Effects of α-ketoglutarate on energy status in the intestinal mucosa of weaned piglets chronically challenged with lipopolysaccharide

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(Received 20 July 2010 – Revised 7 January 2011 – Accepted 10 January 2011 – First published online 23 February 2011)

Abstract

The present study determined whether α-ketoglutarate (AKG) might affect the expression of AMP-activated protein kinase (AMPK) and energy status in the intestinal mucosa of piglets challenged with Escherichia coli lipopolysaccharide (LPS). A total of eighteen piglets (weaned at 21 d of age) were allocated to one of three treatments: (1) non-challenged (control); (2) LPS-challenged (LPS); (3) LPS + 1% AKG (LPS + AKG). Piglets in the control and LPS groups were fed a maize- and soyabean meal-based diet, and the LPS + AKG group was fed the basal diet supplemented with 1% AKG. On days 10, 12, 14 and 16 of the trial, piglets in the LPS and LPS + AKG groups were challenged with LPS (80 μg/kg body weight), whereas piglets in the control group received the same volume of sterile saline. Pigs were euthanised 24 h after the last administration of LPS or saline to obtain intestinal mucosae for biochemical analysis. Compared with the control group, LPS administration decreased (P<0·05) the oxidation of AKG, oleic acid, glutamine and glucose in enterocytes, decreased concentrations of ATP in the duodenal and jejunal mucosae and decreased adenylate energy charge (AMP:ATP ratio) in the jejunal and ileal mucosae. Additionally, LPS treatment reduced (P<0·05) mucosal concentrations of phosphorylated AMPK in the jejunum and ileum as well as acetyl-CoA carboxylase in all segments of the small intestine. The adverse effects of LPS were reversed by AKG. Collectively, these results indicate that dietary supplementation with 1% AKG beneficially modulates the AMPK signalling pathway to improve energy status in the small intestine of LPS-challenged piglets.

Key words: α-Ketoglutarate; Intestinal mucosa; AMP-activated protein kinase signalling; Piglets; Lipopolysaccharide

α-Ketoglutarate (AKG) is an intermediate in the citric acid cycle as well as a precursor of glutamate and glutamine(2,3), therefore potentially playing an important role in intestinal energy metabolism(2,3). Emerging evidence shows that, similar to glutamine(4), AKG can regulate gene expression and the mammalian target of rapamycin signalling pathway in the pig intestine(5). There is also a suggestion that AKG may have a sparing effect on glutamate and aspartate in cells by serving as a fuel source(6,7), and these amino acids are important for arginine metabolism in young pigs(8). Consistent with these previous reports, we have recently demonstrated that dietary supplementation with 1% AKG improved small-intestinal histological morphology and absorptive function in weaning piglets challenged with lipopolysaccharide (LPS)(5). However, little is known about the molecular mechanisms responsible for the action of AKG on the small intestine. In the present study, we focused on small-intestinal energy status, because the gut has a high requirement for ATP to support intestinal integrity, function (including nutrient digestion and absorption) and health(1,3).

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase consisting of three subunits (α, β and γ) and widely exists in eukaryotic cells(9). AMPK is activated by phosphorylation of the α-subunit at threonine 172(10) and serves as a sensor of cellular energy status(11). AMPK phosphorylates and inactivates one of its downstream target enzymes, acetyl-CoA carboxylase (ACC)(12), which converts acetyl-CoA into malonyl-CoA (an inhibitor of fatty acid oxidation and a substrate for fatty acid synthesis). The present investigation tested the hypothesis that AKG could affect the expression

Abbreviations: ACC, acetyl-CoA carboxylase; AEC, adenylate energy charge; AMPK, AMP-activated protein kinase; AKG, α-ketoglutarate; LPS, lipopolysaccharide; TBS, Tris-buffered saline; TBS-T, TBS/0·1% Tween 20.

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of total AMPK and ACC proteins, phosphorylated levels of AMPK and ACC, substrate (fatty acid, glucose and glutamine) oxidation, as well as mucosal concentrations of ATP, ADP and AMP. The study presented here is a continuation of our previously published study(5).

Materials and methods

Experimental design

The animal protocol for the present study was approved by the Animal Care and Use Committee of Hubei Province. All piglets used in this experiment were born naturally at term (114 d of gestation). A total of eighteen crossbred (Duroc × Large White × Landrace, 7-25 (sd 0-23) kg) female pigs weaned at 21 (sd 1) d of age from two litters were assigned by weight and litter to one of three treatment groups: (1) non-challenged (control group), piglets fed the basal diet and receiving an intraperitoneal administration of sterile saline); (2) LPS-challenged control (LPS group, piglets fed the basal diet and receiving an intraperitoneal administration of Escherichia coli LPS); (3) LPS + 1·0 % AKG (LPS + AKG group, piglets fed the basal diet supplemented with 1·0 % AKG and receiving an intraperitoneal administration of LPS). After removal from sows, piglets were housed individually in 1·20 × 1·10 m² stainless-steel kennels and maintained in an environmentally controlled room (22–25°C)(5,13). During the experimental period, pigs had free access to drinking-water and a maize- and soyabean meal-based diet(5) to meet the experimental period, pigs had free access to drinking-water and function in LPS-challenged pigs(5).

Each of the three treatment groups had six pigs. LPS was dissolved in sterile saline. In the AKG diet, 1·0 % maize starch in the basal diet was replaced by 1·0 % AKG (purity ≥ 99·8 %), and AKG (powder) was well mixed with the basal diet. All diets were isoenergetic. The dosage of 1 % AKG was chosen, because it could improve intestinal morphology and function in LPS-challenged pigs(5).

The first 3 d (days 0–3 post-weaning) was an adjustment period for weanling pigs to adapt to a solid food, and sample collection was performed in the following 16 d. On days 10, 12, 14 and 16 of the trial, overnight-fasted piglets in the LPS and LPS + AKG groups were administered intraperitoneally with LPS at 80 μg/kg body weight, whereas pigs in the control group were injected intraperitoneally with the same volume of the vehicle (sterile saline). LPS (Escherichia coli serotype O55:B5; Sigma Chemical, Inc., St Louis, MO, USA) was dissolved in a sterile physiological solution (500 μg LPS/l saline). During days 0–10 of the trial (pre-challenge), all pigs were allowed free access to feed and drinking-water. In order to exclude the possible effects of LPS-induced food intake reduction on gastrointestinal characteristics of the pigs, during days 10–16 of the trial (post-challenge), the control and LPS + AKG piglets were fed the same amount of food/kg body weight as LPS piglets. On day 17 (24 h after the last LPS challenge or saline administration), all pigs were euthanised under anaesthesia with an intravenous injection of sodium pentobarbital (50 mg/kg body weight)(5).

Intestinal sample collection

The pig abdomen was opened immediately, and the whole gastrointestinal tract was immediately exposed(5). The small intestine was dissected free of the mesentery and sampled on a chilled stainless-steel tray. The 10 cm segments were cut at every distal duodenum, mid-jejunum and mid-ileum, respectively. The intestinal segments were opened longitudinally, and the contents were flushed with ice-cold PBS. Mucosal samples were rapidly collected by scraping using a sterile glass microscope slide, rapidly frozen in liquid N₂, and then stored at −80°C until analysis. All samples were collected within 10 min after the pigs were killed.

Determination of substrate oxidation in enterocytes

Jejunal enterocytes were isolated from the control, LPS-treated and LPS + AKG-treated pigs, using Ca-free Krebs bicarbonate buffer(14). The viability of enterocytes, as assessed by trypan blue exclusion, did not differ among the treatment groups of

| Table 1. Composition and nutrient contents of the basal diet (air-dry basis) |
|-------------------------------|------------------|
| Items                        | Content          |
| Maize                        | 57-88            |
| Soyabean meal                | 20-98            |
| Wheat middlings              | 3-00             |
| Fishmeal                     | 5-00             |
| Dried whey                   | 3-00             |
| Soya protein concentrate     | 2-50             |
| CaHPO₄                        | 1-25             |
| Premix*                      | 1-00             |
| Limestone                    | 0-69             |
| Soya oil                     | 2-50             |
| Acidifier                    | 0-30             |
| NaCl                         | 0-30             |
| Mould inhibitor              | 0-10             |
| Choline chloride             | 0-20             |
| L-Lysine-HCl (78·8 % lysine)  | 0-25             |
| ß-Methionine (99 % methionine)| 0-05             |
| Maize starch†                | 1-00             |

* Premix provided the following amounts of vitamins and trace minerals/kg of the complete diet: Fe, 100 mg (FeSO₄·H₂O); Cu, 150 mg (CuSO₄·5H₂O); Mn, 40 mg (MnSO₄·2H₂O); Zn, 100 mg (ZnSO₄·7H₂O); I, 0-5 mg (KI); Se, 0-3 mg (Na₂SeO₃·5H₂O); retinol acetate, 10·800 IU (3·66 mg); cholecalciferol, 4000 IU (0·10 mg); ß-α-tocopheryl acetate, 40 IU (36·4 mg); vitamin K₃, 4 mg; thiamin, 6 mg; riboflavin, 12 mg; pyridoxine, 6 mg; vitmain B₁₂, 0·05 mg; biotin, 0·2 mg; folic acid, 2 mg; niacin, 50 mg; calcium pantothenate, 25 mg.
† In the ß-ketoglutarate (AKG) diet, 1-0 % maize starch was replaced by 1·0 % AKG. All diets were isoenergetic.
‡ Calculated value.
§ Analysed value.
pigs (92–94%). Cells were incubated at 37°C for 30 min in a complete oxygenated (95% O₂/5% CO₂) medium (pH 7.4) containing 5 mM-d-glucose, 0.5 mM-oleic acid, 2 mM-L-glutamine and other amino acids at physiological levels found in the pig plasma. The medium contained d-[U-14C]glucose, [1-14C]oleic acid or [U-14C]AKG. Some media also included 2 mM-AKG + [U-14C]AKG. At the end of a 30 min period of incubation, 14CO₂ was collected in 0.2 ml of NCS-II, and its radioactivity was determined using a Packard liquid scintillation counter. The specific radioactivity of CO₂ production from d-[U-14C]glucose, [1-14C]oleic acid, d-[U-14C]AKG, l-[U-14C]glutamine or [U-14C]AKG in the incubation medium was used to calculate the rates of CO₂ production from d-[U-14C]glucose, [1-14C]oleic acid, l-[U-14C]glutamine or [U-14C]AKG, respectively.

**Determination of mucosal ATP, ADP and AMP**

Frozen mucosal samples (50–100 mg) were homogenised with 1 ml pre-cooling 1:5 m-perchloric acid in an ice-bath. The homogenates were centrifuged at 3000 g for 10 min at 4°C. An aliquot of the supernatant fluid (0.5 ml) was neutralised with 0.2 ml of 2M-potassium carbonate in ice, and its radioactivity was determined using a Packard liquid scintillation counter. The specific radioactivity of ATP, ADP and AMP was determined using a Packard scintillation counter.

**Western blot analysis**

Approximately 100 mg of the mucosal samples were quickly placed into the pre-cooling 2 ml centrifuge tubes, mixed with 1 ml of a pre-cooling mixture of radioimmunoprecipitation assay lysis buffer (SC-24948; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and phosphate inhibitor cocktail (P2850; Sigma Chemical, Inc.), vortexed for 30 s and homogenised in an ice-bath. The homogenate was centrifuged at 12000 g for 15 min at 4°C. The supernatant fluid was divided into aliquots for Western blot and protein analyses. The concentration of protein was measured using a bichinonic acid protein assay kit (Sigma Chemical, Inc.), and then the supernatant fluid was diluted to the same concentrations. For measuring the levels of total and phosphorylated AMPKα, total ACC and phosphorylated ACC-β, 2 × SDS sample buffer (2 ml of 0.5 mM-Tris, pH 6.8, 2 ml of glycerol, 2 ml of 10% SDS, 0.2 ml of β-mercaptoethanol, 0.4 ml of a 4% solution of bromophenol blue and 1.4 ml of water) was added to the diluted supernatant in a 1:1 ratio, boiled for 5 min and cooled on ice before use. Mucosal protein (150 µg/sample for AMPK and 90 µg/sample for ACC) was subjected to SDS-PAGE using 10% (for AMPK) or 6% (for ACC) polyacrylamide resolving gels and transferred to the polyvinylidene difluoride membranes in transfer buffer (25 mM-Tris base, 0.2 mM-glycine and 20% methanol, pH 8.5). Thereafter, the blotted membranes were washed with 25 ml Tris-buffered saline (TBS), which was prepared in 1 litre of 10× TBS: 24.2 g Tris base and 80 g NaCl (pH adjusted to 7.6 with HCl). The membranes were blocked with 5% non-fat dry milk in TBS/0.1% Tween 20 (TBS-T) for 1 h at room temperature with slow agitation followed by three washes in TBS-T. The membranes were incubated overnight at 4°C either with anti-phospho-AMPKα (Thr172) (no. 2535, rabbit mAb; Cell Signaling, Danvers, MA, USA) and anti-AMPKα (no. 2532; Cell Signaling) or with anti-phospho-ACC-β (Ser79) (no. 3661; Cell Signaling) and anti-ACC (no. 3662; Cell Signaling) with TBS-T containing 5% bovine serum albumin, at a dilution of 1:1000. The membranes were washed three times with TBS-T and incubated with a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, 1:5000; Sigma Chemical, Inc.) at room temperature for 1 h. Blots were developed using an enhanced chemiluminescence substrate (SuperSignal; Pierce Biotechnology, Rockford, IL, USA), visualised using an imaging system (Bio-Rad VersaDoc, Model-5000; Bio-Rad, Hercules, CA, USA) and quantified using Quantity One software.

**Statistical analysis**

Values are expressed as means and standard deviations. Experimental data were analysed by one-way ANOVA. Differences among treatment means were determined by Duncan’s multiple range test. All statistical analyses were performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). P ≤ 0.05 was taken to indicate significance.

**Results**

**Oxidation of substrates**

Rates of CO₂ production from all carbons of AKG, glucose and glutamine as well as from the carbon 1 of oleic acid in pig enterocytes are shown in Table 2. Administration of LPS to pigs decreased (P < 0.05) the oxidation of all the four substrates. Dietary supplementation with AKG to the LPS-treated pigs partially ameliorated (P < 0.05) the reduction in the oxidation of AKG, glucose, glutamine and oleic acid in enterocytes.
Data on the concentrations of ATP, ADP and AMP in the intestinal mucosa are summarised in Table 3. Compared with the control group, LPS piglets exhibited a decrease (P<0.05) in AEC but decreased (P<0.05) in the ratio of AMP:ATP in the jejunum and ileum, and an increase (P<0.05) in the ratio of AMP:ATP in the ileum (Table 3). In comparison with the LPS piglets, AKG supplementation increased (P<0.05) the concentrations of ATP in the mucosae of the duodenum and jejunum by 44 and 25%, respectively. The concentrations of ADP, AMP and total adenine nucleotide in the duodenal mucosa of LPS piglets were increased (P<0.05) by 58, 18 and 44%, respectively. Compared with the control group, LPS piglets exhibited a decrease (P<0.05) in AEC in the jejunum and ileum, and an increase (P<0.05) in the ratio of AMP:ATP in the jejunum (P<0.05) and ileum (Table 3). In comparison with the LPS piglets, dietary supplementation with 1% AKG increased (P<0.05) AEC but decreased (P<0.05) AMP:ATP ratios in the mucosae of the jejunum and ileum (Table 3).
Phosphorylated levels for AMP-activated protein kinase and acetyl-CoA carboxylase

Phosphorylation at Thr172 for the α-isofrm of AMPK is illustrated in Fig. 1. LPS challenge resulted in an increase (P<0.05) in the ratio of phosphorylated AMPK-α:AMPK-α in the jejunum and ileal mucosa, compared with the control piglets. Relative to LPS piglets receiving no LPS, dietary supplementation with 1% AKG decreased (P<0.05) the phosphorylated AMPK-α:AMPK-α in the jejunum and ileum to the value observed for the control group. However, in the duodenum, the levels of phosphorylated AMPK-α did not differ (P>0.05) among the control, LPS and LPS+AKG piglets.

Phosphorylation at Ser79 for ACC-β and total ACC-β protein is illustrated in Fig. 2. Compared with the control group, the ratio of phosphorylated ACC-β:ACC-β in the duodenal, jejunal and ileal mucosa of LPS piglets was increased (P<0.05) by 38, 49 and 40%, respectively. Compared with the LPS group, dietary supplementation with AKG did not affect (P>0.05) the phosphorylated ACC-β:ACC-β in the small-intestinal mucosa of LPS piglets.

Discussion

There is growing interest in the nutritional regulation of mucosal inflammation in both clinical medicine and animal production, because the gastrointestinal tract is the first defence against diet-derived pathogens. AKG displays remarkable metabolic and regulatory versatility in the pig intestine, but little is known about its significance in gut signalling or function. The results of the present study indicate, for the first time to our knowledge, that AKG beneficially prevented LPS-induced alterations in substrate oxidation (Table 2), cellular energy status (Table 3) and protein levels for phosphorylated AMPK and ACC-β (Fig. 1) in the piglet small intestine.

LPS is a molecule found in the membrane of all gram-negative bacteria and can induce alterations in gastrointestinal metabolism. In addition, LPS increased heat shock protein 70 expression in the pig small intestine, which is an indicative of oxidative stress in intestinal cells. Moreover, LPS can cause mitochondrial injury in the small intestine. Extending these previous reports, we observed that LPS treatment reduced ATP concentrations and AEC, while altering the cellular energy status in piglet intestinal mucosa (Table 3). This is in keeping with our new findings that the intestinal oxidation of glucose, glutamine and fatty acids was reduced in response to LPS administration (Table 2). Thus, the LPS-challenged weanling piglets provide a useful animal model to develop nutritional interventions for improving energy status in the gut.

The energy charge of the adenyl pool is a better measure of the energy state of a tissue than the level of a single nucleotide. ATP hydrolysis can increase the cellular concentration of ADP, which is converted by adenylate kinase (2 ADP ↔ ATP + AMP) to ATP and AMP. Another novel and important finding of the present study is that AKG supplementation resulted in (1) increased ATP, (2) reduced AMP:ATP and (3) increased AEC in the pig small intestine. These three lines of evidence indicate that dietary supplementation with AKG could modulate the adenine nucleotide pool and support the notion that AKG beneficially alleviates the LPS-induced damage of intestinal energy metabolism. The results reveal a hitherto unrecognised role for AKG in improving mitochondrial function in the small intestine.
Lambert et al.\textsuperscript{(25)} reported that 80% of dietary AKG was oxidised by the small intestine in young pigs, and the remaining AKG was utilised by other splanchnic tissues. Thus, dietary supplementation with AKG did not increase its concentrations in the porcine portal blood\textsuperscript{(25)} Because AKG is extensively metabolised in the first-pass by the intestinal mucosa and virtually does not enter the portal circulation\textsuperscript{(25)}, we suggest that AKG exerts its nutritional benefits in weanling piglets primarily at the small-intestinal level. The possibility that AKG may also affect other splanchnic tissues cannot be ruled out.

AMPK activity in mammalian cells can be regulated by stimuli that affect cellular ATP levels\textsuperscript{(21)}. For example, hypoxia, which occurs in the small intestine of LPS-treated animals\textsuperscript{(21)}, leads to the activation of AMPK via an increase in the AMP:ATP ratio\textsuperscript{(26)}. When activated, AMPK switches on catastrophic pathways for ATP regeneration, such as the oxidation of glucose, amino acids and fatty acids, while switching off ATP-requiring pathways, such as fatty acid and TAG synthesis\textsuperscript{(27)}. To our knowledge, this is the first study indicating that AKG may regulate AMPK signalling in the intestinal mucosa. At present, it is not known whether AKG directly or indirectly phosphorylates AMPK. AKG may act as an energy substrate or a signalling molecule, or both. It is also possible that the metabolism of AKG in enterocytes may regulate its intracellular concentration and thus its effects on cellular targets (e.g. AMPK). Either the elucidation of these mechanisms for the actions of AKG on the gut via AMPK activation and energy metabolism or the determination of whether ATP depletion in enterocytes may induce the activation of AMPK would require complex biochemical studies, and such studies are beyond the scope of the present study. However, emerging evidence shows that AMPK is an upstream molecule in the mammalian target of rapamycin signalling pathway\textsuperscript{(28)}. Thus, we speculate that possibly through the mammalian target of rapamycin activation\textsuperscript{(5)}, AKG stimulates AMPK phosphorylation and oxidation of energy substrates in the intestinal mucosa (Table 2), thereby enhancing ATP supply and supporting cell function. Further studies are warranted to test this hypothesis.

Phosphorylated AMPK-\(\alpha\) (active) AMPK could inhibit ACC activity in the skeletal muscle and liver by phosphorylating ACC-\(\beta\) (namely increasing the phosphorylated level of ACC)\textsuperscript{(29)}, but a role for ACC in enterocyte metabolism is unknown. Interestingly, there have been reports suggesting that, in colonic epithelial cells (colonocytes), conversion of acetyl-CoA to malonyl-CoA may play an important role in lipogenesis\textsuperscript{(30,31)}. ACC activation is a rate-controlling step in the conversion of acetyl-CoA to malonyl-CoA\textsuperscript{(32)}. Malonyl-CoA inhibits carnitine palmitoyl-CoA transferase-1, which transports long-chain fatty acyl-CoA from the cytoplasm into the mitochondria for oxidation\textsuperscript{(50)}. Thus, a fall in malonyl-CoA concentration in response to the increased levels of phosphorylated AMPK and phosphorylated ACC may lead to increased oxidation of fatty acids. However, we found that LPS treatment reduced the oxidation of oleic acid in enterocytes (Table 2). This result may be explained by mitochondrial injury or dysfunction in the small intestine of endotoxin-challenged pigs, thereby impairing Krebs cycle activity and the mitochondrial electron transport system. Consistent with our finding is the report that LPS treatment reduced the activity of aconitase in rat myocardial tissue\textsuperscript{(33)}. Aconitase is a target of NO\textsuperscript{(34)}, and increased NO production by inducible NO synthase in response to LPS treatment could be a mechanism responsible for the reduced oxidation of substrates in the enterocytes of LPS-challenged pigs. Notably, oxidation of oleic acid in the enterocytes of LPS-treated pigs was increased by AKG supplementation, indicating the improvement of mitochondrial integrity and function.

In summary, dietary supplementation with AKG beneficially improves the energy status of the intestinal mucosa as well as AMPK activation and ACC inactivation in the intestinal mucosa of LPS-challenged pigs. These novel findings have important implications for the development of new interventions to ameliorate gut injury and dysfunction in animals. Further research is warranted to understand how AKG affects the AMPK signalling in the gut.

**Acknowledgements**

The present study was jointly supported by the National Natural Science Foundation of China (no. 30871801, 30901041, 30901040, 30928018), the Program for Innovative Research Groups of Hubei Provincial Natural Science Foundation (no. 2007ABC009), National Research Initiative Competitive Grants from the Animal Growth and Nutrient Utilization Program (no. 2008-35206-18764) of the USDA National Institute of Food and Agriculture, Texas AgriLife Research (H-8200), and the Thousand-People Talent program at China Agricultural University. Y. H. and G. W. designed the study and wrote the manuscript. K. Y., L. W., B. D., D. F., Y. L., H. Z., J. L. and Y. Li collected and analysed the experimental results. P. K. and Y. Y. participated in the experiments and revised the manuscript. All authors contributed to data interpretation and approved the final version of the manuscript. There is no conflict of interest that should be disclosed.

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