Amino Acids (2012) 42:2491–2500
DOI 10.1007/s00726-011-1060-6

ORIGINAL ARTICLE

Alpha-ketoglutarate inhibits glutamine degradation and enhances protein synthesis in intestinal porcine epithelial cells

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Received: 6 December 2010 / Accepted: 9 August 2011 / Published online: 23 August 2011

Abstract α-Ketoglutarate (AKG) is a key intermediate in glutamine metabolism. Emerging evidence shows beneficial effects of AKG on clinical and experimental nutrition, particularly with respect to intestinal growth and integrity. However, the underlying mechanisms are unknown. Intestinal porcine epithelial cells (IPEC-1) were used to test the hypothesis that AKG inhibits glutamine degradation and enhances protein synthesis. IPEC-1 cells were cultured for 3 days in Dulbecco's modified Eagle's-F12 Ham medium (DMEM-F12) containing 0, 0.2, 0.5 or 2 mM of AKG. At the end of the 3-day culture, cells were used to determine L-[U-^{14}C]glutamine utilization, protein concentration, protein synthesis, and the total and phosphorylated levels of the mammalian target of the rapamycin (mTOR), ribosomal protein S6 kinase-1 (S6K1) and eukaryotic initiation factor (eIF) 4E-binding protein-1 (4E-BP1). Compared with 0 mM of AKG (control), 0.2 and 0.5 mM of AKG dose-dependently reduced ($P < 0.05$) glutamine degradation and the production of glutamate, alanine and aspartate in IPEC-1 cells. Addition of 0.5 and 2 mM of AKG to culture medium enhanced protein synthesis ($P < 0.05$) by 78 and 101% without affecting protein degradation, compared to the control group. Rapamycin (50 nM; a potent inhibitor of mTOR) attenuated the stimulatory effect of AKG on protein synthesis. Consistent with these metabolic data, the addition of 0.5 or 2 mM of AKG to culture medium increased ($P < 0.05$) the phosphorylated levels of mTOR, S6k1 and 4E-BP1 proteins. Collectively, these results indicate that AKG can spare glutamine and activate the mTOR signaling pathway to stimulate protein synthesis in intestinal epithelial cells.

Keywords α-Ketoglutarate · Intestinal cells · mTOR signaling · Protein synthesis

Abbreviations

AKG	α-Ketoglutarate
BSA	Bovine serum albumin
DMEM-F12	Dulbecco's modified Eagle's F12 Ham medium
FBS	Fetal bovine serum
IPEC-1	Intestinal porcine epithelial cells-1
mTOR	Mammalian target of rapamycin
4E-BP1	4E-Binding protein-1
S6K1	70-kDa Ribosomal protein S6 kinase-1

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Introduction

Alpha-ketoglutarate (AKG) is a key intermediate in L-glutamine catabolism via phosphate-activated glutaminase, glutamate transaminase and glutamate dehydrogenase (Boutry et al. 2011; Wu 2009). When AKG enters the tricarboxylic acid cycle, it is oxidized by AKG dehydrogenase (Blachier et al. 2009; Junghans et al. 2006). Thus, AKG may be able to inhibit glutamine utilization by intestinal cells. Emerging evidence shows beneficial effects of AKG in clinical and experimental nutrition, particularly with respect to intestinal growth and integrity (Hou et al. 2011; Pierzynowski and Sjodin 1998). For example, enteral administration of AKG reduces infectious complications after trauma and surgery in humans in the early postoperative phase (Junghans et al. 2006), whereas AKG improves nitrogen balance in burn patients (De Bandt et al. 1998) and the healing of the small intestine after radiation (Kalfarentzos et al. 1996). Additionally, dietary supplementation with 1% AKG can alleviate mucosal damage and increase the absorptive function of the small intestine in the endotoxin-challenged piglet (Hou et al. 2010), which is an excellent animal model for studying human nutrition and metabolism (Flynn et al. 2009; Suryawan et al. 2009; Wang et al. 2009a; Wilson et al. 2011).

Beneficial effects of AKG may be explained by its key role in the metabolism of amino acids (Chen et al. 2009; Wu and Morris 1998), particularly glutamine (Wu 2010; Xi et al. 2011). Glutamine is known to stimulate protein synthesis in intestinal cells via activating the mammalian target of rapamycin (mTOR) signaling pathway (Palii et al. 2009; Rhoads and Wu 2009; Wu et al. 2011a). Moreover, oxidation of AKG through the tricarboxylic acid cycle can provide large amounts of ATP (Kristensen et al. 2002; Pierzynowski and Sjodin 1998; Wu 1998), thereby possibly sparing glutamine and glutamate for use by metabolic pathways including mTOR activation, protein synthesis and arginine production (Geng et al. 2011; Wu et al. 2011b). The present study was conducted to test this hypothesis using intestinal porcine epithelial cells (IPEC-1), which metabolize glutamine in a similar way to enterocytes freshly isolated from neonatal pigs (Haynes et al. 2009).

Materials and methods

Reagents

Alpha-ketoglutarate was purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibodies to total mTOR, phosphorylated mTOR, total 4EBP1, phosphorylated 4EBP1 and β-actin were purchased from Cell Signaling (Beverly, MA, USA). The antibodies to total S6K1 and phosphorylated S6K1 were produced by Abcam (Cambridge, MA, USA). Dulbecco's modified Eagle's F12 Ham medium (DMEM-F12), fetal bovine serum (FBS) and antibiotics were procured from Invitrogen (Grand Island, NY, USA). Epidermal growth factor was obtained from BD Biosciences (Bedford, MA, USA), and plastic culture plates were manufactured by Corning Inc. (Corning, NY, USA). L-[U-^{14}C]glutamine was procured from American Radiolabeled Chemicals (St. Louis, MO, USA) and purified using anion-exchange chromatography before use (Wu et al. 1991a). Unless indicated, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Culture of IPEC-1 cells

IPEC-1 cells were isolated from the jejunum of newborn pigs (Haynes et al. 2009) and cultured as previously described (Tan et al. 2010). Briefly, the cells were grown in serial passage in uncoated plastic culture flasks (100 mm^2) in DMEM-F12 containing 17.5-mM D-glucose, 2-mM L-glutamine, 0.7-mM L-arginine, 15-mM HEPES (pH 7.4), 5% FBS, insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), epidermal growth factor (5 μg/L), penicillin (50 μg/ml), streptomycin (4 μg/ml) and 0.25 μg/ml of amphotericin B (fungizone®). The medium was changed every 2 days. At confluence, cells were trypsinized and seeded in 6-well cell culture plates with approximately 1×10^4 cells per well and maintained at 37°C in a 5% CO_2 incubator. After 16-h incubation, the cells were cultured in a special medium (Table 1) containing 0, 0.2, 0.5, 2 or 5 mM of AKG for 3 days. This medium contains physiological concentrations of amino acids found in pig plasma (Wu et al. 1997). There were eight independent replicates per AKG dose. The number of independent observations ($n = 8$) per treatment was determined on the basis of statistical power calculation (Fu et al. 2010). Concentrations of amino acids and glucose in this special medium are given in Table 1. The medium was changed every 24 h. After 3 days of culture, the medium was collected for analysis of amino acids using HPLC (Li et al. 2009b, 2011).

Determination of glutamine catabolism in IPEC-1 cells cultured with AKG

Cells (5×10^6/ml) were cultured as described above (Table 1) except that the medium contained 5 mM of D-glucose and 2 mM of L-[U-^{14}C]glutamine (250 dpm/nmol). At the end of 3-h culture, cells and medium were obtained for analysis of ^{14}C-labeled glutamine, glutamate, alanine and aspartate, using anion-exchange chromatography (Wu et al. 1991b). In separate experiments, $^{14}CO_2$ produced

Table 1 Cell culture medium for metabolic studies

Component	mg/L
$CaCl_2 \cdot 2H_2O$	265
$Fe(NO_3)_3 \cdot 9H_2O$	0.10
$MgSO_4$	97.67
KCl	400
$NaHCO_3$	3,700
NaCl	6,400
NaH_2PO_4	109
Alanine (350 μM)	31.2
L-Arginine·HCl (100 μM)	21.1
Asparagine (50 μM)	7.0
Aspartic acid (20 μM)	3.0
L-Cystine·2HCl (75 μM)	24.0
Glutamic acid (75 μM)	11.0
L-Glutamine[a] (500 μM)	73.1
Glycine (250 μM)	19.0
L-Histidine·HCl·H_2O (100 μM)	21.0
L-Isoleucine (150 μM)	20.0
L-Leucine (200 μM)	26.2
L-Lysine·HCl (200 μM)	36.0
L-Methionine (75 μM)	12.0
L-Phenylalanine (100 μM)	17.0
L-Proline (200 μM)	23.0
L-Serine (200 μM)	21.0
Taurine (100 μM)	13.0
L-Threonine (200 μM)	16.0
L-Tryptophan (75 μM)	20.0
L-Tyrosine·2Na·$2H_2O$ (100 μM)	26.0
L-Valine (250 μM)	30.0
Choline chloride	4.0
Folic acid	4.0
Myo-inositol	7.2
Niacinamide	4.0
D-Pantothenic acid·0.5 Ca	4.0
Pyridoxine·HCl	4.0
Riboflavin	0.4
Thiamine·HCl	4.0
D-Glucose (5 mM)	900
Pyruvic acid·Na (100 μM)	11.0

This special medium, which was prepared by Gibco (Carlsbad, CA, USA), was used to measure protein turnover as well as glutamine and AKG metabolism in IPEC-1 cells

[a] Unless indicated in text

from L-[U-^{14}C]glutamine oxidation was collected in 0.2 ml of Soluene (Dai et al. 2010) for analysis of radioactivity by a liquid scintillation counter (Flynn et al. 2010). Intracellular specific activity of L-[U-^{14}C]glutamine was determined (Wu 1997) and used to calculate rates of production of its metabolites.

Determination of amino acid concentrations in IPEC cells cultured with AKG

Cells (10×10^6/ml) were cultured as described above (Table 1) except that the medium contained 5 mM of D-glucose, 2-mM glutamine and 0–5 mM of AKG. This concentration of glutamine was chosen because it was found to be present in the lumen of the piglet small intestine (Wu and Knabe 1995; Wang et al. 2008). At the end of 3 h of culture, the medium was removed and cells were washed rapidly three times with phosphate-buffered saline. Cells were then lysed with 0.5 ml of 1.5 M $HClO_4$, and the extracts were analyzed for amino acids by HPLC (Li et al. 2009b). Concentrations of amino acids in IPEC-1 cells were based on cell volume (0.538 ± 0.036 μl/10^6 cells, mean ± SEM, $n = 12$), which was determined using ^3H_2O, as described by Wu and Flynn (1995).

Determination of AKG catabolism in IPEC-1 cells

Cells (5×10^6/ml) were cultured as described above (Table 1) except that the medium contained 5-mM D-glucose, 2-mM L-glutamine and 0–5 mM of [U-^{14}C]AKG (150 dpm/nmol). At the end of 3-h culture, cells and medium were obtained for analysis of ^{14}C-labeled glutamine, glutamate, alanine and aspartate, using anion-exchange chromatography (Wu et al. 1991b). Ammonia in the medium was analyzed using glutamate dehydrogenase (Wu 1995). In separate experiments, $^{14}CO_2$ produced from [U-^{14}C]AKG oxidation was collected in 0.2-ml Soluene (Dai et al. 2010) for analysis of radioactivity by a liquid scintillation counter (Flynn et al. 2010). The specific activity of [U-^{14}C]AKG in medium was used to calculate the rates of production of its metabolites.

Determination of protein turnover in IPEC-1 cells

Protein synthesis was measured using [^{3}H]phenylalanine, as described by Tan et al. (2010). Briefly, IPEC-1 cells (1×10^5) were seeded in 6-well cell culture plates with DMEM-F12 medium containing 17.5-mM D-glucose, 2-mM L-Glutamine, 0.7-mM L-arginine, 15-mM HEPES (pH 7.4), 5% FBS, insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), epidermal growth factor (5 μg/L), penicillin (50 μg/ml), streptomycin (4 μg/ml) and 0.25 μg/ml of amphotericin B (fungizone®). After 16-h culture, cells were placed in a fresh special medium (Table 1) containing 0, 0.2, 0.5 or 2 mM of AKG. The medium was changed every 24 h. After a 3-day period of culture, cells were washed once with 2 ml of AKG-free medium. Then, cells were cultured for 3 h in 2 ml of DMEM containing 1-mM L-phenylalanine plus 0.1 μCi L-[ring-2,4-^{3}H]phenylalanine (American Radiolabeled Chemicals) and 0, 0.2,

0.5 or 2 mM of AKG. In some experiments, the culture medium contained 50 nM of rapamycin, a potent inhibitor of mTOR (Kim et al. 2008). At the end of a 3-h culture period, the medium was collected and cells were rapidly washed three times with 2 ml of ice-cold PBS. The cell pellet was mixed with 2 ml of 2% trichloroacetic acid (TCA) (Wu and Thompson 1990). Cells in each well were scraped and then the whole solution was collected into a 15-ml tube and centrifuged at 3,000×*g* for 5 min. The supernatant fluid was discarded. The cell pellet was washed three times with 5 ml of 2% TCA and dried in air at 37°C. After the pellet was dissolved in 0.5 ml of 1 M NaOH, 0.4 ml of the solution was transferred to a 20-ml Scintillation vial containing 15 ml of Hionic Fluor Scintillation cocktail (PerkinElmer, MA, USA). The ^{3}H-phenylalanine radioactivity was determined by a liquid scintillation counter after overnight standing at room temperature.

For determining protein degradation, IPEC-1 cells were cultured for 3 days in 2 ml of AKG-free medium (Table 1) containing 0, 0.2, 0.5 or 2 mM of AKG. Beginning on day 4, cells were cultured for 24 h in 2 ml of AKG-free medium (Table 1) containing 0.1 mM of L-phenylalanine plus L-[^{3}H]phenylalanine (0.8 μCi/well) and 0, 0.2, 0.5 or 2 mM of AKG. After the 24-h culture to label cellular proteins, cells were washed three times with 2 ml of AKG-free medium containing 1 mM of L-phenylalanine to deplete intracellular free [^{3}H]phenylalanine. The cells were then cultured for 3 h in 2 ml of AKG-free medium (Table 1) containing 0, 0.2, 0.5 or 2 mM of AKG; in some experiments, the culture medium contained 50 nM of rapamycin. At the end of a 3-h culture period, the medium was collected, the cells were rapidly washed three times with 2 ml of ice-cold phosphate-buffered saline, and 2 ml of 2% TCA was added to each well. The whole TCA extract was collected into a 15-ml tube and centrifuged at 3,000×*g* for 5 min. The supernatant fluid was removed and the pellet was washed three times with 5 ml of 2% TCA and dried in air at 37°C. After the pellet was dissolved in 0.5 ml of 1 M NaOH, 0.4 ml of the solution was transferred to a 20-ml scintillation vial containing 15-ml Hionic Fluor Scintillation cocktail for ^{3}H measurement. For determining [^{3}H]phenylalanine released from prelabeled proteins into culture medium, the collected medium was centrifuged at 3,000×*g* for 2 min to remove any dead cells. An aliquot (1 ml) of the supernatant fluid was transferred to a 15-ml tube containing 2 ml of 2% TCA. After the tubes were centrifuged at 3,000×*g* for 5 min, all the supernatant fluid was transferred to a 20-ml scintillation vial containing 15-ml Hionic Fluor Scintillation cocktail for ^{3}H measurement. The percentage of protein-bound [^{3}H]phenylalanine released into the culture medium (namely, [^{3}H]phenylalanine in medium/[^{3}H]phenylalanine in cell proteins × 100) was calculated to indicate protein degradation in IPEC-1 cells.

Western blot analysis

Cells were cultured for 3 days in a medium containing 0, 0.2, 0.5 or 2 mM of AKG, as described above. After the 3-day culture, cells were obtained for analysis of protein (Li et al. 2008) and Western blotting (Tan et al. 2010). Briefly, cells in 24-well culture plates were gently washed with calcium-containing Dulbecco's phosphate-buffered saline and viable cells were counted using a counting chamber (Dekaney et al. 2008). Cells were collected and lysed in 0.5-ml buffer [20-mM HEPES, pH 7.4, 2-mM EGTA, 50-mM NaF, 100-mM KCl, 0.2-mM EDTA, 50-mM β-glycerophosphate, 1-mM dithiothreitol (DTT), 0.1-mM phenylmethylsulfonyl fluoride (PMSF), 1-mM benzamidine and 0.5-mM sodium vanadate) with a Polytron homogenizer and centrifuged at 10,000×*g* for 10 min at 4°C, as described by Li et al. (2008). Protein concentrations in cell lysates were measured using the BCA method and bovine serum albumin as standard. All samples were adjusted to an equal concentration. The supernatant fluid (containing cell proteins) was then diluted with 2× sodium dodecyl sulfate (SDS) sample buffer (0.63 ml of 0.5 M Tris–HCl pH 6.8, 0.42-ml 75% glycerol, 0.125-g SDS, 0.25-ml β-mercaptoethanol, 0.2-ml 0.05% solution of bromphenol blue and 1-ml water to a final volume of 2.5 ml) and heated in boiling water for 5 min. After the solution was cooled on ice, it was used for Western blot analysis. Aliquots of samples with an equal amount of protein were loaded onto SDS–polyacrylamide gels. After separation on 4–12% gels, proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) under 12 V overnight, using the Bio-Rad Transblot apparatus (Hercules, CA). Membranes were blocked in 5% fat-free dry milk in Tris-Tween buffered saline (TTBS; 20-mM Tris/150-mM NaCl, pH 7.5, and 0.1% Tween-20) for 3 h and then incubated with the following primary antibodies overnight at 4°C with gentle rocking: mTOR (Cell Signaling, 1:1,000), phosphorylated mTOR (Ser2448) (Cell Signaling, 1:1,000), 4EBP1 (Cell Signaling, 1:1,000), phosphorylated 4EBP1 (Ser65) (Cell Signaling, 1:1,000), S6K1 (Abcam, 1:1,000), phosphorylated S6K1 (Thr389) (Abcam, 1:1,000) or β-actin (Cell Signaling, 1:1,000). After washing three times with TTBS, the membranes were incubated at room temperature for 3 h with secondary antibodies at 1:50,000 (horseradish peroxidase-conjugated goat anti-rabbit IgG, Cell Signaling or peroxidase-labeled rabbit anti-goat IgG, Kirkegaard & Perry Lab, Gaithersburg, MD). 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). All protein measurements were normalized to β-actin (Yin

et al. 2010a) and all data were expressed as the relative values to those of cells cultured with 0-mM AKG.

Statistical analysis

Results are expressed as mean ± SEM. The statistical analysis was performed by one-way ANOVA using SPSS 13.0 (SPSS Inc., Chicago, IL). Differences among treatment means were determined using the Student–Newman–Keuls multiple comparison test. P values ≤ 0.05 were taken to indicate significance.

Results

Effects of AKG on glutamine catabolism in IPEC-1 cells

IPEC-1 cells readily utilized glutamine and produced ammonia, glutamate, alanine, aspartate and CO_2 (Table 2). There was no detectable production of proline from glutamine in the cells. Likewise, there was little formation of glutamate from AKG and ammonia in IPEC-1 cells due to a low activity of glutamate dehydrogenase. Increasing concentrations of AKG from 0 to 2 mM in culture medium inhibited ($P < 0.05$) glutamine utilization and decreased ($P < 0.05$) the formation of ammonia, glutamate, alanine, aspartate and CO_2 from glutamine in a dose-dependent manner (Table 2). Rates of glutamine metabolism in IPEC-1 cells did not differ ($P > 0.05$) between 2 and 5 mM of AKG.

Effects of AKG on concentrations of amino acids in IPEC-1 cells

When IPEC-1 cells were cultured with 2-mM glutamine and only 0.1-mM glutamate, large amounts of intracellular glutamate were derived from glutamine degradation via phosphate-activated glutaminase (Table 3). Among all the measured amino acids, glutamate had the highest concentration in cells. Increasing concentrations of AKG in culture medium from 0 to 2 mM increased ($P < 0.05$) intracellular concentrations of glutamine, glutamate, alanine and aspartate in a dose-dependent manner (Table 3). Concentrations of the amino acids in IPEC-1 cells did not differ ($P > 0.05$) between 2 and 5 mM of AKG.

Metabolism of AKG in IPEC-1 cells

Extracellular AKG was readily metabolized to CO_2, glutamate, alanine and aspartate in IPEC-1 cells, with CO_2 being the major product of AKG (Table 4). There was detectable formation of glutamine, proline or asparagine from AKG in these cells. Increasing concentrations of AKG in culture medium from 0 to 5 mM increased ($P < 0.05$) the production of CO_2, glutamate, alanine and aspartate from AKG in a dose-dependent manner (Table 4).

Effects of AKG on cell proliferation and protein turnover in IPEC-1 cells

At day 0 of culture, the number of IPEC-1 cells in each well was 1×10^5 for all wells. At the end of the 3-day culture, the numbers of cells ($\times 10^5$) per well were 2.31, 2.54, 3.75 and 4.16 (pooled SEM = 0.20), respectively, in the presence of 0, 0.2, 0.5 and 2 mM of AKG. IPEC-1 proliferation increased ($P < 0.05$) progressively with increasing concentrations of AKG in culture medium, with the values for 0.5 and 2 mM of AKG being greater ($P < 0.05$) than those for 0 and 0.2 mM of AKG.

Effects of AKG on protein synthesis and protein degradation in IPEC-1 cells are summarized in Table 5. Compared with the control group, addition of 0.5 and 2 mM of AKG to the culture medium increased ($P < 0.05$)

Table 2 Effects of AKG on glutamine catabolism in IPEC-1 cells cultured for 3 h in the presence of 2 mM of glutamine plus 0–5 mM of AKG

	Concentrations of AKG in culture medium (mM)					Pooled SEM
	0	0.2	0.5	2	5	
Net utilization of glutamine and production of its metabolites (nmol/mg protein/h)						
GLN	−224.5[a]	−170.3[b]	−148.2[c]	−106.3[d]	−102.1[d]	8.7
Ammonia	156.3[a]	124.7[b]	91.0[c]	69.1[d]	67.2[d]	6.6
GLU	102.5[a]	83.9[b]	62.7[c]	48.5[d]	46.4[d]	3.9
ALA	25.8[a]	20.4[b]	16.6[c]	11.9[d]	11.3[d]	1.2
ASP	21.6[a]	18.3[b]	14.0[c]	10.2[d]	10.5[d]	1.0
CO_2	64.9[a]	50.2[b]	37.5[c]	22.8[c]	21.3[c]	2.4

SEM, standard error of the mean, $n = 8$. Culture medium contained 2 mM of L-[U-^{14}C]glutamine and 0–5 mM of AKG. The symbol (−) denotes net utilization of glutamine

[a–d] Values within a row with different letters differ ($P < 0.05$)

Table 3 Intracellular concentrations of amino acids in IPEC cells cultured for 3 h in the presence of 2 mM of glutamine plus 0–5 mM of AKG

	Concentrations of AKG in culture medium (mM)					Pooled SEM
	0	0.2	0.5	2	5	
GLN (mM)	1.71[d]	2.03[c]	2.48[b]	3.03[a]	3.12[a]	0.10
GLU (mM)	3.69[d]	4.11[c]	4.63[b]	5.28[a]	5.34[a]	0.13
ALA (mM)	1.93[d]	2.16[c]	2.44[b]	2.87[a]	2.92[a]	0.06
ASP (mM)	1.32[d]	1.65[c]	2.05[b]	2.56[a]	2.61[a]	0.05
PRO (mM)	1.66	1.72	1.76	1.63	1.85	0.08

Values are means plus pooled SEM, $n = 8$

[a–d] Values within a row with different letters differ ($P < 0.05$)

Table 4 Metabolism of [U-^{14}C]AKG in IPEC cells

Product from [^{14}C]AKG	Concentrations of AKG in culture medium (mM)				Pooled SEM
	0.2	0.5	2	5	
Production of metabolites from AKG (nmol/mg protein/h)					
[^{14}C]GLU	0.76[c]	1.02[b]	1.43[a]	1.46[a]	0.07
[^{14}C]ALA	0.10[c]	0.18[b]	0.39[a]	0.41[a]	0.01
[^{14}C]ASP	0.08[d]	0.15[c]	0.32[a]	0.35[a]	0.01
$^{14}CO_2$	17.2[d]	31.4[c]	89.6[b]	147.2[a]	6.6

Values are means plus pooled SEM, $n = 8$

[a–d] Values within a row with different letters differ ($P < 0.05$)

Table 5 Effects of AKG on protein synthesis and degradation in IPEC-1 cells

	Concentrations of AKG in culture medium (mM)				Pooled SEM
	0	0.2	0.5	2	
Protein synthesis (nmol Phe/mg protein/h)	29.6[b]	32.4[b]	52.8[a]	59.5[a]	4.1
Protein degradation (%/h)	2.83	2.65	2.70	2.77	0.23

SEM, standard error of the mean, $n = 8$

[a–c] Values within a row with different letters differ ($P < 0.05$)

Table 6 Effects of rapamycin (an inhibitor of mTOR) on protein synthesis and degradation in IPEC-1 cells

	0 mM of AKG	2 mM of AKG	50 nM of rapamycin	2 mM of AKG + 50 nM rapamycin	Pooled SEM
Protein synthesis (nmol Phe/mg protein/h)	30.2[b]	60.7[a]	9.18[c]	29.5[b]	3.6
Protein degradation (%/h)	2.56[b]	2.48[b]	3.09[a]	3.04[a]	0.21

SEM, standard error of the mean, $n = 8$

[a–c] Values within a row with different letters differ ($P < 0.05$)

protein synthesis in IPEC-1 cells (Table 5). Rates of protein synthesis in IPEC-1 cells did not differ ($P > 0.05$) between 0.5 and 2 mM of AKG. Increasing concentrations of AKG in culture medium from 0 to 2 mM did not affect ($P > 0.05$) protein degradation in IPEC-1 cells (Table 5).

Effects of rapamycin on protein turnover in IPEC-1 cells

Effects of rapamycin on protein synthesis and protein degradation in IPEC-1 cells cultured in the presence or absence of 2-mM AKG are summarized in Table 6.

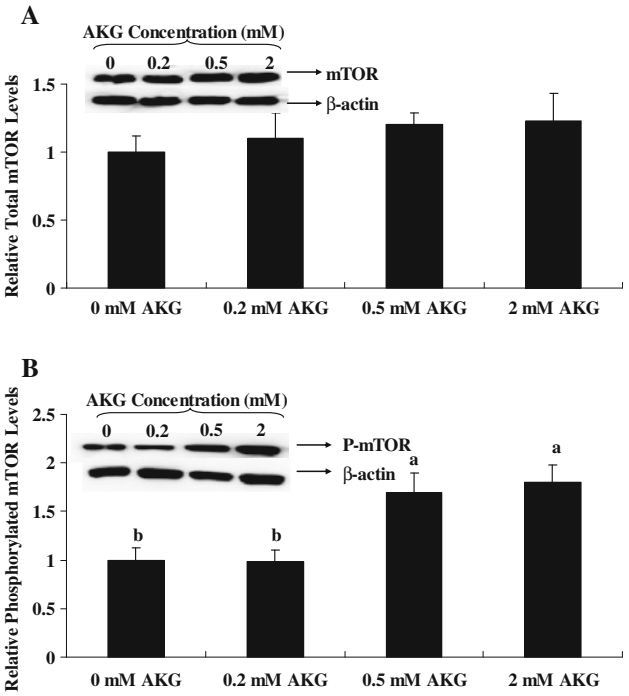

Fig. 1 Relative protein levels for total mTOR (**a**) and phosphorylated mTOR (**b**). IPEC-1 cells were cultured for 3 days in a medium containing 0, 0.2, 0.5 or 2 mM of AKG. At the end of the 3-day culture, cells were used for analysis of total mTOR and phosphorylated mTOR proteins using Western blotting techniques. Data are expressed as means ± SEM, $n = 8$. Values within a row with different letters differ ($P < 0.05$)

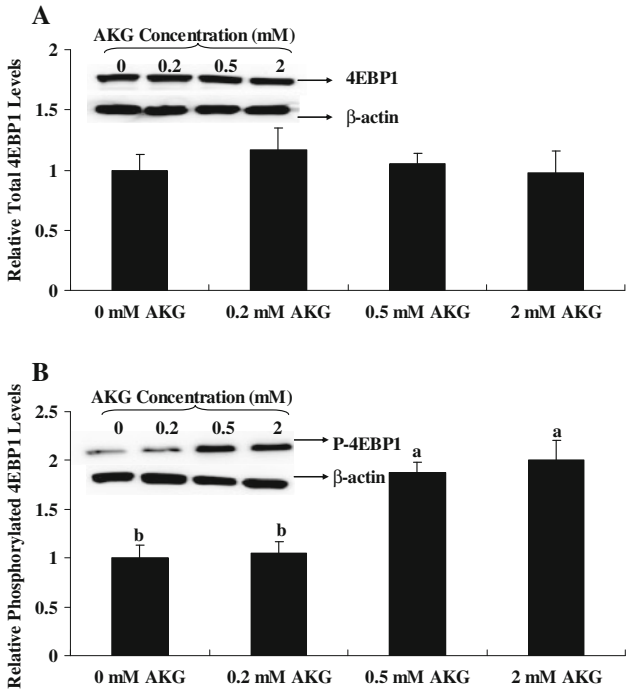

Fig. 2 Relative protein levels for total 4EBP1 (**a**) and phosphorylated 4EBP1 (**b**). IPEC-1 cells were cultured for 3 days in a medium containing 0, 0.2, 0.5 or 2 mM of AKG. At the end of the 3-day culture, cells were used for analysis of total 4EBP1 and phosphorylated 4EBP1 proteins using Western blotting techniques. Data are expressed as means ± SEM, $n = 8$. Values within a row with different letters differ ($P < 0.05$)

Compared with the control group (no rapamycin and no AKG), addition of 50 nM of rapamycin to the culture medium inhibited ($P < 0.05$) protein synthesis, but enhanced ($P < 0.05$) protein degradation in IPEC-1 cells. The rate of protein synthesis in the presence of 2 mM AKG plus 50 nM of rapamycin was lower ($P < 0.05$) than that in the presence of 2 mM AKG alone, but was higher ($P < 0.05$) than the value obtained in the presence of 50 nM of rapamycin alone.

Effects of AKG on mTOR, 4E-BP1 and S6K1 protein levels in IPEC-1 cells

Relative protein levels for total and phosphorylated mTOR, 4EBP1 and S6K1 in IPEC-1 cells are shown in Figs. 1, 2 and 3. Compared with the 0-mM AKG group, addition of 0.5 and 2 mM of AKG to culture medium increased ($P < 0.05$): (a) protein levels for phosphorylated mTOR in IPEC-1 cells by 71 and 80%, respectively (Fig. 1b); (b) protein levels for phosphorylated 4EBP1 by 88 and 101%, respectively (Fig. 2b); and (c) protein levels for phosphorylated S6K1 by 48 and 59%, respectively (Fig. 3b). In contrast, the addition of 0.2-mM AKG had no effect ($P > 0.05$) on protein levels for phosphorylated mTOR, 4EBP1 and S6K1. Moreover, AKG at 0.2, 0.5 and 2 mM did not affect ($P > 0.05$) protein levels for total mTOR, 4EBP1 and S6K1 in the cells, compared to the control group.

Discussion

The gastrointestinal tract actively participates in nitrogen recycling in the gut and plays an important role in nutrition and health (Bergen and Wu 2009; Wu 1998). Many stressful conditions, including fetal growth retardation, early weaning, infection and inflammation, are associated with intestinal mucosal injury and dysfunction, diarrhea and reduced growth (Wang et al. 2009b, 2010; Wu et al. 2009). There is evidence that nutritional modulation (e.g., AKG supplementation) can ameliorate damage of the small intestine in compromised neonates (Hou et al. 2010, 2011). This low-cost method has far-reaching significance for both human medicine and animal management. At present, the underlying mechanisms for the beneficial effects of AKG on the intestine remain elusive.

AKG has multiple pathways for utilization by intestinal cells (Table 3). Of particular note is the extensive

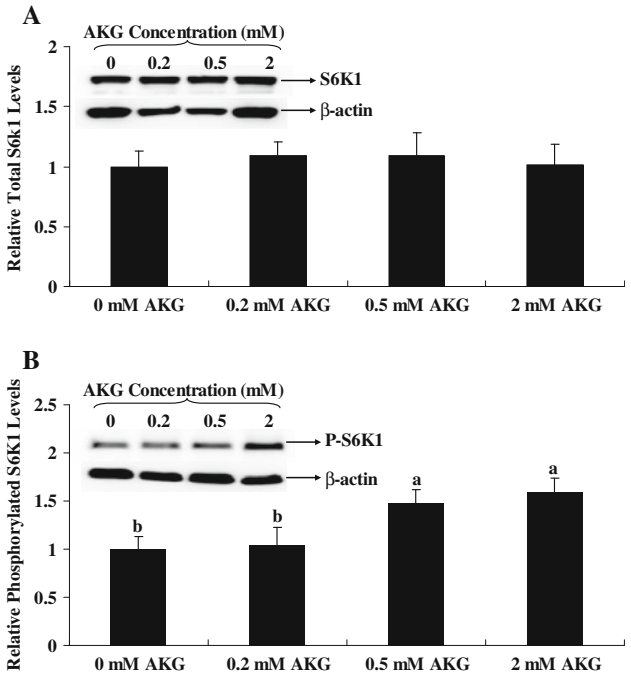

Fig. 3 Relative protein levels for total S6K1 (**a**) and phosphorylated S6K1 (**b**). IPEC-1 cells were cultured for 3 days in a medium containing 0, 0.2, 0.5 or 2 mM of AKG. At the end of the 3-day culture, cells were used for analysis of total S6K1 and phosphorylated S6K1 proteins using Western blotting techniques. Data are expressed as means ± SEM, $n = 8$. Values within a row with different letters differ ($P < 0.05$)

oxidation of AKG by the small intestine of neonates, including piglets (Lambert et al. 2002, 2006). Results of the present study indicate that AKG was rapidly oxidized in porcine intestinal epithelial cells (Table 4). This data is consistent with the presence of a high activity of AKG dehydrogenase in these cells (Wu et al. 2005; Yin et al. 2010b), which facilitates oxidation of AKG via the tricarboxylic acid cycle (Blachier et al. 2009). Furthermore, we found that addition of AKG to culture medium reduced glutamine catabolism and increased intracellular concentrations of both glutamate and glutamine in a dose-dependent manner (Table 3). Such a result can be explained by an inhibition of glutamate catabolism via glutamate transaminases or glutamate dehydrogenase by AKG, which is a product of both enzymes, leading to accumulation of glutamate in cells (Table 3). Because phosphate-activated glutaminase in intestinal cells is sensitive to inhibition by glutamate (Curthoys and Watford 1995), accumulation of glutamate in IPEC-1 cells would result in inhibition of glutamine degradation and, therefore, elevated concentrations of glutamine in the cells (Table 3). Identifying the complex mechanisms responsible for these effects of AKG are resource- and labor-intensive, and is beyond the scope of the current study. It is possible that AKG inhibits glutamine transport by cells, affect the expression of phosphate-dependent glutaminase or directly inhibit the catalytic activity of glutaminase. Future research is warranted to address these issues.

It has been suggested that AKG plays a particular role as an oxidative fuel for the gut, and, therefore, may spare such metabolic fuels as glutamate and aspartate (Junghans et al. 2006; Sliwa 2006). Here, we found that AKG at 0.2–5 mM reduced glutamine utilization by IPEC-1 cells in a dose-dependent manner. This observation does not appear to be consistent with the reports from in vivo studies (Kristensen et al. 2002; Lambert et al. 2002) that the net portal appearance of glutamate and glutamine was not affected by dietary AKG supplementation. However, in these studies, the supplemental doses of AKG in the diets might not have yielded a sufficiently high concentration of AKG in the lumen of the pig small intestine to affect intestinal glutamine or glutamate metabolism. Unfortunately, no data on AKG concentrations in the lumen of the pig small intestine were reported by Kristensen et al. (2002) or Lambert et al. (2002). Additionally, glutamine and glutamate are also metabolized by bacteria in the lumen of the pig small intestine (Dai et al. 2010, 2011) other than intestinal mucosal cells (Stoll et al. 1999; Wu et al. 2005), and the entry of these two amino acids into the portal vein may not necessarily reflect the effects of AKG on their utilization by enterocytes. We cannot rule out the possibility that in the in vivo studies involving growing pigs, a quantity of glutamine formed from AKG may enter the portal circulation. However, because glutamine in plasma has a short physiological half-life (0.6–0.7 h) and because the endogenous flux of glutamine is particularly high in young pigs (Wu et al. 2011a), a significant increase in the circulating levels of glutamine may not be readily detected in response to AKG supplementation.

Glutamine, which is beneficial for enterocytes (Ko et al. 1993; Papaconstantinou et al. 2000), is known to activate the mTOR pathway in the intestine (Rhoads et al. 1997; Rhoads and Wu 2009). Thus, by increasing concentrations of glutamine in cells, AKG may affect the mTOR signaling, a major mechanism for the regulation of protein synthesis in cells (Jobgen et al. 2009; Li et al. 2009a; Odenlund et al. 2009). Therefore, mTOR plays a crucial role in the control of cell growth and proliferation (Davis et al. 2002; Sarbassov et al. 2005). The mTOR signaling pathway includes its two downstream target proteins: S6K1 and 4EBP1 (Suryawan and Davis 2011). The results of the current work indicate that elevation of extracellular AKG resulted in increases in the phosphorylated (active state) levels of mTOR, 4EBP1 and S6K1 in intestinal cells (Fig. 1, 2, 3), thereby promoting the initiation of protein synthesis (Frank et al. 2006, 2007). The activation of mTOR signaling (Figs. 1, 2, 3) is consistent with our finding that the addition of 0.5 and 2 mM of AKG to the culture medium enhanced protein synthesis in IPEC-1 cells

(Table 5) and the number of cells at the end of the 3-day culture (the Results section) when compared with 0 or 0.2 mM of AKG. Interestingly, although AKG can modulate mTOR activation (Fig. 1), the stimulatory effect of AKG on protein synthesis is not only mediated by rapamycin-sensitive signaling (Table 6).

In contrast to arginine (Tan et al. 2010), AKG did not affect protein degradation in enterocytes (Table 5). Thus, enhanced phosphorylation of the mTOR protein alone may not be sufficient to influence intracellular protein degradation in the small intestine. However, inactivation of mTOR by 50-nM rapamycin clearly led to enhanced proteolysis in IPEC-1 cells (Table 6). These results provided the first line of evidence to support an important role for mTOR in inhibiting intracellular protein degradation. To our knowledge, this is the first study to evaluate the effects of AKG on protein turnover in intestinal epithelial cells. Collectively, our findings provide a biochemical basis to explain the previous reports that dietary supplementation with AKG increased jejunal villus height, intestinal mass and whole-body growth in young pigs (Hu et al. 2008; Hou et al. 2010).

In summary, AKG is extensively oxidized to CO_2 by IPEC-1 cells. Addition of AKG to culture medium inhibits glutamine catabolism, increases intracellular concentrations of glutamine in the cells, enhances phosphorylation of mTOR, 4EBP1 and S6K1 proteins, and stimulates protein synthesis in these cells. These novel findings not only aid in understanding the mode of actions of AKG in intestinal cells, but also have important implications for clinical nutrition and animal production.

Acknowledgments This research was jointly supported by the National Natural Science Foundation of China (30901041, 30901040, 30928018, 30972156, 30871801, 30828024, 30828025, 30771558 and 30700581), National 863 Project (2008AA10Z316), National Basic Research Project (2009CB118800), the CAS/SAFEA International Partnership Program for Creative Research Teams, the Program for Innovative Research Groups of Hubei Provincial Natural Science Foundation (Grant No. 2007ABC009), Texas AgriLife Research, American Heart Association (10GRNT4480020) and the Thousand-People-Talent program at the China Agricultural University.

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