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ORIGINAL ARTICLE

Catabolism of nutritionally essential amino acids in developing porcine enterocytes

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Abstract This study was conducted using the piglet model to test the hypothesis that mucosal cells of the neonatal small intestine can degrade nutritionally essential amino acids (EAA). Enterocytes were isolated from the jejunum of 0-, 7-, 14-, and 21-day-old pigs, and incubated for 45 min in Krebs buffer containing plasma concentrations of amino acids and one of the following L-[1-^{14}C]- or L-[U-^{14}C]-amino acids plus unlabeled tracees at 0.5, 2, or 5 mM: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. In these cells, branched-chain amino acids (BCAA) were extensively transaminated and 15–50% of decarboxylated branched-chain α-ketoacids (BCKA) were oxidized to CO_2 depending on the age of piglets. BCAA transamination increased but their decarboxylation decreased between 0 and 14 days of age. Addition of 1 and 2 mM α-ketoglutarate to incubation medium dose-dependently stimulated BCAA transamination without affecting their decarboxylation. Western blot analysis revealed that the abundance of mitochondrial BCAA aminotransferase declined but cytosolic BCAA aminotransferase increased between 0 and 14 days of age, with the cytosolic protein being the major isoform in 7- to 21-day-old pigs. BCKA dehydrogenase protein existed primarily as the phosphorylated (inactive) form in enterocytes of newborn pigs and its levels were markedly reduced in older pigs. All measured parameters of BCAA metabolism did not differ between 14- and 21-day-old pigs. In contrast to BCAA, catabolism of methionine and phenylalanine was negligible and that of other EAA was absent in enterocytes from all ages of piglets due to the lack of key enzymes. These results indicate that enterocytes are an important site for substantial degradation of BCAA but not other EAA in the neonatal gut.

Keywords Amino acids · Catabolism · Enterocytes · Pigs

Abbreviations

AA	Amino acids
BCAA	Branched-chain amino acids
BCAT	Branched-chain amino acid aminotransferase
BCKA	Branched-chain α-ketoacids
BCKAD	Branched-chain α-ketoacid dehydrogenase
EAA	Essential amino acids
α-KG	α-Ketoglutarate
KIC	Ketoisocaproic acid
NEAA	Nutritionally nonessential amino acids

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Introduction

An exciting new development in protein nutrition is the discovery of extensive first-pass catabolism of all dietary amino acids (AA) [including nutritionally essential AA (EAA)] in the small intestine of both humans and animals (Riedijk et al. 2007; Stoll et al. 1998; Wu 1998). This finding has important implications for understanding the efficiency of utilization of dietary AA. Studies using the pig, an excellent model for studying human nutrition (Haynes et al. 2009; Suryawan et al. 2008; Tan et al. 2008), have established the biochemical bases for the metabolism of nutritionally nonessential AA (NEAA) in enterocytes (Flynn et al. 2008; Wu 1998). However, a role for intestinal mucosal cells in degrading EAA has not been well defined.

We recently reported that enterocytes of postweaning 50-day-old pigs actively degrade branched-chain AA (BCAA) but not other EAA (Chen et al. 2007). This is consistent with a relatively high activity of BCAA aminotransferase (BCAT) (Wu 1998). Interestingly, oxidation of branched-chain α-ketoacids (BCKA) in mucosal cells is low due to a low activity of BCKA decarboxylase (BCKAD) (Chen et al. 2007). Because there are developmental changes in intestinal metabolism of NEAA (Wu 1998), it is unknown whether this phenomenon is also true for EAA. The present study was conducted with the piglet model to test the hypothesis that mucosal cells of the neonatal small intestine can degrade EAA.

Materials and methods

Materials

HPLC-grade water and methanol were procured from Fisher Scientific (Houston, TX). The following L-[1-^{14}C]- and L-[U-^{14}C]-labeled AA were obtained from American Radiolabeled Chemicals (St. Louis, MO): histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Before use, ^{14}C-labeled leucine, isoleucine, valine, methionine, phenylalanine, and tryptophan were purified using AG 1-X8 (acetate form, 200–400 mesh) as resin bed (0.6 × 6 cm) and deionized water (2 ml) as eluting solvent (Self et al. 2004). ^{14}C-Labeled histidine and lysine were purified using thin-layer chromatography on silica gel G with *n*-butanol–acetic acid–water (80:20:20; v/v) as a solvent (Brenner et al. 1969). [1-^{14}C]α-Ketoisocaproic acid (KIC) was obtained from Amersham Corporation (Arlington Height, IL). Immediately before use, [1-^{14}C]KIC was purified by incubation at 25°C with 100 µl of 1.5 M $HClO_4$ for 30 min, followed by neutralization with 50 µl of 2 M K_2CO_3 (Chen et al. 2007). In our preliminary studies, we found that exceedingly large amounts of $^{14}CO_2$ could be produced from L-[1-^{14}C] or L-[U-^{14}C]-labeled EAA if they were used, without purification, for incubation with pig enterocytes. Soluene-350, a strong organic base formulated for compatibility with liquid scintillation cocktails and wet tissue solubilization, was obtained from Perkin Elmer. AG 1-X8, Tris, glycine, sodium dodecyl sulfate (SDS), Triton X-100, Tween-20, and nitrocellulose membranes were obtained from BioRad (Hercules, CA). 3-(*N*-morpholino)-propanesulfonic acid (MOPS), SDS running buffer (20X), and NuPage 10% Bis–Tris gel (15-lane) were purchased from Invitrogen (Carlsbad, CA). The BCA Protein assay kit and SuperSignal® West Dura Extended Duration Substrate were purchased from Pierce (Rockford, IL, USA). Rabbit anti-mitochondrial and cytosolic BCAT were generously provided by Dr. Susan Hutson (Wake Forest University, Winston-Salem, NC). Rat anti-BCKAD E1α antibody and rat anti-phosphorylated form of BCKAD E1α antibody were gifts of Dr. Christopher Lynch (Pennsylvania State University, Hershey, PA). All other chemicals were purchased from Sigma Chemicals (St. Louis, MO).

Animals, collection of tissues, and isolation of jejunal enterocytes

Newborn pigs (0-day-old) were used within 1 h of birth without suckling, whereas 7- to 21-day-old pigs were freely nursed by their sows. Liver and skeletal (semitendinosus) muscle were rapidly obtained from anesthetized piglets, placed in liquid nitrogen, and stored at –80°C, as previously described (Wu et al. 2007). Enterocytes were prepared from the mid-jejunum of 0-, 7-, 14- and 21-day-old healthy pigs (offspring of Yorkshire × Landrace sows and Duroc × Hampshire boars), using Ca^{2+}-free Krebs buffer (Wu et al. 1995). After isolation, cells were washed 3 times with Ca^{2+}-containing Krebs buffer and then used for metabolic studies. This work was approved by Texas A&M University's Institutional Animal Care and Use Committee.

Determination of EAA catabolism in enterocytes

Metabolic studies were performed as previously described (Wu 1997). Briefly, enterocytes (25 mg protein) were incubated at 37°C for 45 min in 2-ml oxygenated (95% O_2/5% CO_2) Krebs buffer (pH 7.4) containing 1% bovine serum albumin, 5 mM D-glucose, plasma concentrations of AA (Flynn et al. 2000), and one of the following L-[1-^{14}C]- or L-[U-^{14}C]-labeled AA plus unlabeled tracees at 0.5, 2, or 5 mM (150 dpm/nmol): histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. The medium also contained 0 or 5 mM L-cycloserine [an inhibitor of transamination in cells

(Wu et al. 1989)]; or 1 or 2 mM α-ketoglutarate (α-KG). Media containing all components but no cells were run as blanks. Incubations were initiated by addition of cells. After a 45-min incubation period, the evolved $^{14}CO_2$ was collected in 0.2 ml NCS-II after the medium was acidified with 0.2 ml of 1.5 M $HClO_4$ (Wu 1997). The second collection of $^{14}CO_2$ was performed by addition of 0.7 ml of 30% (v/v) H_2O_2 to the incubation medium to decarboxylate α-ketoacids (Wu and Thompson 1987). AA (including cysteine and homocysteine) and potential products (e.g., histamine and homocysteine) in medium and cell extracts were determined by HPLC (Kohli et al. 2004; Ou et al. 2007; Wu et al. 1997, 2007). Anion-exchange chromatography was employed to detect ^{14}C-labeled tricarboxylic-acid-cycle intermediates (Wu and Thompson 1988). ^{14}C radioactivity was measured using a liquid scintillation counter (Self et al. 2004). The intracellular specific activities of ^{14}C-AA were determined using HPLC, as described by Wu (1997). In some experiments, enterocytes were incubated with (1) one of the following L-[1-^{14}C]- or L-[U-^{14}C]-AA plus unlabeled tracees at 2 mM: histidine, lysine, methionine, phenylalanine, threonine, and tryptophan; and (2) 0 or 2 mM each of BCAA (leucine, isoleucine, and valine), as described above, except that the basal medium contained no AA.

Determination of enzyme activities

Enterocytes, liver, and skeletal muscle (~0.5 g) were homogenized in 2 ml of buffer consisting of 50 mM Hepes (pH 7.5), 3 mM EDTA, 5 mM dithiothreitol, 2% (v/v) Triton X-100, and 0.1% (w/v) protease inhibitor (aprotinin, chymostatin, pepstatin A, and phenylmethylsulfonyl fluoride) (Wu 1997). For BCKAD assays, the homogenization buffer also contained 1 mM potassium fluoride. Homogenates were centrifuged at 600*g* for 10 min and the supernatant fluid was subjected to three cycles of freezing in liquid nitrogen and thawing in a 30°C water bath.

Activities of enzymes were determined as previously described (Chen et al. 2007; Wu et al. 1991; Wu and Thompson 1989b). The assay mixture for BCAT consisted of 0.5 ml of 50 mM Tris/HCl buffer (pH 8.6), 0.1 ml of 1.6 mM pyridoxal phosphate, 0.2 ml of 50 mM α-KG, 1 ml of 10 mM L-leucine plus 0.1 μCi of L-[1-^{14}C]leucine, and 0.2 ml of tissue extract (0.5 and 1 mg protein). The BCKAD assay mixture consisted of 0.1 ml of 20 mM $MgCl_2$, 0.1 ml of 10 mM dithiothreitol, 0.1 ml of 4 mM thiamine pyrophosphate plus 4 mM coenzyme A plus 10 mM NAD, 0.4 ml of 50 mM potassium phosphate buffer (pH 7.5), 0.1 ml of tissue extract (0.5 and 1 mg protein), and 0.1 ml of 1 mM potassium fluoride. Potassium fluoride (an inhibitor of protein kinase) was used to assess BCKAD activity in its active state. All samples were preincubated for 10 min in a 30°C water bath, after which 0.1 ml of 10 mM KIC plus 0.1 μCi [1-^{14}C]KIC was added into all tubes.

The assay mixture for glutamine transaminase K consisted of 100 μl of 200 mM sodium borate (pH 8.5), 100 μl of 40 mM glutamine, 100 μl of 4 mM sodium phenylpyruvate, and 100 μl of tissue extract (0.5 and 1 mg protein). The assay mixture for glutamine transaminase L contained 100 μl of 200 mM sodium borate (pH 8.5), 100 μl of 40 mM L-albizzin, 100 μl of 4 mM α-keto-γ-methiolbutyrate, and 100 μl of tissue extract (0.5 and 1 mg protein). The assay mixture (1 ml) for phenylalanine hydroxylase consisted of 0.1 mM Hepes buffer, 1 mM NADPH, 0.5 mM tetrahydrobiopterin (BH4), 1 mM vitamin C (a stabilizer of BH4), 0 or 10 mM L-phenylalanine, and enzyme extracts (0.5 and 1 mg protein); the reaction was terminated at 5, 10, and 15 min and the assay extract was analyzed for tyrosine using HPLC (Wu and Meininger 2008).

The assay mixture (0.2 ml) for lysine: α-KG reductase consisted of 20 mM Hepes (pH 7.4), 25 mM L-lysine, 0.5 mM NADPH, 0 or 7.5 mM α-KG, and tissue extract (0.25 and 0.5 mg protein). The assay mixture (0.2 ml) for saccharopine dehydrogenase assay contained 35 mM Tris/HCl (pH 9.4), 1.8 mM NAD, 0 or 2 mM saccharopine, and tissue extract (0.25 and 0.5 mg protein). The assay mixture (0.2 ml) for threonine dehydrogenase consisted of 50 mM Tris/HCl (pH 8.4), 0 or 25 mM L-threonine, 2 mM NAD, 0.5 mM pyridoxal phosphate, and tissue extract (0.25 or 0.5 mg protein). The assay mixture (0.2 ml) for threonine dehydratase contained 30 mM potassium phosphate buffer (pH 8.2), 0.3 mM EDTA, 0.5 mM NADPH, 10 mM α-KG, glutamate dehydrogenase, 0.1 mM pyridoxal phosphate, 0 or 25 mM L-threonine, and tissue extract (0.25 or 0.5 mg protein). In all of the enzyme assays, absorbance at 340 nm was determined at 0, 5, 10, and 15 min using a Molecular Devices Microplate Reader (Sunnyvale, CA).

Western blot analysis of BCAT, BCKAD and glutamine synthetase proteins

Enterocytes were pulverized in liquid nitrogen and homogenized lysis buffer containing 20 mM Tris–HCl (pH 7.4), 50 mM NaCl, 50 mM NaF, 50 mM EDTA, 1% Triton X-100, 1× protease inhibitor cocktail and 11× phosphatase inhibitor cocktail (Calbiochem, La Jolla, CA). Proteins in homogenates were analyzed using the BCA Protein Assay Kit (Pierce). The samples were subsequently diluted with 21× Laemmli buffer (125 mM Tris–HCl pH 6.8, 4% w/v SDS, 10% 2-mercaptoethanol, 12% glycerol, and 0.004% w/v bromphenol blue) and heated in boiling water for 5 min. NuPage 10% Bis–Tris gel from Invitrogen was used

for SDS–PAGE separation of proteins. Proteins were transferred to a nitrocellulose membrane (Bio-Rad) under 12 V overnight, using the Bio-Rad Transblot apparatus. Membranes were blocked in 5% fat-free dry milk in TTBS (20 mM Tris/150 mM NaCl, pH 7.5, and 0.1% Tween-20) for 3 h and then incubated with one of the following primary antibodies overnight at 4°C with gentle shaking: antibodies for mitochondrial BCAT (1:10,000), cytosolic BCAT (1:10,000), total BCKAD E1α (1:10,000), phosphorylated BCKAD E1α (1:50,000), and glutamine synthetase (1:5,000). After being washed 3 times with TTBS, the membranes were incubated at room temperature for 2–3 h with secondary antibodies (peroxidase-labeled donkey anti-rat, anti-rabbit or anti-mouse IgG, Jackson Immuno Research, 1:50,000). Finally, the membranes were washed with TTBS, followed by development using SuperSignal West Dura Extended Duration Substrate according to the manufacturer's instructions (Pierce, Rockford, IL). The signals were detected on Fujifilm LAS-3000 (Tokyo, Japan). Results were expressed as relative pixel intensities (arbitrary unit, AU). Equal numbers of samples from each group of piglets were run on the same gel to ensure consistency. Values from replicate gels were normalized to an arbitrary value for a pooled sample included on every gel (Li et al. 2008).

Calculations and statistical analysis

Rates of catabolism of ^{14}C-AA were calculated based on their intracellular specific radioactivities (Wu and Thompson 1987; Wu 1997). Data were analyzed by one-way analysis of variance and the Student–Newman–Keul's multiple comparison test, using the Statistical Analysis System (SAS Institute, Cary, NC). Probability values ≤ 0.05 were taken to indicate statistical significance.

Results

BCAA degradation in enterocytes

BCAA transamination and oxidative decarboxylation in enterocytes, measured with leucine, were linear with 45-min incubation. For example, in enterocytes from 14-day-old pigs, net transamination of leucine was 1.46 ± 0.11, 3.06 ± 0.24 and 4.65 ± 0.37, net KIC release was 1.03 ± 0.09, 2.25 ± 0.17 and 3.38 ± 0.22, and oxidative decarboxylation of leucine was 0.43 ± 0.05, 0.81 ± 0.17 and 1.28 ± 0.09 nmol product/mg protein, respectively, at the end of 15, 30 and 45 min of incubation. These results indicated the biochemical viability of pig enterocytes under the in vitro conditions used. BCAA were extensively transaminated in enterocytes from all ages of piglets, with 15–50% of decarboxylated BCKA being oxidized to CO_2 depending on the age of piglets (Table 1). Between 0 and 14 days of age, BCAA transamination increased ($P < 0.01$) but their decarboxylation decreased ($P < 0.05$) progressively. In enterocytes from 14-day-old pigs, rates of BCAA catabolism increased ($P < 0.01$) with increasing their extracellular concentrations from 0.5 to 5 mM (Table 2). Similar results were obtained for cells from 0-, 7-, and 21-day-old pigs (data not shown). Addition of 1 and 2 mM α-KG to incubation medium dose-dependently stimulated BCAA transamination without affecting their decarboxylation (Table 3), whereas addition of 1 and 5 mM L-cycloserine to incubation medium inhibited ($P < 0.01$) BCAA transamination by 54 and 92%, respectively. All measured parameters of BCAA metabolism did not differ between 14- and 21-day-old pigs. In contrast to BCAT and BCKAD, glutamine synthetase protein was very weakly expressed in enterocytes from all ages o the piglets (data not shown).

Degradation of other EAA in enterocytes

Catabolism of methionine and phenylalanine was negligible in enterocytes from 0- to 21-day-old piglets (Table 4). The rates of $^{14}CO_2$ production from [1-^{14}C]methionine and [1-^{14}C]phenylalanine were similar to those from [U-^{14}C]methionine and [U-^{14}C]phenylalanine, respectively, indicating that the non-carboxyl carbons of either AA did not undergo further oxidation in the cells (Table 4). In the absence of physiological levels of AA from incubation medium, the rates of methionine and phenylalanine transamination at 2 mM were also negligible in pig enterocytes (84–106 pmol/mg protein per 45 min; mean ± SEM, $n = 8$) but were ~130% higher ($P < 0.01$) than the values obtained in the presence of all other AA. Addition of 5 mM L-cycloserine to incubation medium completely abolished the degradation of methionine and phenylalanine. Of particulate note, there was no production of $^{14}CO_2$ from L-[1-^{14}C] or L-[U-^{14}C]-labeled histidine, lysine, threonine, and tryptophan in pig enterocytes incubated in the presence or absence of plasma AA. Further, enterocytes could not convert ^{14}C-labeled histidine, lysine, methionine, phenylalanine, threonine or tryptophan into tricarboxylic-acid-cycle intermediates or nitrogenous products. For example, there was no production of (1) cysteine and homocysteine from methionine; (2) tyrosine from phenylalanine; or (3) histamine from histidine in enterocytes of 0- to 21-day-old pigs.

Activities of enzymes involved in EAA metabolism

Enterocytes, like skeletal muscle and liver, contained activities of BCAT, BCKAD, lysine:α-KG reductase,

Table 1 BCAA catabolism in enterocytes of 0- to 21-day-old pigs

Age of piglets (Days)	CO_2 from all carbons (a)	CO_2 from carbon-1 (b)	Net release of BCKA (c)	Net transamination (d = b + c)	Percentage of transaminated BCAA released as BCKA (%) (c/d × 100%)	Percentage of decarboxylated BCAA oxidized as CO_2 (a–b)/nb × 100%
2 mM L-Leucine						
0	3.15 ± 0.16^{b}	1.86 ± 0.07^{a}	1.15 ± 0.05^{c}	3.02 ± 0.09^{c}	38.2 ± 1.0^{c}	14.0 ± 0.7^{c}
7	3.96 ± 0.20^{a}	1.42 ± 0.05^{b}	2.06 ± 0.07^{b}	3.48 ± 0.10^{b}	59.0 ± 1.8^{b}	36.0 ± 1.3^{b}
14	4.02 ± 0.26^{a}	1.16 ± 0.04^{c}	3.35 ± 0.15^{a}	4.51 ± 0.13^{a}	74.0 ± 1.6^{a}	49.2 ± 2.4^{a}
21	4.24 ± 0.23^{a}	1.25 ± 0.04^{c}	3.48 ± 0.12^{a}	4.73 ± 0.12^{a}	73.5 ± 2.0^{a}	47.8 ± 1.7^{a}
2 mM L-Isoleucine						
0	2.92 ± 0.12^{b}	1.65 ± 0.06^{a}	1.07 ± 0.06^{c}	2.71 ± 0.07^{c}	39.6 ± 1.2^{c}	15.2 ± 0.8^{c}
7	3.68 ± 0.15^{a}	1.27 ± 0.06^{b}	1.83 ± 0.08^{b}	3.12 ± 0.11^{b}	58.4 ± 1.5^{b}	37.8 ± 1.6^{b}
14	3.50 ± 0.19^{a}	0.97 ± 0.05^{c}	3.08 ± 0.17^{a}	4.06 ± 0.18^{a}	75.7 ± 1.6^{a}	52.0 ± 2.9^{a}
21	3.79 ± 0.17^{a}	1.09 ± 0.05^{c}	3.30 ± 0.13^{a}	4.40 ± 0.16^{a}	74.8 ± 2.3^{a}	49.6 ± 2.0^{a}
2 mM L-Valine						
0	1.64 ± 0.08^{b}	1.03 ± 0.05^{a}	0.65 ± 0.03^{c}	1.68 ± 0.06^{c}	38.8 ± 1.7^{c}	14.6 ± 0.6^{c}
7	2.08 ± 0.10^{a}	0.86 ± 0.04^{b}	1.13 ± 0.06^{b}	1.98 ± 0.07^{b}	56.9 ± 1.7^{b}	35.6 ± 2.0^{b}
14	2.14 ± 0.15^{a}	0.71 ± 0.03^{c}	1.92 ± 0.12^{a}	2.63 ± 0.09^{a}	73.2 ± 2.0^{a}	50.5 ± 2.6^{a}
21	2.25 ± 0.11^{a}	0.74 ± 0.03^{c}	2.11 ± 0.09^{a}	2.85 ± 0.10^{a}	74.1 ± 2.8^{a}	51.2 ± 1.7^{a}

Values, expressed as nmol product/mg protein per 45 min, are mean ± SEM, $n = 8$. In the formula for calculating the percentage of decarboxylated BCAA oxidized as CO_2, n represents the number of non-carboxyl carbons (namely, $n = 5$ for leucine and isoleucine; $n = 4$ for valine). The BCKA for leucine, isoleucine, and valine are α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV), and α-ketoisovaleric acid (KIV), respectively

a–c: Means within a column of each BCAA without a common superscript differ ($P < 0.05$)

Table 2 Concentration-dependent increase of BCAA catabolism in enterocytes of 14-day-old pigs

Medium [BCAA] (mM)	CO_2 from all carbons (a)	CO_2 from carbon-1 (b)	Net release of BCKA (c)	Net transamination (d = b + c)	Percentage of transaminated BCAA released as BCKA (%) (c/d × 100%)	Percentage of decarboxylated BCAA oxidized as CO_2 (a-b)/nb × 100%
L-Leucine						
0.5	1.68 ± 0.11^{c}	0.47 ± 0.02^{c}	1.39 ± 0.07^{c}	1.86 ± 0.08^{a}	74.6 ± 2.3	51.7 ± 1.4^{a}
2	4.18 ± 0.29^{b}	1.28 ± 0.07^{b}	3.42 ± 0.17^{b}	4.71 ± 0.20^{a}	72.8 ± 2.0	49.3 ± 1.2^{ab}
5	7.07 ± 0.37^{a}	2.14 ± 0.12^{a}	6.04 ± 0.32^{a}	8.18 ± 0.35^{a}	73.6 ± 1.8	46.1 ± 1.0^{b}
L-Isoleucine						
0.5	1.55 ± 0.15^{c}	0.43 ± 0.03^{c}	1.15 ± 0.13^{c}	1.60 ± 0.10^{c}	72.0 ± 2.1	52.1 ± 1.6^{a}
2	3.64 ± 0.21^{b}	1.06 ± 0.06^{b}	3.13 ± 0.20^{b}	4.20 ± 0.23^{b}	74.5 ± 1.8	48.8 ± 1.5^{ab}
5	6.35 ± 0.34^{a}	1.94 ± 0.09^{a}	4.98 ± 0.27^{a}	6.93 ± 0.31^{a}	71.7 ± 1.9	45.5 ± 1.2^{b}
L-Valine						
0.5	0.94 ± 0.11^{c}	0.31 ± 0.02^{c}	0.80 ± 0.05^{c}	1.12 ± 0.06^{c}	71.4 ± 2.6	51.0 ± 1.9^{a}
2	2.18 ± 0.18^{b}	0.73 ± 0.04^{b}	2.07 ± 0.16^{b}	2.80 ± 0.17^{b}	74.0 ± 2.3	49.7 ± 1.7^{ab}
5	3.49 ± 0.29^{a}	1.25 ± 0.06^{a}	3.28 ± 0.19^{a}	4.54 ± 0.20^{a}	72.2 ± 2.5	44.9 ± 1.6^{b}

Values, expressed as nmol product/mg protein per 45 min, are mean ± SEM, $n = 8$. In the formula for calculating the percentage of decarboxylated BCAA oxidized as CO_2, n represents the number of non-carboxyl carbons (namely, $n = 5$ for leucine and isoleucine; $n = 4$ for valine)

a–c: Means within a column of each BCAA without a common superscript differ ($P < 0.05$)

glutamine aminotransferase K, and glutamine aminotransferase L (Table 5). BCAT activity in enterocytes was much higher ($P < 0.01$) than that in liver but lower ($P < 0.01$) than the value for skeletal muscle, whereas BCKAD activity was much ($P < 0.01$) lower in enterocytes compared with liver and muscle. Intestinal BCAT activity

Table 3 Effect of α-KG on BCAA transamination and decarboxylation in enterocytes of 14-day-old pigs

[α-KG] (mM)	Net transamination	Decarboxylation	Net release of BCKA
2 mM L-Leucine			
0	4.46 ± 0.12[c]	1.16 ± 0.06	3.29 ± 0.10[c]
1	5.66 ± 0.18[b]	1.12 ± 0.07	4.54 ± 0.15[b]
2	6.45 ± 0.24[a]	1.09 ± 0.06	5.36 ± 0.22[a]
2 mM L-Isoleucine			
0	4.08 ± 0.14[c]	1.03 ± 0.05	3.05 ± 0.12[c]
1	5.10 ± 0.18[b]	1.08 ± 0.06	4.01 ± 0.17[b]
2	5.92 ± 0.26[a]	0.99 ± 0.07	4.92 ± 0.25[a]
2 mM L-Valine			
0	2.86 ± 0.10[c]	0.72 ± 0.05	2.15 ± 0.09[c]
1	3.57 ± 0.12[b]	0.72 ± 0.06	2.85 ± 0.11[b]
2	4.28 ± 0.14[a]	0.71 ± 0.05	3.56 ± 0.14[a]

Values, expressed as nmol product/mg protein per 45 min, are mean ± SEM, $n = 8$

a–c: Means within a column of each BCAA without a common superscript differ ($P < 0.05$)

Table 4 Catabolism of methionine and phenylalanine in enterocytes of 0- to 21-day-old pigs

Age of piglets (Days)	CO_2 from all carbons	CO_2 from carbon-1	Net release of α-ketoacid
2 mM L-Methionine			
0	3.8 ± 0.3[c]	3.7 ± 0.4[c]	9.4 ± 0.7[c]
7	8.1 ± 0.6[b]	8.1 ± 0.7[b]	15.0 ± 1.1[b]
14	10.6 ± 0.9[a]	10.4 ± 0.8[a]	24.6 ± 1.6[a]
21	11.3 ± 1.3[a]	11.2 ± 1.4[a]	25.8 ± 1.9[a]
2 mM L-Phenylalanine			
0	5.2 ± 0.6[c]	5.1 ± 0.6[c]	13.4 ± 1.0[c]
7	9.5 ± 0.7[b]	9.3 ± 0.8[b]	20.6 ± 1.3[b]
14	12.8 ± 1.1[a]	12.7 ± 1.0[a]	32.5 ± 1.8[a]
21	13.6 ± 1.5[a]	13.4 ± 1.6[a]	34.4 ± 2.3[a]

Values, expressed as pmol product/mg protein per 45 min, are mean ± SEM, $n = 8$. The α-ketoacids of methionine and phenylalanine are α-keto-γ-methiolbutyric acid and phenylpyruvic acid, respectively

a–c: Means within a column of each AA without a common superscript differ ($P < 0.05$)

increased ($P < 0.01$) but BCKAD activity decreased ($P < 0.01$) progressively between 0 and 14 days of age. Activities of these enzymes did not differ between 14- and 21-day-old pigs. There was no detectable activity of threonine dehydrogenase, threonine dehydratase, and phenylalanine hydroxylase in enterocytes or skeletal muscle

Table 5 Activities of enzymes in enterocytes, liver and skeletal muscle of 0- to 21-day-old pigs

Age of piglets (Days)	Enterocytes	Skeletal muscle	Liver
BCAT			
0	1.37 ± 0.06[c]	2.73 ± 0.11[c]	0.32 ± 0.02[c]
7	1.84 ± 0.09[b]	3.58 ± 0.16[b]	0.43 ± 0.02[b]
14	2.36 ± 0.14[a]	4.72 ± 0.25[a]	0.57 ± 0.03[a]
21	2.49 ± 0.16[a]	4.89 ± 0.29[a]	0.60 ± 0.03[a]
BCKAD			
0	0.83 ± 0.06[a]	1.12 ± 0.08[c]	1.43 ± 0.10[c]
7	0.61 ± 0.04[b]	1.41 ± 0.11[b]	2.57 ± 0.13[b]
14	0.43 ± 0.03[c]	1.93 ± 0.13[a]	3.12 ± 0.19[a]
21	0.45 ± 0.03[c]	1.86 ± 0.08[a]	3.04 ± 0.16[a]
Lysine:α-KG reductase			
0	0.10 ± 0.02[b]	0.42 ± 0.03[c]	1.93 ± 0.12[d]
7	0.23 ± 0.03[a]	0.64 ± 0.04[b]	2.85 ± 0.16[c]
14	0.27 ± 0.03[a]	0.90 ± 0.07[a]	4.02 ± 0.21[b]
21	0.29 ± 0.04[a]	0.94 ± 0.06[a]	4.86 ± 0.25[a]
Saccharopine dehydrogenase			
0	ND	0.16 ± 0.02	0.52 ± 0.04[d]
7	ND	0.19 ± 0.03	0.84 ± 0.06[c]
14	ND	0.18 ± 0.02	1.19 ± 0.09[b]
21	ND	0.22 ± 0.03	1.57 ± 0.11[a]
Glutamine transaminase K			
0	0.28 ± 0.03[c]	0.25 ± 0.02[c]	0.22 ± 0.03[c]
7	0.44 ± 0.05[b]	0.37 ± 0.03[b]	0.41 ± 0.04[b]
14	0.57 ± 0.06[a]	0.56 ± 0.05[a]	0.54 ± 0.06[a]
21	0.62 ± 0.05[a]	0.58 ± 0.04[a]	0.51 ± 0.05[a]
Glutamine transaminase L			
0	0.10 ± 0.01[c]	0.23 ± 0.02[c]	0.39 ± 0.04[c]
7	0.21 ± 0.02[b]	0.52 ± 0.04[b]	0.74 ± 0.06[b]
14	0.32 ± 0.04[a]	0.73 ± 0.07[a]	0.98 ± 0.10[a]
21	0.29 ± 0.03[a]	0.76 ± 0.06[a]	1.05 ± 0.08[a]

Values, expressed as nmol/mg protein per min, are mean ± SEM, $n = 8$

ND, not detected (<0.01 nmol/mg protein per min)

a–d: Means within a column of each enzyme without a common superscript differ ($P < 0.05$)

(<0.01 nmol/mg protein per min). Threonine dehydratase activity in liver was 0.10 ± 0.01, 0.17 ± 0.02, 0.22 ± 0.02, and 0.29 ± 0.03 nmol/mg protein per min in 0-, 7-, 14-, and 21-day-old pugs, respectively ($P < 0.01$). Threonine dehydrogenase activity in liver was 0.94 ± 0.06, 1.66 ± 0.09, 2.05 ± 0.14, and 2.78 ± 0.19 nmol/mg protein per min in 0-, 7-, 14-, and 21-day-old pigs, respectively ($P < 0.01$). Phenylalanine hydroxylase activity in liver was 0.58 ± 0.04, 1.14 ± 0.07, 1.52 ± 0.09, and 1.96 ± 0.12 nmol/mg protein per min in 0-, 7-, 14-, and 21-day-old pigs, respectively ($P < 0.01$).

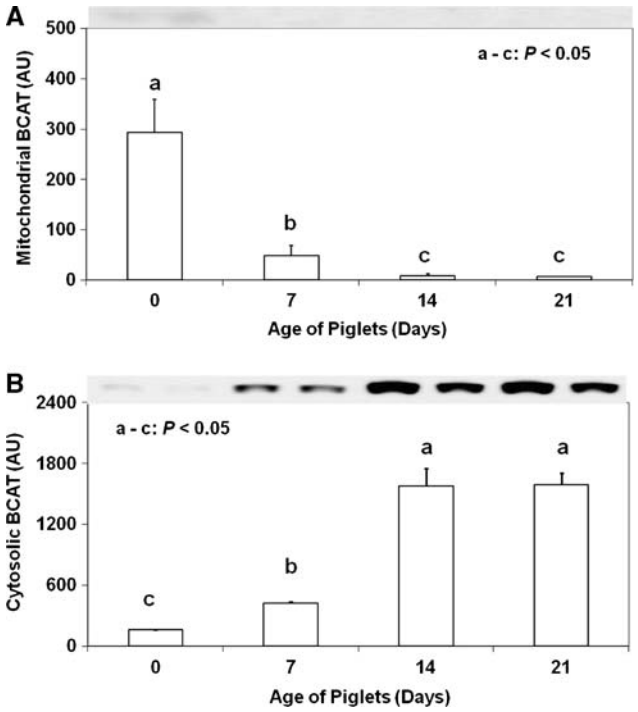

Fig. 1 Abundance of BCAT in mitochondria (*Panel A*) and cytoplasm (*Panel B*) of enterocytes isolated from 0-, 7-, 14- and 21-day-old pigs. Soluble tissue lysate proteins (18 μg) were separated in 10% polyacylamide gels. Antibodies against mitochondrial and cytosolic BCAT were used for the western blot analysis of protein levels. Representative blots are shown. Data in the *bar graph* are means ± SEM, $n = 8$

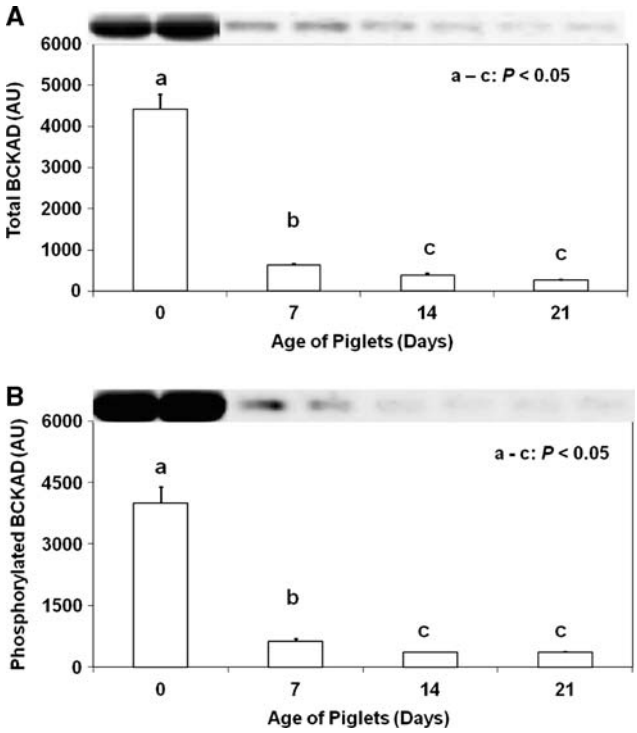

Fig. 2 Abundance of total BCKAD (*Panel A*) and phosphorylated BCKAD (*Panel B*) in enterocytes of 0-, 7-, 14- and 21-day-old pigs. Soluble tissue lysate proteins (9 μg) were separated in 10% polyacylamide gels. Antibodies against BCKAD E1α subunit and phosphorylated form of E1α were used for western blot analysis. Representative blots are shown. Data in the *bar graph* are means ± SEM, $n = 8$

BCAT, BCKAD and glutamine synthetase proteins in enterocytes

Western blot analysis revealed that the abundance of mitochondrial BCAT declined ($P < 0.01$) but that of cytosolic BCAT isoform increased ($P < 0.01$) in enterocytes between 0 and 14 days of age (Fig. 1). The mitochondrial isoform of BCAT protein was barely detectable in cells from 14- and 21-day-old pigs, whereas the cytosolic protein was the major isoform in 7- to 21-day-old pigs. BCKA dehydrogenase protein (measured as the E1α subunit) existed primarily as the phosphorylated (inactive) form in enterocytes of newborn pigs and its levels were markedly reduced ($P < 0.05$) in older pigs (Fig. 2). BCAT or BCKAD protein in enterocytes did not differ between 14- and 21-day-old pigs (Fig. 1). In contrast to BCAT and BCKAD, glutamine synthetase protein was very weakly expressed in enterocytes from 0- to 21-day-old pigs (data not shown).

Discussion

The small intestine contains both BCAT and BCKAD activities for degrading BCAA (Wu 1998). Remarkably, in milk protein-fed piglets, 40% of leucine, 30% of isoleucine and 40% of valine in the diet are extracted by the portal-drained viscera in first-pass metabolism, with less than 20% of the extracted BCAA utilized for intestinal mucosal protein synthesis (Stoll et al. 1998). Here, we reported both BCAA transamination and BCKA oxidative decarboxylation in enterocytes of healthy preweaning pigs. Indeed, rates of BCAA transamination in piglet enterocytes were comparable to those in skeletal muscles of young rats and chickens (Goldberg and Chang 1978; Wu and Thompson 1988). However, compared with glutamine (Wu et al. 1995; 2008b), oxidation of BCKA was limited in enterocytes of healthy suckling piglets (Table 1). Thus, it is unlikely that BCAA are quantitatively significant fuels for the piglet small intestine.

Mucosal catabolism of BCAA may function to: (1) provide nitrogen for the synthesis of both alanine and glutamate (Fig. 3); (2) generate BCKA which may inhibit proteolysis in enterocytes as reported for skeletal muscle (Nakashima et al. 2007); (3) modulate the balance of AA that enter the portal vein and appear in the blood circulation; and (4) regulate nitric oxide-dependent local and systemic blood flow (Kakoki et al. 2006). Because of the

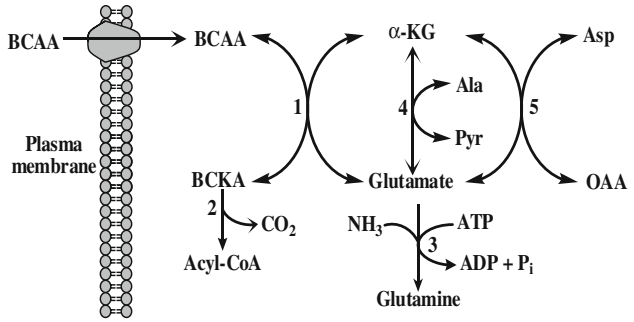

Fig. 3 BCAA catabolism in enterocytes. BCAA enter cells through specific transporters on the plasma membrane. Enzymes catalyzing the indicated reactions are: (1) BCAT; (2) BCKAD; (3) glutamine synthetase; (4) glutamate-pyruvate transaminase; and (5) glutamate-oxaloacetate transaminase. Glutamine synthesis is limited in enterocytes of 0- to 21-day-old pigs. Ala, alanine; Asp, aspartate; Gln, glutamine; OAA, oxaloacetate; Pyr, pyruvate

near absence of BCAA degradation in liver due to a limited BCAT activity (Harper et al. 1984), the small intestine may play an important role in regulating plasma BCAA concentrations in organisms. It is noteworthy that Kakoki et al. (2006) recently reported that infusion of leucine to the kidney of normal rats increased renal vascular resistance and that increasing extracellular concentrations of leucine can inhibit NO synthesis in endothelial cells. Interestingly, the results of recent studies indicate a positive correlation between circulating levels of leucine and insulin resistance in mice (She et al. 2007), rats (Jobgen et al. 2009; Wu et al. 2007) and humans (Chevalier et al. 2006). Thus, in view of a crucial role for nitric oxide in mediating vascular insulin sensitivity (Wu and Meininger 2009), elevation of BCAA levels may lead to endothelial dysfunction by inhibiting the synthesis of nitric oxide. Therefore, we suggest that first-pass metabolism in the gut mucosa can reduce the entry of dietary BCAA into the systemic circulation, which has both nutritional and physiological importance for maintaining cardiovascular function and whole-body homeostasis.

Methionine, phenylalanine, lysine, threonine, and histidine were traditionally considered not to be catabolized by the intestinal mucosa (Wu 1998). However, Stoll et al. (1998) reported that 50% of dietary lysine and methionine, 45% of dietary phenylalanine, and 60% of dietary threonine were extracted in first-pass by the portal drained viscera of milk protein–fed pigs, with one-third of the extracted EAA catabolized by the small intestine. Interestingly, van Goudoever et al. (2000) found that intestinal oxidation of enteral lysine contributed one-third of total body lysine oxidation in growing pigs fed a high-protein diet. More recently, Riedijk et al. (2007) reported that the piglet intestine actively degraded enterally administered methionine. However, direct evidence for oxidation of these EAA in mucosal cells is lacking.

We recently demonstrated that transamination of methionine and phenylalanine in enterocytes of postweaning pigs was negligible in the presence of physiological levels of AA (Chen et al. 2007). The enzymes responsible for initiating the limited degradation of methionine and phenylalanine may include BCAT and possibly glutamine transaminases L and K (Wu and Thompson 1989a). All of these enzymes can use methionine and phenylalanine as substrates only when BCAA and other AA are absent (Wu and Thompson 1989b; Wu et al. 1991). In support of this view, we found that addition of leucine, isoleucine and valine (2 mM each) to incubation medium completely inhibited the transamination of methionine and phenylalanine in enterocytes of preweaning (the present study) and postweaning (Chen et al. 2007) pigs, as reported for rat and chicken in skeletal muscle (Wu and Thompson 1989a). Additionally, there is no activity of phenylalanine hydroxylase in pig enterocytes (the present study). Collectively, these results suggest that BCAT is responsible for initiating the limited catabolism of methionine and phenylalanine in pig enterocytes. It is noteworthy that a quantitatively very small amount of methionine is used for the synthesis of polyamines in intestinal mucosal cells (Dekaney et al. 2008; Sugita et al. 2007; Wang 2007), as reported for other cell types (Kahana et al. 2007; Shantz and Levin 2007).

Emerging evidence shows a lack of quantitatively significant oxidation of histidine, lysine, threonine and tryptophan in enterocytes of postweaning growing pigs (Chen et al. 2007). Similar results were obtained for newborn and sow-reared preweaning pigs (the present study). Consistent with our metabolic data, enterocytes lack the key enzymes responsible for degradation of these EAA, including threonine dehydrogenase, threonine dehydratase, saccharopine dehydrogenase and phenylalanine hydroxylase (the present study). This failure was not an artifact because activities of these enzymes were readily detected in the piglet liver under the same assay conditions (Table 5).

Our findings have important implications for protein nutrition and health. First, extensive in vivo catabolism of histidine, lysine, methionine, phenylalanine, threonine or tryptophan by the small intestine (Stoll et al. 1998) may result from the action of luminal microbes in the intestinal mucosa (Blachier et al. 2007; Kuc et al. 2008; Saito et al. 2007). Thus, dietary supplementation with antibiotics can markedly enhance growth performance and protein deposition in skeletal muscle of young pigs (Deng et al. 2007). Manipulation of AA metabolism in intestinal lumenal microorganisms may provide an attractive means to improve the efficiency of utilization of dietary protein, as exemplified by the current use of prebiotics and probiotics in both animal and human nutrition (Kong et al. 2007; Lin

et al. 2008). Second, increased intestinal mucosal mass in neonates receiving dietary supplementation of gut trophic factors [e.g., glutamine, proline and polyamines (Flynn et al. 2008; Rhoads and Wu 2008; Wang et al. 2008)] will not result in enhancement of catabolism of histidine, lysine, methionine, phenylalanine, threonine and tryptophan by the gut. Rather, there is a positive relationship between intestinal mucosal mass and production of citrulline (the precursor of arginine, an EAA for young mammals (Wu et al. 2004, 2008a) in both piglets (Berkeveld et al. 2008) and infants (Rhoads et al. 2005). Because BCAA are usually abundant in plant or animal protein-based diets (Mateo et al. 2007, 2008), degradation of these AA by enterocytes via transamination will not negatively impact protein nutrition in organisms. Thus, in contrast to the previous suggestion (Stoll et al. 1998; Wu 1998), it is unlikely that a large mass of the intestinal mucosa will reduce nutritional efficiency of dietary protein in animals or humans. Third, stimulation of BCAA transamination by α-KG in enterocytes (Table 3), as reported for muscle mitochondria (Hutson et al. 1980), may help reduce excess BCAA in the gut and the blood circulation. This may provide a potentially attractive means to decrease the elevated levels of circulating BCAA and improve insulin sensitivity in obese subjects. Finally, it is noteworthy that dietary α-KG is completely catabolized in the piglet small intestine in Lamberr et al. (2006). Whether α-KG metabolism mediates the enhancement of piglet growth (Andersen et al. 2008) and contributes to leucine and gaseous signaling in the intestine (Li et al. 2009; Liao et al. 2008; Rhoads and Wu 2008) warrants further investigation.

In conclusion, BCAA are actively transaminated in enterocytes of 0- to 21-day-old pigs, with most of the resultant BCKA being released into the extracellular space. These metabolic data are consistent with a high BCAT activity but a low BCKAD activity in these cells. Intestinal BCAA catabolism may play an important role in regulating the balance of dietary AA that enter the portal vein and may have enormous nutritional and physiological significance. In contrast, the degradation of methionine and phenylalanine is negligible and that of other EAA is absent in enterocytes of healthy piglets. Knowledge about intestinal AA metabolism is crucial for understanding and improving the efficiency of utilization of dietary protein and AA in animals and humans.

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