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ORIGINAL ARTICLE

Dietary α-ketoglutarate supplementation ameliorates intestinal injury in lipopolysaccharide-challenged piglets

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Abstract Neonates are at increased risk for inflammatory bowel disease, but effective prevention and treatments are currently limited. This study was conducted with the lipopolysaccharide (LPS)-challenged piglet model to determine the effects of dietary supplementation with α-ketoglutarate (AKG) on the intestinal morphology and function. Eighteen 24-day-old pigs (weaned at 21 days of age) were assigned randomly to control, LPS, and LPS + AKG groups. The piglets in the control and LPS groups were fed a corn- and soybean meal-based diet, whereas the LPS + AKG group was fed the basal diet supplemented with 1% AKG. On days 10, 12, 14, and 16, piglets in the LPS and LPS + AKG groups received intraperitoneal administration of LPS (80 μg/kg BW), whereas piglets in the control group received the same volume of saline. On day 16, D-xylose was orally administrated to all pigs at the dose of 0.1 g/kg BW, 2 h after LPS or saline injection, and blood samples were collected 3 h thereafter. Twenty-four hours post-administration of LPS or saline, pigs were killed to obtain intestinal mucosae for analysis. Compared with the control group, LPS challenge reduced ($P < 0.05$) protein levels, the ratio of villus height to crypt depth, and the ratio of phosphorylated mTOR to total mTOR in duodenal, jejunal, and ileal mucosa. These adverse effects of LPS were attenuated ($P < 0.05$) by AKG supplementation. Moreover, AKG prevented the LPS-induced increase in intestinal HSP70 expression. Collectively, these novel results indicate that dietary supplementation with 1% AKG activates the mTOR signaling, alleviates the mucosal damage, and improves the absorptive function of the small intestine in LPS-challenged piglets. The findings not only help understand the mode of AKGs actions in the neonatal gut but also have important implications for infant nutrition under inflammatory conditions.

Keywords α-Ketoglutarate · Intestine · mTOR signaling · Piglets · Lipopolysaccharide

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Abbreviations

AKG	α-ketoglutarate
BSA	Bovine serum albumin
BW	Body weight
HSP 70	Heat shock protein 70
LPS	Lipopolysaccharide
mTOR	Mammalian target of rapamycin
PBS	Phosphate buffered saline
SEM	Standard error of the mean

Introduction

The gastrointestinal tract is important as the first defense of the body against bacteria-derived endogenous and exogenous harmful agents (Blachier et al. 2007; Eklou-Lawson et al. 2009; Wang et al. 2009b). Stresses, such as

early-weaning, infection and inflammation, result in intestinal mucosal injury and dysfunction, diarrhea, reduced growth, and economic loss (Blikslager et al. 2007; Bergen and Wu 2009; Liu et al. 2008). There is evidence that nutritional modulation (e.g. glutamine and arginine) can ameliorate damage of the small intestine in compromised neonates (Flynn et al. 2009; Han et al. 2009; Wu et al. 1996; 2009). Relatively high costs of these nutrients provide an impetus for us to identify cheaper replacements in swine production, and one of them may be α-ketoglutarate (AKG) (Hu et al. 2009; Liu et al. 2009).

AKG plays a central role in the citric acid cycle and an intermediate in the oxidation of glutamine and glutamate in the small intestine (Wu 2009). Exogenous AKG can be converted to glutamate and glutamine in many tissues (Pierzynowski and Sjodin 1998; Kristensen et al. 2002), amino acids that are major fuels for the intestinal mucosa (Elango et al. 2009; Wu 1998). Accordingly, dietary supplementation with AKG could improve intestinal morphology and function in healthy young pigs without lipopolysaccharide (LPS) challenge (Hu et al. 2009). At present, little is known about molecular mechanisms for the action of AKG on the small intestine.

Glutamine, arginine, and leucine can regulate gene expression and activate the mammalian target of rapamycin (mTOR) signaling pathway to maintain intestinal integrity and function (Haynes et al. 2009; Palii et al. 2009; Rhoads and Wu 2009). mTOR, also known as FK506 binding protein 12-rapamycin associated protein 1, is a highly conserved serine/threonine protein kinase and a master regulator of protein synthesis in cells, including enterocytes (Liao et al. 2008). In response to stimulation by certain nutrients and growth factors, mTOR phosphorylates eIF4E-binding protein-1 (4E-BP1), and ribosomal protein S6 kinase-1 (S6K1), thereby promoting the initiation of polypeptide formation. Interestingly, the metabolism of these amino acids is affected by AKG (Chen et al. 2009; Wu and Morris 1998). Thus, we hypothesized that AKG might regulate mTOR activation and mucosal function in the small intestine of LPS-challenged pigs, an excellent model for studying nutrition and metabolism of human infants (Davis et al. 1998; Frank et al. 2007; Suryawan et al. 2009).

Materials and methods

Animal care and diets

The animal use protocol for this research was approved by the Animal Care and Use Committee of Hubei Province. All pigs used in this experiment were born naturally at term (114 days of gestation). Eighteen crossbred healthy female piglets (Duroc × Landrace × Yorkshire) were reared by sows and then weaned at 21 days of age. After weaning, piglets were housed individually in stainless steel metabolic cages (1.20 × 1.10 m^2) and maintained at an ambient temperature of 22–25°C in an environmentally controlled room (He et al. 2009) without electric light between 8:00 AM and 8:00 PM. Each cage was equipped with a feeder and a nipple waterer to allow piglets free access to feed and drinking water (Tan et al. 2009b). Piglets were fed every 4 h between 8:00 AM and 8:00 PM a corn- and soybean meal-based diet (Table 1). No feed was provided to piglets at other times. This feeding strategy (more frequent provision of food at a small amount for each feeding) was adopted to prevent intestinal necrosis and improve the efficiency of utilization of dietary nutrients for piglet growth.

Experimental design

All weanling piglets had free access to the basal diet (Table 1) between days 21 and 24 of age (days 0–3 postweaning) for adapting to solid food. At 24 days of age, piglets (7.25 ± 0.23 kg body weight) were assigned randomly into three treatment groups: (1) non-challenged control (control, piglets fed the basal diet and receiving intraperitoneal administration of sterile saline); (2) LPS-challenged control (LPS group, piglets fed the basal diet and receiving intraperitoneal administration of *Escherichia coli* LPS); and (3) LPS + 1.0% AKG (AKG group, piglets fed the basal diet supplemented with 1.0% AKG and receiving intraperitoneal administration of LPS). AKG (powder) was well mixed with the basal diet. The dosage of 1% AKG was chosen because it was found in our preliminary study to be more effective in improving histological morphology and function of the pig small intestine compared with 0.5 and 2% AKG (Hu et al. 2008, 2009). LPS was dissolved in sterile saline. There were six pigs per group. In the AKG diet, 1.0% cornstarch in the basal diet was replaced by 1.0% AKG (purity ≥99.8%). All diets were isocaloric. On days 10, 12, 14, and 16 postweaning, overnight fasted piglets in the LPS group and AKG group received intraperitoneal administration of LPS (*Escherichia coli* serotype 055:B5; Sigma Chemical Inc., St. Louis, MO, USA) at the dose of 80 μg/kg BW, whereas piglets in the control group received intraperitoneal administration of the same volume of sterile saline. During days 0–10 postweaning (pre-challenge), all the piglets had free access to feed and drinking water. To exclude a possible effect of LPS-induced reduction in food intake on the piglet intestine, the control and AKG piglets were pair-fed during days 10–16 postweaning (post-challenge with LPS) the same amount of feed per kg body weight as LPS piglets. On day 16, D-xylose was orally administrated to piglets at the dose of 0.1 g/kg BW (infused with 10% D-xylose at 1 mL/kg BW) 2 h after LPS or saline

Table 1 Composition and nutrient contents of the basal diet (air-dry basis)

Items	Content
Ingredients (%)	
Corn	57.88
Soybean meal	20.98
Wheat Middling	3.00
Fish meal	5.00
Dried whey	3.00
Soy protein concentrate	2.50
CaHPO[d]	1.25
Premix[a]	1.00
Limestone	0.69
Soy oil	2.50
Acidifier	0.30
NaCl	0.30
Mold inhibitor	0.10
Choline chloride	0.20
L-Lysine·HCl (78.8% lysine)	0.25
DL-Methionine (99% methionine)	0.05
Cornstarch[b]	1.00
Nutrients composition	
Digestible energy[c] (MJ/kg)	14.22
Crude protein (%)[d]	20.90
Total lysine (%)[c]	1.15
Total methionine (%)[c]	0.30
Total methionine + cystine (%)[c]	0.65
Total threonine (%)[c]	0.74
Total tryptophan (%)[c]	0.21
Calcium (%)[d]	0.70
Total phosphorus (%)[d]	0.60
Available phosphorus (%)[c]	0.32

[a] Premix provided the following amounts of vitamins and trace minerals per kilogram of the complete diet: Fe 100 mg ($FeSO_4 \cdot H_2O$), Cu 150 mg ($CuSO_4 \cdot 5H_2O$), Mn 40 mg ($MnSO_4 \cdot 5H_2O$), Zn 100 mg ($ZnSO_4 \cdot 7H_2O$), I 0.5 mg (KI), Se 0.3 mg ($Na_2SeO_3 \cdot 5H_2O$), retinol acetate 10 800 IU, cholecalciferol 4000 IU, DL-α-tocopheryl acetate 40 IU, vitamin K_3 4 mg, thiamin 6 mg, riboflavin 12 mg, pyridoxine 6 mg, vitamin B_{12} 0.05 mg, biotin 0.2 mg, folic acid 2 mg, niacin 50 mg, D-calcium pantothenate 25 mg

[b] In the AKG diet, 1.0% cornstarch was replaced by 1.0% AKG. All diets were isocaloric

[c] Calculated value

[d] Analyzed value

administration. Absorption of D-xylose into plasma is a useful marker of in vivo intestinal integrity in pigs (Mansoori et al. 2009). Twenty-four hours post-injection of LPS (on day 17), all piglets were killed under anesthesia with an intravenous injection of pentobarbital sodium (50 mg/kg BW) for the evaluation of small intestinal morphology and the biochemical analysis of the intestinal mucosa (Deng et al. 2009).

Blood sample collection

On day 16 of the trial, 1 h after infusion of D-xylose (3 h after LPS or saline administration), blood samples were collected from anterior vena cava into heparinized vacuum tubes (Becton–Dickinson Vacutainer System, Franklin Lake, NJ, USA), as we described (Kong et al. 2009). Blood samples were centrifuged at 3,000×*g* for 10 min at 4°C to obtain plasma (Tan et al. 2009a). Plasma was stored at −80°C until analysis.

Intestinal sample collection

The pig abdomen was opened immediately from sternum to pubis, and the whole gastrointestinal tract was immediately exposed (Li et al. 2009b). The small intestine was dissected free of the mesentery and placed on a chilled stainless steel tray. The 5-, 10- and 10-cm segments were cut at distal duodenum, mid-jejunum, and mid-ileum, respectively (Wang et al. 2008). The 5-cm intestinal segments were processed, embedded, and stained according to the procedures of Luna (1968). The segments were flushed gently with ice-cold phosphate-buffered saline (PBS, pH 7.4) and then placed in 10% fresh, chilled formalin solution for histological measurements. The 10-cm intestinal segments were opened longitudinally and the contents were flushed with ice-cold PBS (Wang et al. 2008). Mucosa was collected by scraping using a sterile glass microscope slide (Wang et al. 2009a), rapidly frozen in liquid nitrogen, and stored at −80°C until analysis. All samples were collected within 20 min after killing.

Intestinal morphology

Intestinal segments for the morphometric study were prepared according to Nabuurs et al. (1993). After a 24-h fixation, the tissue samples were removed from formalin solution and then dehydrated using increasing concentrations of ethanol (70 to 100%) and chloroform. Subsequently, the samples were embedded in paraffin, and then placed in a refrigerator to make the paraffin sufficiently rigid. The segments were sliced into 4-μm crosscut sections using a microtome (American Optical Co., Scientific Instrument Div., Buffalo, NY, USA), and stained with hematoxylin and eosin. Morphometric measurements were performed with a light microscope with a computer-assisted morphometric system (BioScan Optimetric, BioScan Inc., Edmonds, WA, USA). Measurements were taken in ten, well-oriented villi and crypts from each intestinal tissue section of each piglet (Wu et al. 1996). The villus height (the distance from the villus tip to crypt mouth) and the associated crypt depth (the distance from the crypt mouth to base) were measured.

Determination of D-xylose in plasma

D-xylose in plasma was determined as described by Mansoori et al. (2009). Briefly, 50 μL of the collected plasma was added to 5 mL of the phloroglucinol color reagent solution (Sigma Chemical Inc., St. Louis, MO, USA) and heated at 100°C for 4 min. The samples were allowed to cool to room temperature in a water bath. Xylose standard solutions were prepared by dissolving D-xylose in saturated benzoic acid (prepared in deionized water) to obtain 0, 0.7, 1.3, 2.6 mmol/L. They were added to the color reagent solution alongside the samples. The absorbance of samples and standard solutions at 554 nm were measured using a spectrophotometer (Model 6100, Jenway Ltd., Felsted, Dunmow, CM6 3LB, Essex, England, UK). The standard solution of 0 mmol/L D-xylose was considered as blank (Eberts et al. 1979).

Analysis of amino acids in the jejunal mucosa

Amino acids in jejunal mucosa were analyzed by HPLC (Wu and Meininger 2008; Li et al. 2009b). Briefly, the frozen mucosa sample (~100 mg) was homogenized with a tissue homogenizer in 1 mL of 1.5 mol/L ice-cold perchloric acid, followed by addition of 0.5 mL of 2 mol/L potassium carbonate. Tubes were vortexed and centrifuged at 3,000×*g* for 5 min to obtain the supernatant fluid for analysis. The chromatographic system consisted of Waters Breeze HPLC system (Waters Corporation, Milford, MA, USA), including 1525 binary HPLC pumps, 2487 Dual-λ Absorbance Detector, 717 plus autosampler, and Breeze system software); Waters XTerra MS C18 column (5 μm × 4.6 mm × 150 mm); mobile phase A (0.1 mol/L sodium acetate, pH 7.2); and mobile phase B (100% methanol).

Measurement of mucosal protein

Frozen intestinal mucosal samples (~0.1 g) were powdered under liquid nitrogen using a mortar and pestle and then homogenized with a tissue homogenizer in 1 mL of ice-cold PBS–EDTA buffer (0.05 mol/L Na_3PO_4, 2.0 mol/L NaCl, 2 mmol/L EDTA, pH 7.4). Protein concentration of small-intestinal mucosal homogenates was measured as described by Lowry et al. (1951) using reagents from Bio-Rad Laboratories (Hercules, CA, USA) and bovine serum albumin as the standard.

Protein immunoblot analysis

Analyses of mTOR and HPS70 proteins were performed by western blotting as described by Yao et al. (2008). Briefly, frozen intestinal mucosal samples were powdered under liquid nitrogen using a mortar and pestle. The powdered samples were homogenized in seven volumes of lysis buffer (20 mmol/L HEPES, pH 7.4, 2 mmol/L EGTA, 50 mmol/L NaF, 100 mmol/L KCl, 0.2 mmol/L EDTA, 50 mmol/L β-glycerophosphate, 1 mmol/L dithiothreitol, 0.1 mmol/L PMSF, 1 mmol/L benzamidine, and 0.5 mmol/L sodium vanadate) with a Polytron homogenizer. The homogenates were centrifuged at 12,000×*g* for 15 min at 4°C. The supernatant was aliquoted into microcentrifuge tubes, to which 2× SDS sample buffer (2 mL of 0.5 mol/L Tris, pH 6.8, 2 mL glycerol, 2 mL of 10% SDS, 0.2 mL of β-mercaptoethanol, 0.4 mL of a 4% solution of bromphenol blue, and 1.4 mL of water) was added in a 1:1 ratio. The samples were boiled for 5 min and cooled on ice before being used for western blot analysis. The proteins (150 μg/sample for mTOR; 50 μg/sample for HSP70 and β-actin) were separated by electrophoresis on a 6% (for mTOR) or 10% (for HSP70 and β-actin) polyacrylamide gel. Proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. Non-fat dry milk in TBS/T buffer (137 mmol/L NaCl, 20 mmol/L Tris–HCl, pH 7.4, 0.1% Tween-20) was used to block filters for at least 1 h at room temperature. Membranes were incubated with primary antibodies: phosphorylated (Ser^{2448}) and total mTOR (rabbit monoclonal antibodies from Cell Signaling Technology, Inc., Danvers, MA, USA, dilution 1:1,000 in primary antibody dilution buffer), HSP70 (mouse monoclonal antibody from Stressgen Bioreagents, Columbia, Canada, dilution 1:2,000 in primary antibody dilution buffer) or β-actin (mouse monoclonal antibody from Sigma–Aldrich Inc., St. Louis, MO, USA; dilution 1:5,000 in primary antibody dilution buffer). The primary antibody dilution buffer was 1× TBS, 0.1% Tween-20 with 5% BSA. The membranes were washed three times with TBS-T (1× Tris-buffered saline including 0.1% Tween 20) and incubated for 1 h at room temperature with anti-rabbit (mouse) immunoglobulin G horseradish peroxidase conjugated secondary antibody (purchased from Beijing ZhongShan Golden Bridge Biological Technology Co., Ltd, China; dissolved in 5% non-fat dry milk in TBS-Tween-20 buffer in 1:5,000 dilution). Incubation of primary antibodies was followed by three washes with TBS-T buffer for 10 min, and incubation of the secondary antibodies was followed by five washes for 8 min. Blots were developed using an enhanced chemiluminescence western blotting kit (ECL-plus, Amersham Biosciences, Sweden), visualized using a Gene Genome bioimaging system, and analyzed using GeneTools software (Syngene, Frederick, MD, USA).

Statistical analysis

Results are expressed as means with pooled SEM and analyzed by one-way analysis of variance (ANOVA). Log

transformation of variables was performed when variance of data was not homogenous among treatment groups (Fu et al. 2010). Differences among treatment means were determined by the Duncan's multiple range test. All statistical analyses were performed using SPSS 13.0 software (SPSS Inc. Chicago, IL, USA). Probability values ≤ 0.05 were taken to indicate significance.

Results

Feed intake and growth of piglets

Between days 24 and 34 of age (days 0 and 10 postweaning; prior to LPS challenge), average daily feed intakes of piglets were 755, 751, and 753 g/day (pooled SEM = 45 g/day), respectively, for the control, LPS, and LPS + AKG groups ($P = 0.996$), whereas AKG intake by the AKG-supplemented piglets was 0.69 ± 0.04 g/kg body weight per day. During this pre-challenge period, average daily weight gains of piglets were 367, 373, and 428 g/day (pooled SEM = 52 g/day), respectively, for the control, LPS, and LPS + AKG groups ($P = 0.446$). Between days 34 and 40 of age (days 10 and 16 postweaning; LPS challenge period), average daily feed intakes of piglets or daily weight gains of piglets did not differ among the control, LPS, and LPS + AKG groups (Table 2), and AKG intake by the AKG-supplemented piglets was 0.42 ± 0.06 g/kg body weight per day.

Intestinal morphology

Data for small intestinal morphology are summarized in Table 3. Compared with the control group, LPS piglets exhibited an increase ($P < 0.05$) in crypt depth in the duodenum, jejunum, and ileum, and a decrease ($P < 0.05$) in the ratio of villus height to crypt depth in the duodenum, jejunum, and ileum. In comparison with the LPS piglets, dietary supplementation of 1% AKG decreased ($P < 0.05$) crypt depth in jejunum and ileum, while increasing ($P < 0.05$) the ratio of villus height to crypt depth in the duodenum, jejunum, and ileum.

Concentrations of plasma D-xylose

Concentrations of D-xylose in plasma did not differ ($P > 0.05$) between control and LPS-treated piglets. Dietary supplementation with 1% AKG increased ($P < 0.05$) plasma D-xylose content by 9.7% compared to LPS piglets (Table 2).

Heat shock protein 70 (HSP70) expression

LPS administration resulted in an increase ($P < 0.05$) in HSP70 expression in jejunal and ileal mucosae, compared with control piglets. Regarding LPS piglets, dietary supplementation with 1% AKG decreased ($P < 0.05$) HSP70 expression in jejunum (Fig. 1).

Concentrations of amino acids and protein in jejunal mucosa

Data on concentrations of amino acids and protein in intestinal mucosa are shown in Table 4. The LPS-treated piglets exhibited a 32% greater ($P < 0.05$) level of ornithine in the jejunal mucosa compared with the control group. In addition, LPS treatment decreased ($P < 0.05$) concentrations of protein in the mucosa of duodenum (−19%), jejunum (−5.6%), and ileum (−9.3%) compared with the control piglets. Concentrations of citrulline in jejunal mucosa was 95% greater ($P < 0.05$) in AKG-supplemented piglets than in LPS piglets. In the LPS piglets, AKG supplementation decreased ($P < 0.05$) concentrations of ornithine (−25%), while increasing concentrations of glutamate (+28%) and protein (+11%) in jejunal mucosa. Dietary supplementation with 1% AKG did not affect ($P > 0.05$) jejunal mucosal concentrations of arginine, aspartate, methionine, asparagine, lysine, and alanine (Table 4), as well as isoleucine, leucine, phenylalanine, threonine, tyrosine, and valine (data not shown).

Table 2 Effects of dietary AKG on D-xylose concentrations in plasma, feed intake, and growth performance of piglets after LPS challenge

Variables	Control	LPS	LPS + AKG	Pooled SEM	P-value
D-xylose in plasma (mmol/L)	2.22[ab]	2.17[a]	2.38[b]	0.08	0.046
Body weight at day 16 postweaning (kg)	14.5	14.1	15.1	1.34	0.756
Average daily feed intake between days 10 and 16 postweaning (g/day)	579	581	578	55	0.974
Average daily weight gain between days 10 and 16 postweaning (g/day)	320	275	316	47	0.280

Control (non-challenged control) = piglets fed the basal diet and injected with sterile saline; LPS (LPS challenged control) = piglets fed the basal diet and challenged with *Escherichia coli* LPS; LPS + AKG (LPS + 1.0% AKG) = piglets fed the basal diet supplemented with 1.0% AKG and challenged with LPS

Values within a row with different letters differ ($P < 0.05$)

SEM standard error of the mean $n = 6$

Table 3 Effects of AKG on the intestinal mucosal morphology of piglets after LPS challenge

Items	Control	LPS	LPS + AKG	Pooled SEM	*P*-value
Villus height (μm)					
Duodenum	290^{ab}	268^{a}	327^{b}	25	0.038
Jejunum	336	318	327	16	0.541
Ileum	388^{b}	329^{a}	333^{a}	12	0.026
Crypt depth (μm)					
Duodenum	61^{a}	83^{b}	73^{ab}	6.7	0.013
Jejunum	74^{a}	107^{b}	59^{a}	7.8	0.013
Ileum	68^{a}	81^{b}	65^{a}	5.7	0.022
Villus height/crypt depth					
Duodenum	4.91^{b}	3.43^{a}	4.62^{b}	0.57	0.039
Jejunum	4.59^{b}	2.96^{a}	6.17^{c}	0.54	0.001
Ileum	5.57^{b}	4.00^{a}	4.96^{b}	0.49	0.016

Control (non-challenged control) = piglets fed the basal diet and injected with sterile saline, LPS (LPS challenged control) = piglets fed the basal diet and challenged with *Escherichia coli* LPS, LPS + AKG (LPS + 1.0% AKG) = piglets fed the basal diet supplemented with 1.0% AKG and challenged with LPS

Values within a row with different letters differ ($P < 0.05$)

SEM standard error of the mean $n = 6$

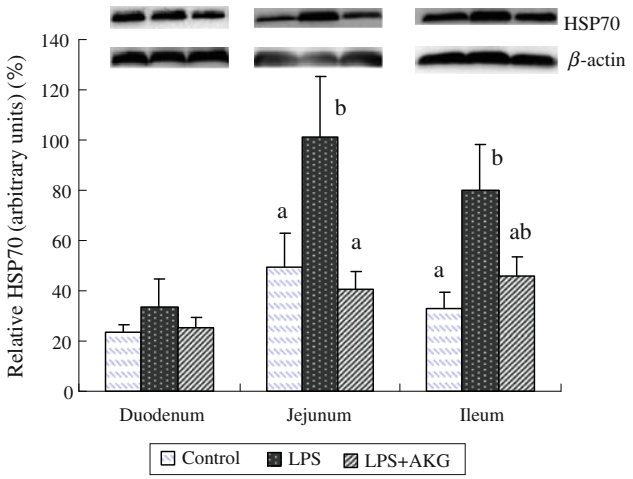

Fig. 1 Relative levels of heat shock protein 70 (HSP70) expressed in small intestinal mucosa of piglets. Mucosal extracts (50 μg protein/sample) were separated by 10% SDS-polyacrylamide gel electrophoresis for determination of HSP70 and β-actin. Values for relative HSP70 were normalized for β-actin. Data are means ± SEM, $n = 6$. Control (non-challenged control) = piglets fed the bsasl diet and injected with sterile saline, LPS (LPS challenged control) = piglets fed the same control diet and challenged with *Escherichia coli* LPS, LPS + AKG (LPS + 1.0% AKG) = piglets fed the basal diet supplemented with 1.0% AKG and challenged with LPS. $^{a,\ b}$Within same intestinal segment, means with different superscripts differ ($P < 0.05$)

Phosphorylation levels for mTOR

Compared with the control group, the ratio of phosphorylated mTOR to mTOR (p-mTOR/mTOR) in duodenal, jejunal, and ileal mucosa of LPS piglets was decreased ($P < 0.05$) by 22, 19, and 23%, respectively. Dietary supplementation with AKG increased ($P < 0.05$) the p-mTOR/mTOR ratio by 47, 47, and 26% ($P < 0.05$), respectively, in comparison with the LPS group (Fig. 2).

Table 4 Effects of dietary AKG supplementation on concentrations of amino acids and protein in the intestinal mucosa of piglets after LPS challenge

Items	Control	LPS	LPS + AKG	Pooled SEM	*P*-value
Amino acids (μmol/L)					
Citrulline	83^{ab}	63^{a}	124^{b}	30	0.050
Ornithine	329^{a}	433^{b}	325^{a}	45	0.031
Glutamate	1,404^{a}	1,365^{a}	1,744^{b}	126	0.016
Arginine	359	362	379	41	0.867
Aspartate	545	474	590	65	0.227
Methionine	109	108	107	14	0.99
Asparagine	311	327	302	41	0.822
Lysine	341	353	339	29	0.86
Alanine	885	1,009	882	79	0.216
Protein (%)					
Duodenum	11.6^{b}	9.38^{a}	10.5^{ab}	0.98	0.049
Jejunum	10.8^{b}	10.0^{a}	11.2^{b}	0.30	0.003
Ileum	8.64^{b}	7.83^{a}	8.16^{ab}	0.32	0.046

Control (non-challenged control) = piglets fed the basal diet and injected with sterile saline, LPS (LPS challenged control) = piglets fed the basal diet and challenged with *Escherichia coli* LPS, LPS + AKG (LPS + 1.0% AKG) = piglets fed the basal diet supplemented with 1.0% AKG and challenged with LPS

Values within a row with different letters differ ($P < 0.05$)

SEM standard error of the mean $n = 6$

Discussion

Weaning of mammals involves complex events, including environmental and dietary stresses that interfere with gut development and adaptation (Lalles et al. 2007). Weaning is one of the most critical developmental stages of the digestive tract when food is changed from maternal milk to a solid diet. This is a period of starvation associated with the absence of the dam, impairment of energy status, and thermoregulation, as shown by behavioral and biochemical changes. Nutrition and gastrointestinal function play a significant role in the survival, adaptation, and growth of the offspring during the transition from suckling to weaning (Le Floc′h and Seve 2000). The process of weaning is divided into the acute phase and adaptive phase. During the first phase, piglets learn how to eat and there is a reduction in feed intake. There is a significant reduction in protein and DNA mass as well as villus height in the small intestine. Increase in digestive enzyme capacity and secretion appears in the adaptive phase of weaning.

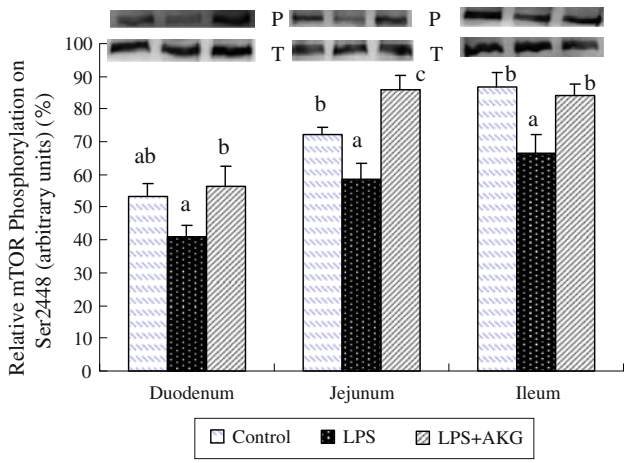

Fig. 2 The phosphorylation state of mTOR in the small intestine mucosa of piglets. Mucosal extracts (150 μg protein/sample) were separated by 6% SDS-polyacrylamide gel electrophoresis for determination of phosphorylation of mTOR (P) at Ser^{2448} and total mTOR (T). Values for phosphorylated mTOR were normalized for total mTOR. Data are means ± SEM, $n = 6$. Control (non-challenged control) = piglets fed the basal diet and injected with sterile saline, LPS (LPS challenged control) = piglets fed the basal diet and challenged with *Escherichia coli* LPS, LPS + AKG (LPS + 1.0% AKG) = piglets fed the basal diet supplemented with 1.0% AKG and challenged with LPS. [a, b, c] Within same intestinal segment,means with different superscripts differ ($P < 0.05$)

AKG is a precursor of glutamine, which is of physiological and nutritional importance for neonates, particularly under stressful or inflammatory conditions (Baker 2009; Wu 2009). Additionally, as an intermediate in the citric acid cycle and an intermediate in the oxidation of glutamate and glutamine, AKG contributes substantially to ATP homeostasis and mucosal integrity in the small intestine (Lambert et al. 2006). Furthermore, as a scavenger of ammonia in the pathway of glutamate synthesis by glutamate dehydrogenase, AKG plays an important role in the endogenous provision of arginine and proline (Hu et al. 2009; Phang et al. 2008; Wu and Morris 1998), which are essential amino acids for young pigs (Kim and Wu 2004, 2009; Wu et al. 2007). These amino acids can also regulate expression of genes in diverse cell types that are crucial for cell metabolism, proliferation, and function (Jobgen et al. 2009; Kohli et al. 2004; Li et al. 2009a; Odenlund et al. 2009). Accordingly, dietary supplementation with 1% AKG increased concentration of protein in the jejunal mucosa (Table 4) and relieved growth depression in weaned piglets challenged by LPS (Liu et al. 2009).

Although AKG has been reported to be extensively metabolized by the pig intestine (Lambert et al. 2002, 2006), little is known about its nutritional significance. A novel and significant observation of the present study was an increase in citrulline concentration in the intestinal mucosa of AKG-supplemented piglets (Table 4). This result indicates a net increase in the formation of this amino acid in the gut of AKG-supplemented pigs. Citrulline is the immediate precursor for the endogenous synthesis of arginine, which is an essential amino acid for young mammals (Wu et al. 2009) and protects intestinal cells from LPS-induced injury (Tan et al. 2009c). Thus, our current finding provided a new basis to explain the beneficial effects of AKG on the intestine. It is possible that AKG is first converted into glutamate, which is subsequently utilized for citrulline synthesis via pyrroline-5-carboxylate synthase (Wu and Morris 1998). Indeed, 64% of the AKG disappeared from the small-intestinal lumen within 1 h (Lambert et al. 2006), suggesting rapid utilization of AKG by the mucosa. Alternatively, glutamine, formed from AKG in extraintestinal tissues is a substrate for intestinal citrulline generation (Wu et al. 1994b), because glutamine in arterial blood is actively taken up by the pig small intestine (Wu et al. 1994a). The contribution of AKG to whole-body glutamine synthesis can be quantitatively important because 10% of the intraduodenally infused AKG is absorbed into the portal circulation (Kristensen et al. 2002; Lambert et al. 2002, 2006).

To our knowledge, this is the first study to evaluate whether dietary AKG supplementation could attenuate gut injury in weaned piglets chronically challenged with *Escherichia coli* LPS. Previous studies have demonstrated that LPS administration results in many of the metabolic derangements of sepsis (Webel et al. 1997; Orellana et al. 2002; Fink and Heard 1990). These include elevations in temperature and heart rate, and symptoms of acute bacterial infection such as anorexia and hypersomnia (Liu et al. 2008). LPS treatment causes epithelial lifting at the tips of villi and reduces the villus height in the mucosa of duodenum, jejunum, and ileum, while increasing crypt depth in the mucosa of ileum (Liu et al. 2008; Lobo et al. 2003; Mercer et al. 1996). A high value of the villus:crypt ratio is a useful indicator of a high capacity for digestion and absorption (Hampson 1986; Montagne et al. 2003). In this regard, it is noteworthy that AKG supplementation resulted in (1) increased villus height; (2) reduced crypt depth; and (3) an increased ratio of villus height to crypt depth (Table 3). These findings support the notion that AKG beneficially alleviates the LPS-induced damage of the intestinal structure. Similarly, dietary supplementation with 1% AKG increased the ratio of villus height to crypt depth in the gut of healthy pigs (Hu 2008). Consistent with this view, dietary supplementation with 1% AKG to LPS-challenged piglets increased the entry of orally administered D-xylose into the blood circulation (Table 2), which is a simple, specific, and sensitive measure of intestinal absorption ability (Rolston and Mathan, 1989). Taken together, these data indicate that AKG improved the ability of the small intestine to absorb nutrients under inflammatory conditions.

Oral administration of glutamine, a product of AKG metabolism, can protect intestinal cells from LPS-induced damage (Haynes et al. 2009). However, AKG may also act through other possible mechanisms. One of these putative mechanisms may involve expression of HSP70 (Li et al. 2004). In response to stress, HSP70 is expressed at elevated levels to promote refolding and prevent aggregation of partially-denatured proteins, thereby protecting cells from injury (Beckmann et al. 1990). This is an adaptive mechanism for allowing organisms to survive heat shock stress. Meanwhile, a high concentration of HSP70 is indicative of oxidative stress in intestinal cells (Sepponen and Poso 2006). Thus, LPS treatment greatly increased HSP70 expression in the pig small intestine (Fig. 1). Notably, dietary supplementation with AKG reduced the elevated concentrations of the HSP70 protein in the jejunal and ileal mucosae of LPS-challenged piglets (Fig. 1), indicating an important role of AKA in ameliorating oxidative stress.

It is possible that AKG may affect expression of key proteins involved in anti-inflammaory responses via the mTOR signaling, a major mechanism for the regulation of protein synthesis in cells (Frank et al. 2006, 2007), thus supporting intestinal growth under septic conditions (Liu et al. 2009). In addition, mTOR plays a crucial role in the control of cell growth and proliferation (Davis et al. 2002; Karinch et al. 1993; Sarbassov et al. 2005). Compelling evidence shows that the mTOR signaling pathway includes the 70-kDa ribosomal protein S6 kinase-1 (S6K1) and eukaryotic initiation factor (eIF) 4E-binding protein-1 (4EBP1) (Liao et al. 2008). We found that dietary supplementation with AKG increased the phosphorylated level of mTOR (the active state of mTOR) in the intestinal mucosa of piglets (Fig. 2). This effect of AKG is expected to increase intestinal protein synthesis in piglets receiving administration of LPS. Accordingly, mucosal protein concentration was greater in AKG-supplemented piglets than in non-supplemented piglets when they were challenged with LPS (Table 4). Future studies are warranted to elucidate the mechanism for the effect of AKG on mTOR signaling. At present, it is unknown whether certain nutrients directly or indirectly phosphorylate mTOR (Wu 2009). Interestingly, findings from recent in vitro studies indicate that these nutrients may stimulate the binding of Rag GTPases to raptor (a protein in the mTOR complex 1), thereby resulting in mTOR activation (Sancak et al. 2008). Future studies are warranted to test this hypothesis using the in vivo piglet model.

In conclusion, dietary supplementation with AKG alleviates intestinal injury and improves intestinal absorption in LPS-challenged piglets. The effects of AKG are associated with reduced oxidative stress (indicated by decreased expression of the HSP70 protein) and increased activation of the mTOR signaling. These findings not only aid in understanding the mode of AKGs actions in the gut of young pigs but also have important implications for improving infant nutrition and management under inflammatory conditions.

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