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ORIGINAL ARTICLE

L-Glutamine deprivation induces autophagy and alters the mTOR and MAPK signaling pathways in porcine intestinal epithelial cells

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Abstract L-Glutamine (Gln) is an essential amino acid for intestinal growth and integrity. However, the underlying molecular mechanisms are not fully known. In the present study, porcine intestinal epithelial cells (IPEC-1) were used to test the hypothesis that autophagy is induced by Gln deprivation and inhibited by Gln supplementation. After a 2-day period of growth in normal medium, IPEC-1 cells were transferred to a Gln-free custom-made DMEM. Cell numbers, the distribution of autophagosomes, the abundance of the protein for an autophagy marker LC3B, as well as abundances of the mTOR and MAPK proteins during an 8-h period were determined. Furthermore, the rescue effect of 5 mM Gln was evaluated. Our results showed that Gln deprivation reduced the cell number, while enhancing the accumulation of autophagosomes and the expression of LC3B-II in IPEC-1 cells within 8 h. The concentrations of Glu, Asp, Cit, Arg, Leu, Ile, Val, Ala, β-Ala, Orn, Phe, Met and Ser in the culture medium were altered by Gln deprivation. Further analysis revealed that Gln deficiency inactivated, but Gln supplementation activated, the mTOR and MAPK/ERK signaling pathways. Collectively, our findings support the notion that Gln deficiency induces autophagy and disturbs amino acid metabolism in intestinal epithelial cells, as well as attenuated their mTOR and MAPK/ERK signaling pathways to inhibit protein synthesis and cell proliferation.

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Abbreviations

Akt	Protein kinase B
AMPK	Adenosine 5′-monophosphate (AMP)-activated protein kinase
ASCT2	Na^+-neutral AA exchanger 2
Bcl-2	B-cell lymphoma 2
DMEM-F12	Dulbecco's modified Eagle's F12 Ham medium
ERK	Extracellular signal-related kinase
FBS	Fetal bovine serum
Gln	L-Glutamine
IPEC-1	Porcine intestinal epithelial cells-1
JNK	Jun N-terminal kinase
LC3B	Light chain 3B
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
S6K1	Ribosomal protein S6 kinase-1

Introduction

L-Glutamine (Gln) is an abundant amino acid in blood and plays a vital role in maintaining gut integrity in both humans and animals (Wu et al. 2011; Watford 1999). Studies have demonstrated that Gln is a major energy substrate for the rapid development of enterocytes and approximately 70 % of Gln in the enteral diet is degraded by rat and pig small intestines during the first pass (Wu 1998; Reeds and Burrin 2001). Furthermore, glutamine metabolism plays multiple roles in regulating intestinal gene expression (Wang et al. 2008), intracellular protein turnover (Xi et al. 2012; Wu et al. 2014), immune function (Ren et al. 2013; Zhong et al. 2012),

cell proliferation (Kim et al. 2013), and apoptosis (Papaconstantinou et al. 1998; Han et al. 2013). Of particular interest, Gln is capable of activating intestinal cell signaling-related kinases (Rhoads and Wu 2009). For example, Gln enhances intestinal cells proliferation via MAPKs (Rhoads et al. 2000), promote intestinal restitution and protein synthesis by activating mTOR signaling (Rhoads and Wu 2009; Kim et al. 2013), and improves cell survival by regulating heat shock proteins in the intestine (Wu et al. 2013c; Larson et al. 2007).

Gln is considered a conditionally essential amino acid under inflammatory and many other stress conditions (Wu et al. 2011; Wu 2013). Illness, burn, injury, and very low birth weight can lead to a significant decrease in plasma level of Gln, and an increase of Gln released from tissue protein degradation (Xi et al. 2011). Gln depletion has negative effects on gut integrity and epithelial function (Motoki et al. 2011; Lin et al. 2012; Papaconstantinou et al. 1998). Several studies have shown the efficacy of Gln supplementation in protecting intestinal epithelial tight junctions, preventing intestinal oxidative injury and atrophy, and stimulating protein synthesis and cell proliferation (Wu et al. 1996; Zhong et al. 2012; Haynes et al. 2009; Li and Neu 2009). Although these findings are encouraging, questions still remain regarding the exact mechanisms whereby Gln deprivation can cause instability of the intestinal epithelial cells and mucosal damage.

Autophagy (hereafter referring to macroautophagy), which is literally defined as self-eating, functions as a bulk protein-degradation system for cellular components that is classically induced by starvation or nutrient deprivation (Patel and Stappenbeck 2013; Mizushima 2007). When cells lack essential nutrients, autophagy is activated to supply amino acids. A classical example is that autophagy can be switched on during amino acid deprivation to maintain the amino acid pool in the rat liver, which is used for glucose synthesis to meet the energy requirements of the brain and erythrocytes (Mortimore and Schworer 1977). In addition, recent studies have indicated that autophagy has a greater variety of physiological and pathophysiological roles, including regulation of cell death, cell proliferation, inflammation, and numerous diseases such as cancer (Mizushima 2005). A series of signaling pathways including PI3K/Akt/mTOR, MAPKs, AMPK and DAPK, are involved in autophagic cell survival and cell death (Codogno and Meijer 2005). The contribution of autophagy is complicated by the fact that autophagy can be either protective or harmful, depending on the biological context (Mizushima 2007).

Interestingly, only certain amino acids are capable of modulating autophagy and their actions are highly cell-specific. Mordier et al. (2000) found that leucine restriction induced autophagy and accelerated the rate of protein breakdown in C2C12 myoblasts though a mTOR-independent way while Wu et al. (2012) reported the induction via targeting of *ULK1*. However, leucine deprivation caused the caspase-dependent apoptotic death of melanoma cells because it failed to activate autophagy (Sheen et al. 2011). There are also conflicting reports about arginine metabolism and autophagy (Garcia-Navas et al. 2012; Angcajas et al. 2014; Savaraj et al. 2010). Furthermore, physiologic concentration of Gln (0.7 mM in plasma) has been reported to increase autophagy by inactivation of mTOR and p38 MAP kinase pathways in rat intestinal epithelial IEC18 cells and human colonic epithelial Caco-2 cells (Sakiyama et al. 2009). However, whether Gln deprivation could induce autophagy and thus impair cell growth via certain signaling pathways remains to be determined.

Based on the foregoing, we hypothesized that autophagy can be induced by Gln deprivation and inhibited by Gln resupplementation in intestinal epithelial cells. This hypothesis was tested in the present study using porcine intestinal epithelial cells (IPEC-1), which metabolize glutamine in a similar way to freshly isolated enterocytes from neonatal pigs (Haynes et al. 2009).

Materials and methods

Reagents

Fetal bovine serum (FBS), dulbecco's modified Eagle's F12 Ham medium (DMEM-F12), Trypsin/EDTA and antibiotics (Penicillin–Streptomycin for Cell Culture) were from GIBCO (Carlsbad, CA, USA). Plastic culture plates were manufactured by Corning Inc. (Corning, NY, USA). High-performance liquid chromatography-grade water and methanol were procured from Fisher Scientific (Houston, TX, USA). L-Glutamine, insulin, and all other chemicals (unless indicated) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture and treatments

The porcine intestinal epithelial cells (IPEC-1) were kindly provided by Dr. Guoyao Wu (Texas A&M University, College Station, TX, USA). IPEC-1 cells were grown in serial passage in uncoated plastic culture flasks (100 cm^2) in DMEM-F12 containing 10 % FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Medium was changed every 2 days. At confluence, cells were trypsinized and seeded in 6-well cell culture plates with approximately 10^5 cells per well and grown in a humidified incubator at 37 °C with 5 % CO_2 and 95 % air. For the experiment of Gln deprivation, after reaching 70–90 % confluence, cells were gently washed with calcium-containing Dulbecco's phosphate-buffered saline and then transferred to a serum- and

Gln-free custom-made DMEM (modified DMEM # 08-5009EF, GIBCO). Concentrations of amino acids (AA) in the modified DMEM were similar to those found in the plasma of young pigs (Flynn et al. 2000). Cells were treated for 0.5, 1, 2, 4, 6, 8, 10, 12, 24 and 48 h. The medium was changed every 24 h. In Gln supplementation experiments, cells were cultured in the Gln-free basal medium supplemented with 5 mM Gln for 1 h after each treatment. Cells were collected, nap-frozen in liquid nitrogen, and stored at −80 °C for protein assays and Western blot analysis.

Determination of cell growth and protein concentrations

To determine effects of Gln on IPEC-1 cell growth, cells were seeded in 96-well cell culture plates with approximately 5×10^3 cells per well for cell counting. In some experiments, 1×10^5 cells were seeded in 6-well cell culture plates for determination of protein concentrations. The cells were grown to 50 % confluence in regular growth DMEM containing 10 % FBS and then changed to the serum- and Gln-free custom-made DMEM for each treatment. Cell numbers were determined using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan). Data acquisition and analysis were performed on a microplate reader using SpectraMax software (Molecular Devices, Inc. CA, USA) at 450 nm. Cellular protein was determined using the bicinchoninic acid (BCA) protein assay reagent (Applygen Technology, Inc., Beijing, China) at 562 nm.

Transmission electron microscopic analysis

The electron microscopy observation of cells was completed at College of Biological Sciences of China Agricultural University. In brief, approximately 10^7 cells were collected and fixed in 2.5 % glutaraldehyde solution diluted with 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h. Cells then were postfixed for 1 h in 0.1 M sodium cacodylate-buffered (pH 7.4) solution containing 1 % OsO_4. After dehydration in an ethanol gradient (70 % ethanol for 20 min, 96 % ethanol for 20 min, 100 % ethanol for 2×20 min), cells were incubated with propylene oxide (2×10 min), impregnated with a mixture of propylenoid/LX-112 (1:1; Ladd Research Industries, Williston, VT, USA) and embedded in LX-112. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined under a Jeol-100 CX II transmission electron microscopy (TEM) at 80 kV. Photographs were captured with a digital camera (Martinet et al. 2006).

Western blot analysis of signaling pathways proteins

RIPA Lysis Buffer C1053 (Applygen Technology, Inc., Beijing, China) with 1× protease inhibitor cocktail and 1× phosphatase inhibitor cocktail was used to lyse IPEC-1 cells. Debris was removed through centrifugation at 12,000×*g* for 15 min at 4 °C and the total protein concentration was detected using the BCA Protein Assay Kit according to the manufacturer's instructions (Applygen Technology, Inc., Beijing, China).

The extracted protein sample (typically 30 μg) was denatured at 95 °C for 5 min in a loading buffer containing 5 % β-mercaptoethanol, separated in parallel by 5–10 % (5–15 % for LC3B and Bcl-2 determination) sodium dodecyl sulfate–polyacrylamide (SDS-PAGE) gel electrophoresis (Bio-Rad, Richmond, CA, USA). Fractionated proteins were elelctroblotted (Bio-Rad) onto a single polyvinylidene difluoride (PVDF) membrane (0.45 μm, Millipore, Billerica, MA, USA). Membranes with transferred proteins were blocked for 1 h at room temperature in blocking buffer containing 5 % nonfat milk in TBST (0.05 % Tween 20, 100 mM Tris–HCl and 150 mM NaCl, pH 7.5). The membranes were first incubated with primary antibodies diluted in 5 % nonfat milk in TBST (5 % BSA for phosphospecific antibodies) for overnight at 4 °C. After washed three times with TBST, the membranes were incubated with secondary antibody (1 h, Applygen Technology, Inc., Beijing, China). Enhanced chemifluorescence detection reagents (ECL-plus, Amersham Biosciences, Sweden) were applied for 3 min, and the chemifluorescence was detected using the Image Quant LAS 4000 mini system (GE Healthcare Bio-sciences AB, Inc., Sweden), and quantified using a gel-imaging system with Image Quant TL software (GE Healthcare Life Science, Inc., USA). At last, all membranes were stripped and re-probed with β-actin (mouse monoclonal, 1:2,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for loading control. All Western blot experiments were performed in triplicate for each cell sample to ensure reproducibility of results. The primary antibodies used are listed in Table 1.

Determination of amino acids by HPLC

The concentrations of amino acid in culture DMEM were determined by HPLC using reverse-phase C18 column after derivatization with o-phthaldialdehyde reagent using the method described by Wu et al. (1997). In brief, the collected medium was centrifuged at 3,000×*g* for 2 min to remove any dead cells. The supernatant fluid (0.5 ml) was acidified with 0.5 ml of 1.5 M $HClO_4$, followed by addition of 0.25 ml of 2 M K_2CO_3. The neutralized extract was analyzed for pre-column derivatization with o-phthaldialdehyde in Waters HPLC (Model Alliance e2695 Separation Module), as described by Dai et al. (2014a). Fluorescence of amino acid-o-phthaldialdehyde derivatives was detected using Waters 2475 Multi λ Fluorescence Detector (Dai et al. 2014b).

Table 1 Information of primary antibodies used for Western blot analysis

Antibody	Catalog no.	Source	Clone	MW (kDa)	Dilution ratio	Company
LC3B	L7543	Rabbit	Poly	16, 18	1:2,000	Sigma
Bcl-2 (50E3)	#2870	Rabbit	Mono	26	1:1,000	Cst
Phospho-AMPKα (Thr172) (40H9)	#2535	Rabbit	Mono	62	1:1,000	Cst
AMPKα	#2532	Rabbit	Poly	62	1:1,000	Cst
Phospho-Akt (Ser473)	#4058	Rabbit	Mono	60	1:1,000	Cst
Akt	#4691	Rabbit	Poly	60	1:1,000	Cst
Phospho-mTOR (Ser2448)	#2971	Rabbit	Poly	289	1:1,000	Cst
mTOR	#2972	Rabbit	Poly	289	1:1,000	Cst
Phospho-p70 S6 kinase (Thr389)	#9205	Rabbit	Poly	70, 85	1:1,000	Cst
p70 S6 Kinase	#9202	Rabbit	Poly	70, 85	1:1,000	Cst
ASCT2/SLC1A5	ARP42247	Rabbit	Poly	60	1:1000	Aviva
Phospho-p44/42 MAPK	#9101	Rabbit	Poly	42, 44	1:1,000	Cst
p44/42 MAPK (Erk1/2)	#9102	Rabbit	Poly	42, 44	1:1,000	Cst
Phospho-p38 MAP kinase	#9211	Rabbit	Poly	43	1:1,000	Cst
p38 MAPK	#9212	Rabbit	Poly	43	1:1,000	Cst
Phospho-SAPK/JNK (Thr183/Tyr185)	#9251	Rabbit	Poly	46, 54	1:1,000	Cst
SAPK/JNK	#9252	Rabbit	Poly	46, 54	1:1,000	Cst

MW molecular weight, *Sigma* sigma-aldrich corporation, *Cst* cell signaling technology, *Aviva* aviva systems biology

Statistical analysis

Values are expressed as mean ± SEM. Results were analyzed by one-way ANOVA using the SAS programs (version 9.0, SAS Institute Inc., Cary, NC, USA). Differences among treatment means were determined using the Student–Newman–Keuls multiple comparison test. Probability values ≤0.05 were taken to indicate statistical significance (Wei et al. 2012).

Results

Gln deprivation inhibits IPEC-1 cell growth

To determine the effect of Gln deprivation on IPEC-1 cell growth, cells were cultured in Gln-free DMEM for 0, 1, 2, 4, 6, 8, 10, 12, 24 and 48 h. The optical density representing the cell number was observed at 450 nm after adding CCK-8 for 2 h. The cell number (expressed as OD_{450}) exhibited a marked decrease in a time-dependent manner in the Gln deprivation group (Gln−) compared with the control group ($P < 0.05$) (Fig. 1). The cells barely grew upon Gln deprivation, compared with the cell number at 0 h.

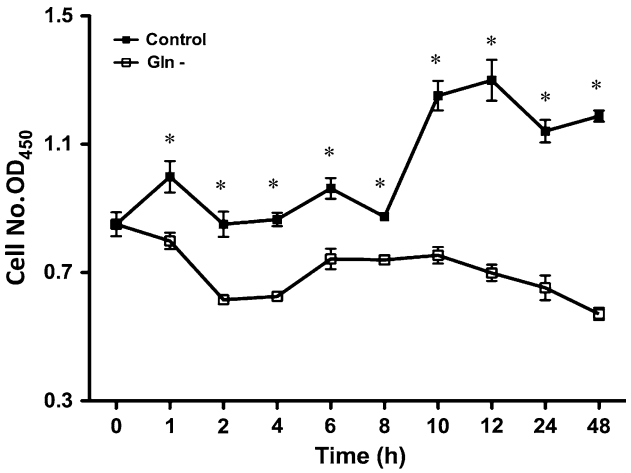

Fig. 1 Growth curves of IPEC-1 cells cultured in control or Gln deprivation (Gln−) medium for 0–48 h. IPEC-1 cells were cultured in normal DMEM/F12 containing 10 % FBS (Control) or in Gln-deprived medium (Gln−) for 0–48 h. Cell numbers were represented as the optical density at 450 nm (OD_{450}) after addition of CCK-8 reagents. Cells were seeded in 96-well cell culture plates, $n = 15$. Data with *error bars* represent mean ± SEM, $^*P < 0.05$

Gln deprivation induces autophagy in IPEC-1 cells

To determine whether Gln deprivation could induce autophagy in IPEC-1 cells, a time course experiment of Gln deprivation was carried out. Western blot analysis revealed an increase ($P < 0.05$) in the autophagy marker LC3B-II in IPEC-1 cells in a time-dependent manner, with the peak value at 8 h after Gln deprivation (Fig. 2a). Cells were then processed for electron microscopy to explore the autophagosomes accumulating at 0, 0.5, 1, 2, 4 and 8 h after deprivation of Gln. Under normal culture conditions

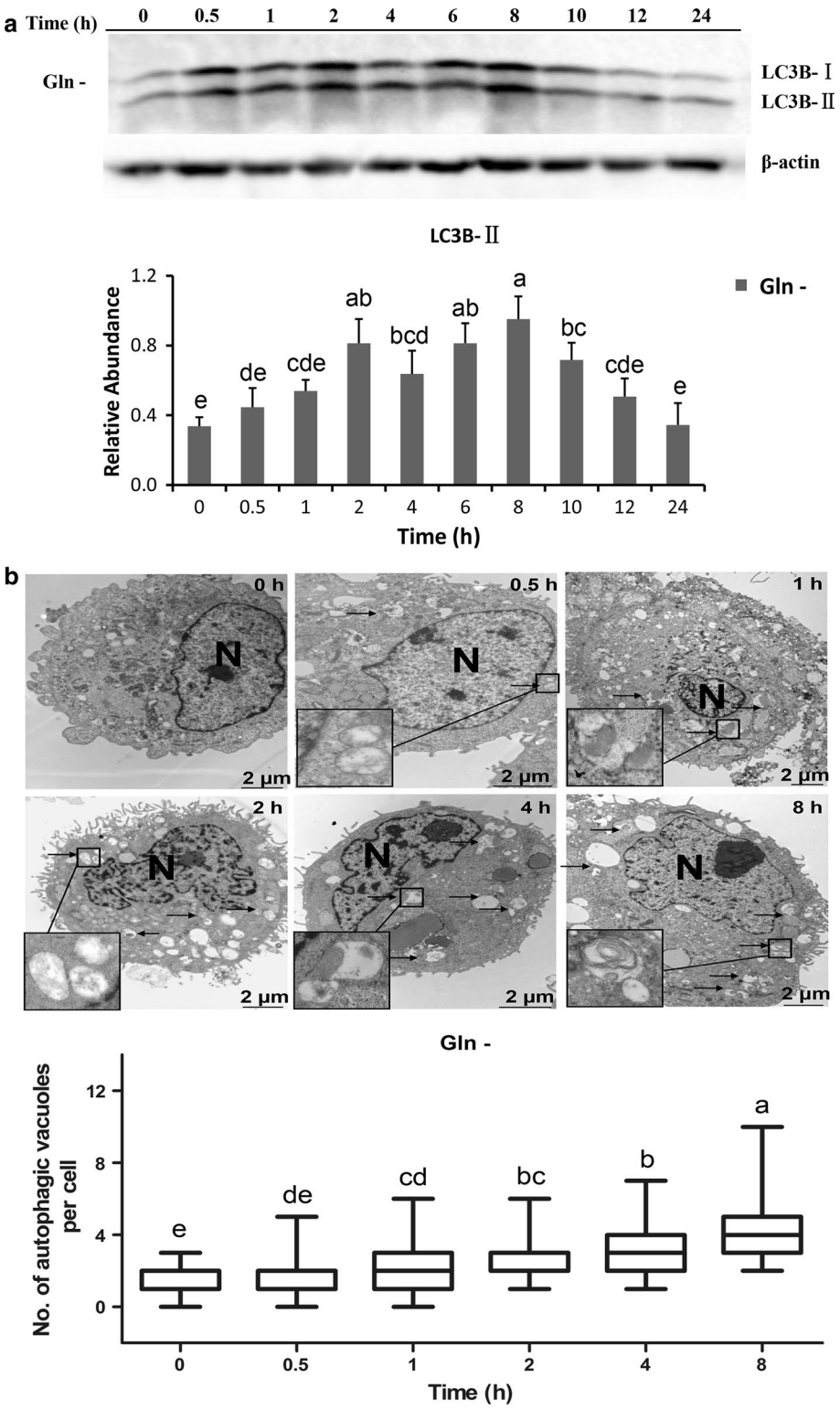

Fig. 2 Gln deprivation induces autophagy in IPEC-1 cells. **a** Western blot analysis and quantification of the autophagy marker LC3B in IPEC-1 cells cultured in Gln-deprived medium (Gln−) for 0–24 h. The autophagy level was reflected by the expression of LC3B-II. Equal loading was assessed by β-actin immunoblotting. All data are representative of at least three independent experiments. Letters without a common letter differ are significantly different among the treatment groups ($P < 0.05$). **b** Representative electron micrographs of IPEC-1 cells cultured in Gln-deprived medium for 0, 0.5, 1, 2, 4 and 8 h. Double-membrane autophagic vacuoles were denoted by *black arrows*. *N* represents nucleus. The number of autophagic vacuoles per cell was calculated and presented below ($n = 40$). Data with error bars represent mean ± SEM. Letters without a common letter differ are significantly different among the treatment groups ($P < 0.05$)

(e.g., 0 h), cells exhibited a stable and low expression of autophagic vacuoles, while Gln deprivation certainly increased autophagy in IPEC-1 cells, with the autophagosomes numbers increasing from 1.3 to 4.2 per cell within 8 h of Gln deprivation (Fig. 2b).

Lower concentrations of free amino acids present in Gln deprivation medium

The concentrations of free amino acids in control and Gln deprivation medium were determined. The concentrations

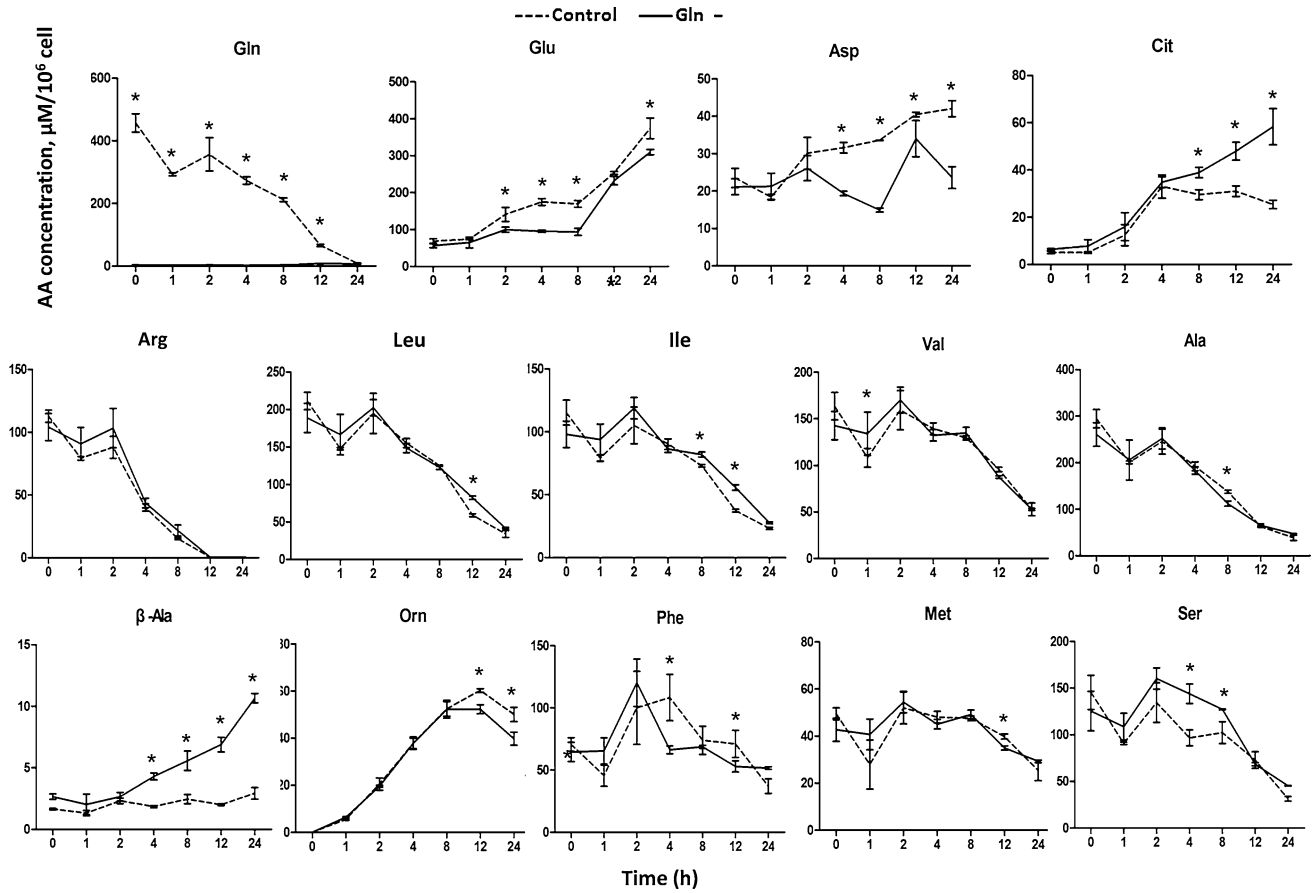

Fig. 3 Comparison of time-dependent changes in free amino acid concentrations in the control and Gln deprivation medium. The medium samples were collected after cell culturing for 0–24 h. The concentrations of 22 amino acids in control and Gln-deprived medium were analyzed by HPLC as described in materials and methods and normalized per 10^6 cells. 14 amino acids included Gln, Glu, Asp, Cit, Arg, Leu, Ile, Val, Ala, β-Ala, Orn, Phe, Met and Ser are shown in the figure. Data with *error bars* represent mean ± SEM of at least three independent experiments. *$P < 0.05$ between the control and Gln deprivation groups

of six amino acids, including Glu, Asp, Ala, Orn, Phe and Met, decreased upon Gln deprivation, while seven amino acids, including Cit, Arg, Leu, Ile, Val, β-Ala and Ser, increased in Gln deprivation group, compared with the control group ($P < 0.05$) (Fig. 3). The differences in AA concentrations between the Gln deprivation group and the control group were increased after 2-h culture.

Gln supplementation enhanced cell number and cellular protein content and repressed autophagy in IPEC-1 cells

Rescue experiments were conducted to test the hypothesis that supplementing 5 mM Gln to the Gln-free basal medium could inhibit autophagy. Notably, after 5 mM Gln was added to the starvation medium for 1 h, this treatment (Gln+) enhanced the cell number ($P < 0.05$) (Fig. 4a). Total protein concentrations of IPEC-1 cells after 0.5, 4 and 8 h Gln deprivation were also increased after Gln supplementation for 1 h ($P < 0.05$) (Fig. 4b). The number of autophagic vacuoles decreased in 2 and 4 h in the Gln+ group, compared with the Gln-group (Fig. 4c). Accordingly, the abundance of the LC3B-II protein was decreased in the Gln+ group ($P < 0.05$) (Fig. 4d). In addition, the cellular level of Bcl-2, a newly identified inhibitor of autophagy, was reduced upon Gln deprivation ($P < 0.05$), while Gln supplementation reversed its suppression ($P < 0.05$) (Fig. 4e).

Activation of AMPK and inactivation of the mTOR signaling pathway during Gln deprivation in IPEC-1 cells

Gln deprivation stimulated AMP kinase activity, with the ratio of p-AMPK to AMPK increasing in a time-dependent manner ($P < 0.05$) (Fig. 5a). Gln deprivation greatly reduced mTOR phosphorylation ($P < 0.05$), while Gln supplementation markedly increased the level of the phosphorylated mTOR ($P < 0.05$) (Fig. 5c). Similarly, the phosphorylated level for the Akt protein was reduced ($P < 0.05$) at 2, 4 and 8 h after deprivation of Gln, whereas Gln

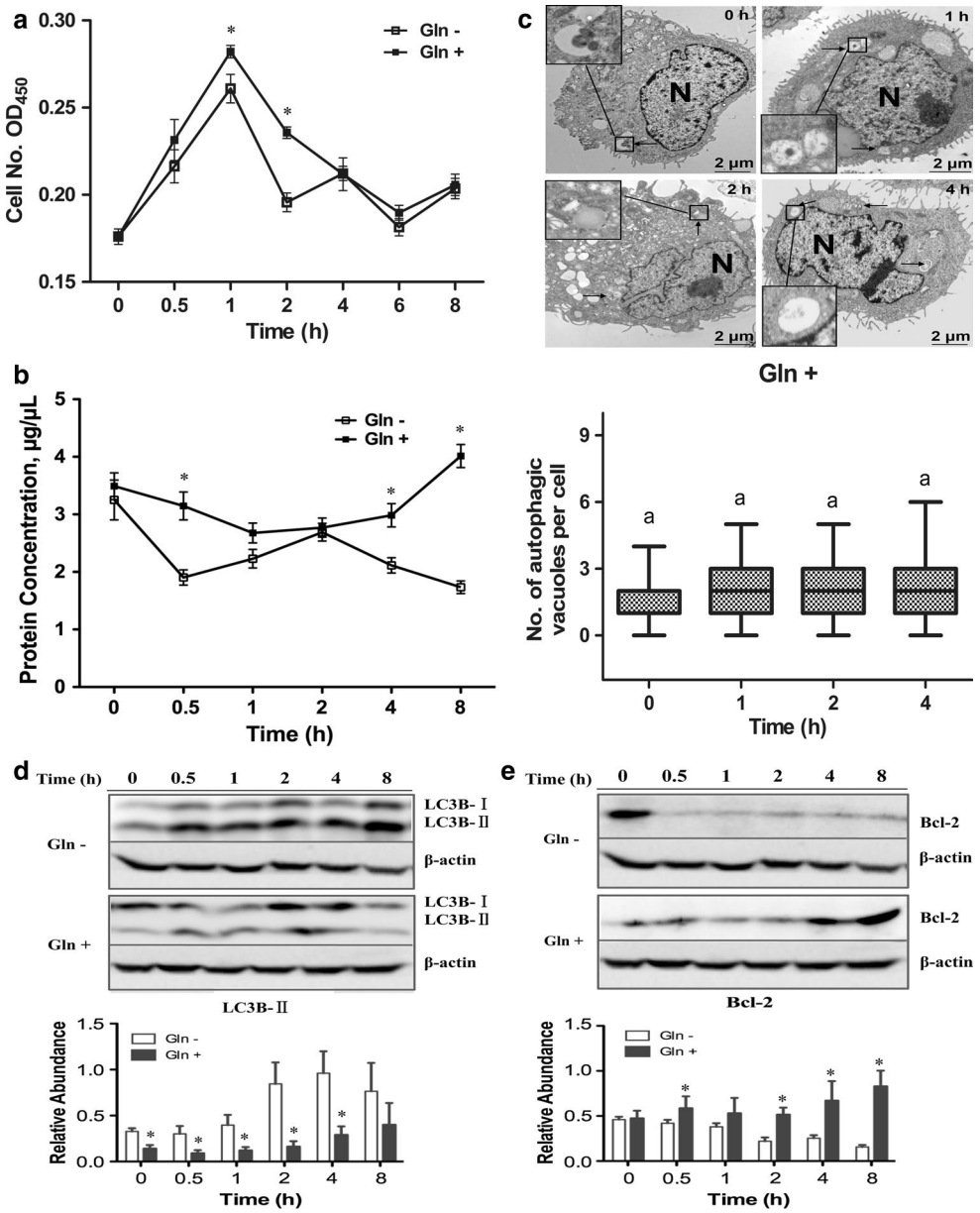

Fig. 4 **a** IPEC-1 cell number in the Gln deprivation group (Gln−) and the Gln supplementation group (Gln+). Cells were cultured in the Gln-deprived medium (Gln−) or Gln-supplemented medium (5 mM Gln) after cultured in the Gln deprivation medium (Gln+) for 0–8 h. Cell numbers were represented as the optical density at 450 nm (OD_{450}) after addition of CCK-8 reagents. Cells were seeded in 96-well cell culture plates, $n = 15$. **b** Total protein concentration of IPEC-1 cells cultured with (Gln−) or (Gln+) medium for 0–8 h. Approximately 3×10^6 cells were collected, 100 μl RIPA lysis buffer were added, and then cellular protein was determined using the BCA protein assay reagent. The standard curve was prepared with BSA in concentration gradient. **c** Representative TEM images of IPEC-1 cells from Gln-supplemented medium (5 mM Gln) after culture in Gln deprivation medium for 0, 1, 2 and 4 h (Gln+). Double-membrane autophagic vacuoles were denoted by *black arrows*. *N* represents nucleus. The number of autophagic vacuoles per cell was calculated and presented below ($n = 25$). Letters without a common letter differ are significantly different among treatment groups ($P < 0.05$). **d** Western blot analysis of LC3B in IPEC-1 cells cultured in Gln deprivation medium (Gln−) and Gln-supplemented medium (5 mM Gln) after culture in Gln deprivation medium for 0–8 h (Gln+). **e** Western blot analysis and quantification of the autophagy inhibitor Bcl-2 in (Gln−) and (Gln+) cells. Equal loading was assessed by β-actin immunoblotting. Data with *error bars* represent mean ± SEM of at least three independent experiments, $^*P < 0.05$

supplementation increased the phosphorylation of Akt at 0, 0.5 and 1 h (Fig. 5b). Similar results were observed for the level of phosphorylated p70S6k ($P < 0.05$) (Fig. 5d).

Gln transporter ASCT2 (also known as SLC1A5), which transports the extracellular Gln into the cell, exhibited a time-dependent decrease in protein abundance in

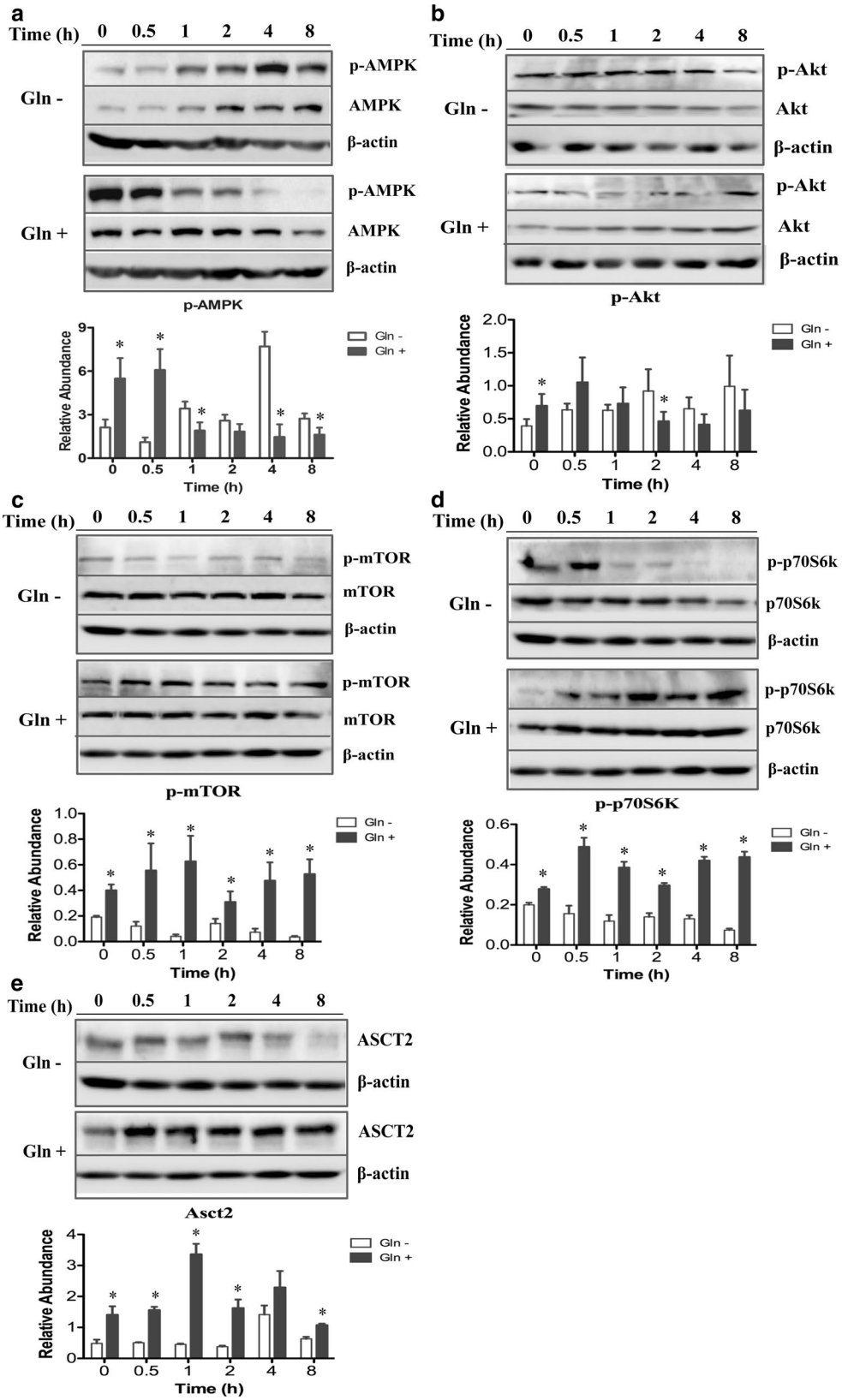

Fig. 5 Gln regulates the AMPK/Akt/mTOR signaling pathway as well as the Gln transporter ASCT2 in IPEC-1 cells. Representative Western blots for phosphorylated AMPK (p-AMPK) and total AMPK (**a**), Akt (**b**), mTOR (**c**) and p70S6 k (**d**) in IPEC-1 cells cultured in Gln deprivation medium (Gln−) and Gln-supplemented medium (5 mM Gln) after culture in Gln deprivation medium for 0–8 h (Gln+). Relative abundance was represented as phosphorylated protein to total protein expression. **e** Western blot analysis and quantification of the Gln transporter ASCT2 in IPEC-1 cells cultured in the Gln-deprived medium (Gln−) and Gln-supplemented medium (5 mM Gln) after culture in Gln deprivation medium for 0–8 h (Gln+). Equal loading was assessed by β-actin immunoblotting. Data with *error bars* represent mean ± SEM of at least three independent experiments, $^*P < 0.05$

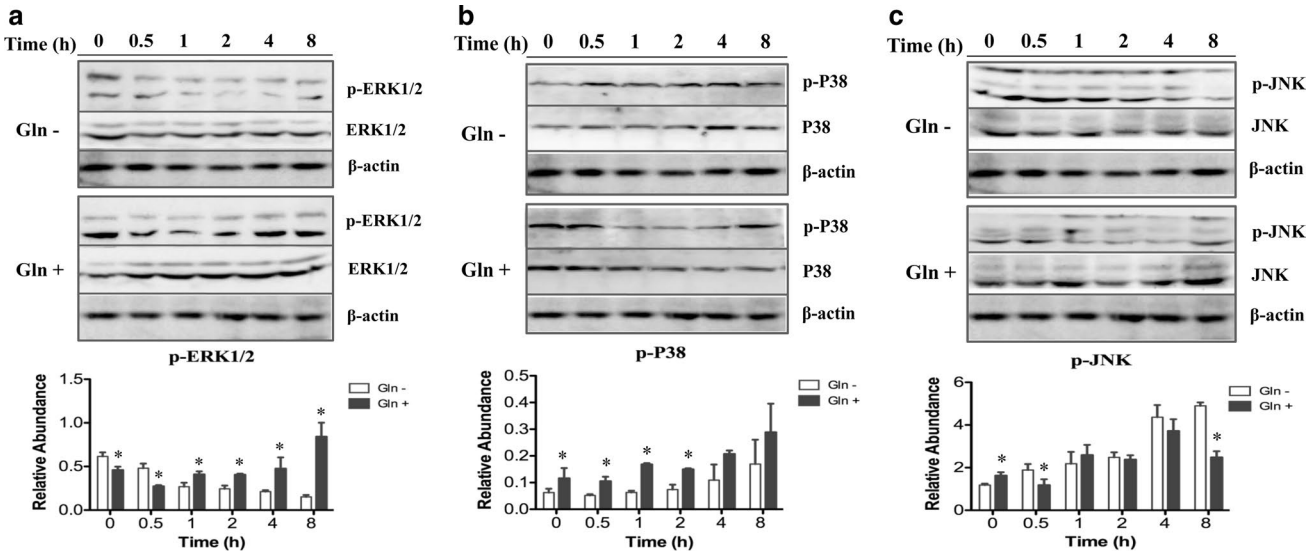

Fig. 6 Gln deprivation altered the MAPK signaling pathways in IPEC-1. Representative Western blots for phosphorylated ERK1/2 (p-ERK1/2) and total ERK1/2 (**a**), JNK (**b**), and P38 (**c**) in IPEC-1 cells cultured in the Gln-deprived medium (Gln−) and Gln-supplemented medium (5 mM Gln) after culture in the Gln-deprived medium for 0–8 h (Gln+). Relative abundance was represented as phosphorylated protein to total protein expression. Equal loading was assessed by β-actin immunoblotting. Data with *error bars* represent mean ± SEM of at least three independent experiments, $^*P < 0.05$

IPEC-1 cells cultured in Gln deprivation medium for 0–8 h ($P < 0.05$). Gln supplementation increased ASCT2 expression remarkably ($P < 0.05$), compared with the absence of Gln (Fig. 5e).

Alteration of the MAPK signaling pathways in autophagic IPEC-1 cells in response to Gln deprivation

Effects of Gln deprivation and supplementation on phosphorylation of the ERK1/2 protein in IPEC-1 cells are illustrated in Fig. 6a. Gln deprivation reduced the phosphorylation of the ERK1/2 protein, with the ratio of p-ERK1/2 to ERK1/2 decreasing in a time-dependent manner ($P < 0.05$). Gln supplementation increased the level of phosphorylated ERK1/2 ($P < 0.05$) (Fig. 6a). However, the phosphorylation level of P38 was elevated in response to either Gln deprivation or Gln supplementation at 0.5, 1 and 2 h after treatment ($P < 0.05$) (Fig. 6b). Similar results were obtained for the JNK protein (Fig. 6c).

Discussion

L-Glutamine is a conditionally essential amino acid for infants and piglets (Wu 1998). The small intestine accounts for one-third of Gln utilization in the body and Gln plays an important role in maintaining gut integrity and mucosal cell proliferation (Mates et al. 2002; Klimberg and Souba 1990; Souba et al. 1985; Wu et al. 2011). However, illness, injury, and low birth weight could lead to a significant decrease of Gln both in the plasma and the intestine (Motoki et al. 2011; Wu et al. 1994). Gln depletion can cause instability of the intestinal epithelial alignment by increased apoptosis (Motoki et al. 2011; Papaconstantinou et al. 1998) or impaired tight junctions (Li and Neu 2009). However, the molecular events triggered by the absence of Gln are largely unknown. Elucidating the mechanisms whereby Gln deprivation affects IPEC-1 cell growth will be helpful for developing new nutritional strategies to improve intestinal health, growth and development.

Autophagy has a greater variety of physiological and pathophysiological roles, including regulation of cell death, cell proliferation, inflammation, and numerous diseases (Mizushima 2005). Certain amino acids are capable of modulating autophagy in a highly cell-specific manner. Given the conflicting results published in the literature, the present study was conducted to test the hypothesis that autophagy can be induced during Gln deprivation and reversed by Gln supplementation in intestinal epithelial cells. Our results demonstrated unequivocally that Gln deprivation induced autophagy, disturbed amino acid metabolism, and inactivated mTOR and MAPK/ERK signaling pathways in IPEC-1 cells.

First, we developed a Gln deprivation model in vitro by culturing IPEC-1 cells in custom-made basal medium that contained physiological levels of all amino acids but no Gln. As previously reported (Papaconstantinou et al. 1998; Motoki et al. 2011), Gln deprivation dramatically

reduced intestinal cell growth. This may result from not only decreased protein synthesis but also increased protein degradation in IPEC-1 cells. In the whole body, autophagy is considered an essential process for cell survival in response to nutrient deprivation and environmental stress. It has long been known that autophagy is induced by amino acid deprivation (Mortimore and Schworer 1977). For example, leucine deprivation accelerates protein breakdown though induction of autophagy in liver and skeletal muscle (Blommaart et al. 1995; Mordier et al. 2000). By examining the protein expression of LC-3B-II and formation of autophagic vacuoles, we found that IPEC-1 cells underwent autophagy in the face of Gln deficiency, which is a major metabolic fuel for enterocytes (Wu 2009). This process causes the atrophy of the small-intestinal mucosa, leading to reduced catabolism of dietary amino acids (Wu 1998) as an adaptive mechanism for protein malnutrition. Importantly, Gln supplementation reduced the abundance of the LC3B-II protein and the number of autophagosome in IPEC-1 cells, emphasizing the importance of this amino acid in intestinal epithelial cell homeostasis. These results are consistent with those from the previous studies involving different models (Nicklin et al. 2009; Ko et al. 2011). It is unlikely that activation of autophagy by Gln deficiency has a protective role for intestinal cell survival, because a marked decrease in the IPEC-1 cell number was observed upon Gln deprivation. The underlying causes may be complex, which may include a decrease of Bcl-2 protein abundance, a protein that functions to inhibit both autophagy and apoptosis (Xu et al. 2013). This implicates that apoptosis of intestinal epithelial cells can be triggered by Gln deprivation alone. In support of this view, there are reports that Gln deficiency increased apoptosis through enhancing fragmentation of DNA and caspase-3 activity in rat intestinal epithelial cell (Larson et al. 2007). Moreover, we found that the expression of tight junction proteins in IPEC-1 cells was reduced in response to Gln deprivation.

The current study also sheds light on the reduction of total protein concentration in intestinal cells during Gln deprivation. As noted above, protein degradation is augmented and protein synthesis is suppressed in IPEC-1 cells via the mTOR cell signaling pathway (Xi et al. 2012). This mechanism may also contribute to the activation of autophagy brought about by Gln deprivation (Yao et al. 2012). Recently, Ezaki et al. (2011) found that amino acids released from hepatic autophagy could enter the blood, thus causing a transient increase in concentrations of free amino acids in the liver and plasma. In our study, we observed seven free amino acids increased in Gln deprivation group probably as a result of autophagy induction. In contrast, concentrations of six free amino acids in culture medium were decreased by Gln deprivation, suggesting increases in the catabolism of these amino acids and/or in their utilization via metabolic pathways other than protein synthesis. Rapid dividing intestinal cells may need more amino acids to maintain cellular viability than their quantities produced by autophagy.

It is well known that Gln stimulates numerous signaling pathways in the intestine (Rhoads and Wu 2009). For example, Gln can activate the mTOR signaling pathway (Nicklin et al. 2009; Fumarola et al. 2005; Nakajo et al. 2005; Chiu et al. 2012), which inhibits autophagy (Wu et al. 2011, 2013a; Ravikumar et al. 2004; Nazio et al. 2013). Gln also stimulates MAPKs in enterocytes (Rhoads and Wu 2009; Wu et al. 2013b). As a major energy sensor, AMP-activated kinase (AMPK) increased immediately in Gln-starving cells. Autophagy is regulated by AMPK activation and AMPK inhibits mTOR-dependent signaling (Inoki et al. 2012; Appuhamy et al. 2014; Alers et al. 2012; Kim et al. 2011). Consistent with the previous reports, our results showed that Gln deprivation reduced the activation of mTOR and its downstream protein p70S6 kinase. Interestingly, the expression of p-mTOR showed a transient increase at 2-h Gln deprivation. An alternative explanation is that amino acids produced from breakdown of autophagic protein in the first 2 h could cause a temporary increase in concentrations of free amino acids (especially glutamine). The increased amino acids as well as p70S6 K might have a feedback effect on signaling upstream of mTOR. Gln deprivation also reduced the phosphorylation of ERK1/2, while slightly enhancing phosphorylated P38 and JNK expression. Of note, Gln supplementation reversed these adverse effects on the cells. Thus, we suggest that Gln deprivation, which resulted in decreased expression of ASCT2/SLC1A5, induces autophagy in intestinal epithelial cells via repressing the mTOR and MAPK signaling pathways.

Whether the effects of Gln may depend on cell type is not known (Rhoads et al. 2000; Carr et al. 2010; Larson et al. 2007; Choi et al. 2012). There were conflicting reports that Gln increases autophagy in intestinal epithelial cells (Sakiyama et al. 2009), which may reflect the use of different models, such as whether the normal or injury tissue/cell is studied, or whether the cells are dividing rapidly or not. Another relevant question is the choice of endpoint measurement. Gln is rapidly degraded by enterocytes (Haynes et al. 2009), and the phosphorylation of S6 k may occur within several minutes but the accumulation of autophagosomes may be completed within 30 min. In addition, changes in intracellular concentrations of Gln metabolites (e.g., glutamate and aspartate) may also regulate cell signaling pathways. Furthermore, High levels of ammonia, which is formed from Gln by phosphate-activated glutaminase, could induce autophagy (Cheong et al. 2011). Therefore, the period and dose of Gln treatment may affect its

effects in intestinal cells. For example, treating cells with 10 mM Gln or even higher (30 g Gln/day in the enteral diet) could be harmful to cells (Heyland et al. 2013; Albrecht and Norenberg 2006). This may explain the controversial issues related to the toxicity of Gln, particularly observed in cultured cells.

In summary, Gln deprivation inactivates the mTOR and MAPK signaling pathways, leading to enhancement of autophagy in IPEC-1 cells. Conversely, supplementation with 5 mM Gln to cultured IPEC-1 cells suppresses autophagy, increases cellular protein content, and promotes cell proliferation. Gln supplementation also reverses most of the deleterious changes in the signaling molecules. These results explain beneficial effects of dietary Gln supplementation on improving the integrity and function of the small intestine under stress conditions.

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Conflict of interest The authors declare that they have no conflict of interest.

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