-1960 (2011)

97. Guoyao Wu, Darrell Knabe, Nick Flynn, Wene Yan and Sean Flynn: Arginine degradation in developing porcine enterocytes. *Am J Physiol Gastrointest Liver Physiol* 271, G913-G919 (1996)

98. Zhaolai Dai, Jin Zhang, Guoyao, and Weiyun Zhu. Utilization of amino acids by bacteria from the pig small intestine. *Amino Acids* 39, 1201-1215 (2010)

99. Zhaolai Dai, Guoyao Wu and Weiyun Zhu: Amino acid metabolism in intestinal bacteria: links between gut ecology and host health. *Front Biosci* 16, 1768-1786 (2011)

100. Yongqing Hou, Lei Wang, Binying Ding, Yulan Liu, Huiling Zhu, Jian Liu, Yongtang Li, Ping Kang, Yulong Yin and Guoyao Wu: Alpha-ketoglutarate and intestinal function. *Front Biosci* 16, 1186-1196 (2011)

101. Cynthia Meininger and Guoyao Wu: Regulation of endothelial cell proliferation by nitric oxide. *Methods Enzymol* 352, 280-295 (2002)

102. Guoyao Wu and Cynthia Meininger: Nitric oxide and vascular insulin resistance. *BioFactors* 35, 21-27 (2009)

103. Junjun Wang, Lixiang Chen, Defa Li, Yulong Yin, Xiaoqiu Wang, Peng Li, Larry Dangott, Weixin Hu and Guoyao Wu: Intrauterine growth restriction affects the proteomes of the small intestine, liver and skeletal muscle in newborn pigs. *J Nutr* 138, 60-66 (2008)

104. Junjun Wang, Guoyao Wu, Huaijun Zhou and Fenglai Wang: Emerging technologies for amino acid nutrition research in the post-genome era. *Amino Acids* 37, 177-186 (2009)

105. Qinghua He, Xiangfeng Kong, Guoyao Wu, Pingping Ren, Huiru Tang, Fuhua Hao, Ruilin Huang, Tiejun Li, Bie Tan, Peng Li, Zhiru Tang, Yulong Yin and Yongning Wu: Metabolomic analysis of the response of growing pigs to dietary L-arginine supplementation. *Amino Acids* 37, 199-208 (2009)

106. Wenjiang Fu, Arnold Stromberg, Kert Viele, Raymond Carroll and Guoyao Wu: Statistics and bioinformatics in nutritional sciences: analysis of complex data in the era of systems biology. *J Nutr Biochem* 21, 561-572 (2010)

107. Marc Rhoads and Guoyao Wu: Glutamine, arginine, and leucine signaling in the intestine. *Amino Acids* 37, 111-122 (2009)

108. JE Wholt, JH Clark, RG Derrig and CL Davis: Valine, leucine, and isoleucine metabolism by lactating bovine mammary tissue. *J Dairy Sci* 60, 1875-1882 (1977)

109. JA Maas, J France, J Dijkstra, A Bannink and BW McBride: Application of a mechanistic model to study competitive inhibition of amino acid uptake by the lactating bovine mammary gland. *J Dairy Sci* 81, 1742-1734 (1998)

110. TR Mackle, DA Dwyer and DE Bauman: Effects of branched-chain amino acids and sodium caseinate on milk protein concentration and tield from dairy cows. *J Dairy Sci* 82, 161-171 (1999)

Abbreviations: BCAA, branched-chain amino acids; BCATc, cytosolic branched-chain amino acid aminotransferase; BCATm, mitochondrial branched-chain amino acid aminotransferase; BCKA, branched-chain α-ketoacids; BCKAD, branched-chain α-ketoacid dehydrogenase; 4EBP1, 4E-binding protein-1; KIC, α-ketoisocaproate; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamycin complex 2; S6K1, 70-kDa ribosomal protein S6 kinase-1.

Key Words: Branched-chain amino acids, Mammary epithelial cells, Milk, Lactation, Review

Send correspondence to: Jian Lei, College of Animal Science and Technology, South China Agricultural University, Guangzhou, China, Tel: 86-0-134 2882 6697, Fax 86-20-38765949, E-mail: sgleijian@163.com