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# Regulation of protein turnover by L-glutamine in porcine intestinal epithelial cells

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**Abstract**

L-Glutamine (Gln) plays an important role in sustaining the intestinal mucosal mass of humans and animals. However, the underlying mechanisms are largely unknown. This study tested the hypothesis that Gln regulates protein turnover in intestinal epithelial cells. Intestinal porcine epithelial cells (IPEC-1) were cultured for 3 h (short-term study) or 96 h (long-term study) in Gln-free Dulbecco's modified Eagle-F12 Ham medium containing 0, 0.5 or 2.0 mM Gln. To determine effects of ammonia (a metabolite of Gln, i.e., 0.18 mM ammonia produced from 2 mM Gln in 3 h) on protein turnover, additional experiments were conducted in which medium contained 0.5 mM Gln and 0, 0.2, 0.5 or 2.0 mM $NH_4Cl$. Variables of analysis included cell growth, protein synthesis, proteolysis and mammalian target of rapamycin (mTOR) signaling. IPEC-1 cell growth increased with extracellular Gln concentrations. Compared with 0 mM Gln, the addition of 0.5 and 2 mM Gln to medium stimulated protein synthesis and inhibited protein degradation in those cells in both the short- and long-term studies. Ammonia (0.05 to 2.0 mM) did not affect protein synthesis, although higher levels of ammonia (0.5 and 2.0 mM) reduced protein degradation in IPEC-1 cells. Consistent with the data on protein turnover, 0.5 and 2 mM Gln increased abundance of phosphorylated eIF4E-binding protein-1 and phosphorylated S6 kinase-1 proteins. Collectively, these results demonstrate that physiological levels of Gln regulate protein turnover independent of ammonia production in intestinal cells through the mTOR signaling pathway.


*Keywords:* Glutamine; Protein turnover; Intestinal cells

## 1. Introduction

L-Glutamine (Gln) is an abundant amino acid in plasma, skeletal muscle, milk and fetal fluids [1,2]. Approximately 70% of Gln in the enteral diet is degraded by rat and pig small intestines in the first pass [3,4]. Therefore, Gln is a major fuel for absorptive epithelial cells of the small intestine. Additionally, in both rats and pigs, Gln can ameliorate intestinal atrophy, enhance the absorption of nutrients, maintain gut function and prevent the entry of luminal pathogenic microorganisms into the systemic circulation [5–8]. Furthermore, Gln stimulates migration and proliferation of intestinal cells to sustain mucosal mass [9–11]. Thus, Gln plays a crucial role in sustaining the mucosal mass of the small intestine in both humans and animals [12]. This is particularly important for infants whose small intestine grows very rapidly during the neonatal period but who are highly susceptible to oxidative injury [4].

Using the pig as an animal model to study human nutrition and metabolism [12–14], we have reported that dietary supplementation with Gln enhanced intestinal growth in neonatal pigs [2,15,16]. However, the underlying mechanisms are largely unknown [8–10]. Gln may increase the activity of the mammalian target of rapamycin (mTOR), a protein kinase that regulates protein synthesis in cells, including lymphocytes and skeletal muscle [17,18]. mTOR phosphorylates eIF4E-binding protein-1 (4EBP1) and ribosomal protein S6 kinase-1 (S6K1), thereby initiating polypeptide formation [19] and possibly inhibition of autophagy, a key event of lysosomal protein degradation [20]. At present, direct evidence for effects of Gln on regulating protein turnover in intestinal cells is lacking. This study tested the hypothesis that increasing extracellular concentrations of Gln may stimulate protein synthesis and decrease proteolysis in intestinal epithelial cells.

## 2. Methods and materials

### 2.1. Reagents

Dulbecco's modified Eagle-F12 Ham medium (DMEM-F12), fetal bovine serum (FBS) and antibiotics were purchased from Invitrogen (Grand Island, NY, USA).

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Epidermal growth factor and selenium were obtained from BD Biosciences (Bedford, MA, 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-[Ring-2,4-$^3$H] phenylalanine and protein extraction reagents were obtained from American Radiolabeled Chemicals (St. Louis, MO, USA) and EMD Biosciences (San Diego, CA, USA), respectively. Unless indicated, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Cell culture

Intestinal porcine epithelial cells (IPEC-1) were isolated from the jejunum of unsuckled newborn pigs, as previously described [15]. The cells were grown in serial passage in uncoated plastic cell culture flasks (75 cm$^2$) with a vent cap in DMEM-F12 containing 17.5 mM D-glucose, 2 mM Gln, 0.7 mM arginine, 15 mM HEPES (pH 7.4), 5% FBS, epidermal growth factor (5 μg/L), insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), penicillin (50 μg/ml), streptomycin (4 μg/ml) and 0.25 μg/ml amphotericin B (Fungizone). Medium was changed every 2 days. All cell cultures were carried out at 37 °C in a 5% $CO_2$ incubator. At confluence, cells were passaged using trypsinization [21].

### 2.3. Determination of cell growth

To determine effects of Gln on IPEC-1 cell growth, cells were seeded in 96-well cell culture plates with approximately 5000 cells per well. After overnight culture, the cells were starved for 6 h in 100 μl of Gln-free Dulbecco's modified Eagle medium (DMEM). The cells were then cultured in 100 μl of Gln-free DMEM containing 5% FBS, 5 mM D-glucose, 0.1 mM sodium pyruvate and 0, 0.5 or 2 mM Gln [22]. The physiological concentrations (μM) of other amino acids in the medium were as follows: L-alanine, 350; L-arginine·HCl, 100; L-asparagine, 50; L-aspartic acid, 20; L-cystine·2HCl, 75; L-glutamic acid, 75; glycine, 250; L-histidine·HCl·$H_2O$, 100; L-isoleucine, 150; L-leucine, 200; L-lysine·HCl, 200; L-methionine, 75; L-phenylalanine, 100; L-proline, 200; L-serine, 200; taurine, 100; L-threonine, 200; L-tryptophan, 75; L-tyrosine·2Na·2$H_2O$, 100 and L-valine, 250. The media were changed every 2 days. At days 0, 2, 4 and 6 of culture, cell numbers were determined using the 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide method [23]. The number of independent experiments ($n$=12) for cell growth assay was determined on the basis of statistical power calculation [24].

## 3. Determination of protein synthesis and degradation

### 3.1. Seeding of cells in culture plates

IPEC-1 cells ($1\times10^5$ cell per well) were seeded in six-well cell culture plates with 2 ml of DMEM-F12 medium. After 16-h culture, the cells were used for studies of intracellular protein turnover, as described by Tan et al. [22]. There were eight independent experiments for each concentration of Gln, based on statistical power calculation [24].

### 3.2. Determination of protein synthesis

After seeding onto plates, IPEC-1 cells were cultured for 4 days in Gln-free DMEM [22] containing 5% FBS and 0.5 mM Gln (short-term study) or 0.5-2 mM Gln (long-term study). The media were changed every 2 days. At the end of the 4-day culture, the medium was removed, and cells were washed twice with 2 ml of Gln-free medium. Cells were then cultured for 3 h in 2 ml DMEM containing 5% FBS and 1 mM L-phenylalanine plus 0.8 μCi L-[ring-2, 4-$^3$H]phenylalanine [22] and 0, 0.5 or 2 mM Gln (both short-term and long-term studies). To determine effects of ammonia (a metabolite of Gln) on protein synthesis, additional experiments were conducted in which DMEM contained 5% FBS, 0.5 mM Gln and 0, 0.2, 0.5 or 2.0 mM $NH_4Cl$. This was based on our observation that the culture of IPEC-1 cells with 0.5 and 2 mM L-glutamine for 3 h resulted in the presence of 0.05 and 0.18 mM ammonia in the medium, respectively, as analyzed using glutamate dehydrogenase [25]. At the end of a 3-h culture period, the medium was collected for analysis of ammonia and amino acids [26], whereas the cells were rapidly washed twice with 2 ml ice-cold PBS. After addition of 2 ml of 2% trichloroacetic acid (TCA) to each well, the cells were scraped, and the whole TCA extract was collected into a 15-ml tube and centrifuged at 3000$g$ for 5 min. The supernatant fluid was discarded, and the cell pellet was washed three times with 5 ml of 2% TCA and dried in air at room temperature. After the pellet was dissolved in 0.5 ml of 1 M NaOH, 0.4 ml of the solution was transferred to a 5-ml scintillation vial containing 4.5 ml Hionic Fluor Scintillation cocktail (PerkinElmer, MA, USA) to determine protein-bound $^3$H-phenylalanine [27]. $^3$H-phenylalanine radioactivity was determined using a liquid scintillation counter after standing overnight at room temperature. An aliquot (0.1 ml) of the cell solution was stored at −20 °C for protein assay using the BCA method. Specific activity of $^3$H-phenylalanine in medium was used to calculate protein synthesis in cells [22].

### 3.3. Determination of protein degradation

After seeding onto plates, IPEC-1 cells were cultured for 3 days in 2 ml of Gln-free DMEM containing 5% FBS and 0.5 mM Gln (short-term study) or 0, 0.5 or 2 mM Gln (long-term study). Beginning on day 4, cells were cultured for 24 h in 2 ml of Gln-free DMEM containing 5% FBS, 0.1 mM L-phenylalanine plus L-[$^3$H]phenylalanine (0.8 μCi/well), and either 0.5 mM Gln (short term) or 0, 0.5 or 2 mM Gln (long term). After the 24-h culture to label cellular proteins, cells were washed three times with 2 ml Gln-free medium to deplete intracellular free [$^3$H]phenylalanine [28]. The cells were then cultured for 3 h in 2 ml DMEM containing 5% FBS, 1 mM L-phenylalanine and 0, 0.5, or 2 mM Gln (both short-term and long-term studies). To determine effects of ammonia on protein synthesis, additional experiments were conducted in which DMEM contained 5% FBS, 0.5 mM Gln and 0, 0.2, 0.5 or 2.0 mM $NH_4Cl$. At the end of 3-h culture, the medium was collected, the cells were rapidly washed three times with 2 ml ice-cold PBS, 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 3000$g$ 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 5-ml scintillation vial containing 4.5 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 3000$g$ 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 3000$g$ for 5 min, 2 ml of the supernatant fluid was transferred to a 5-ml scintillation vial containing 3 ml Hionic Fluor Scintillation cocktail for $^3$H measurement. The percentage of protein-bound [$^3$H] phenylalanine released into 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 [22].

## 4. Western blot analysis of proteins in the mTOR signaling pathways

IPEC-1 cells were cultured in the medium containing 0, 0.5 or 2 mM Gln, as described above. After a 4-day period of culture, the medium was removed, and the cells were washed with Dulbecco's PBS. The CytoBuster protein extraction reagent containing 10 μl/ml protease inhibitor cocktail and 10 μl/ml phosphatase inhibitor cocktail (Novagen, Madison, USA) was added to the cell pellet. After 5 min at room temperature, the cell solution was transferred into a microtube and centrifuged at 16,000$g$ and 4°C for 5 min. The supernatant fluid (cell extract) was stored at −80°C for protein assays and Western blot analysis. Protein concentrations in the cell extract were measured using Pierce BCA Protein Assay Kit and bovine serum albumin as standard. All samples were adjusted to an equal protein concentration. The samples were subsequently diluted with 4× loading buffer [0.1 ml of 1.25 M Tris–HCl (pH 6.8), 0.3 ml 75% glycerol, 0.08 g sodium dodecyl sulfate (SDS), 0.1 ml β-mercaptoethanol, 0.4 ml 0.05% bromphenol blue and 0.1 ml water to a final volume of 1 ml] and heated in boiling water for 5 min. After the solution was cooled on ice, aliquots of samples (28 μg protein for total mTOR, total 4EBP1 and

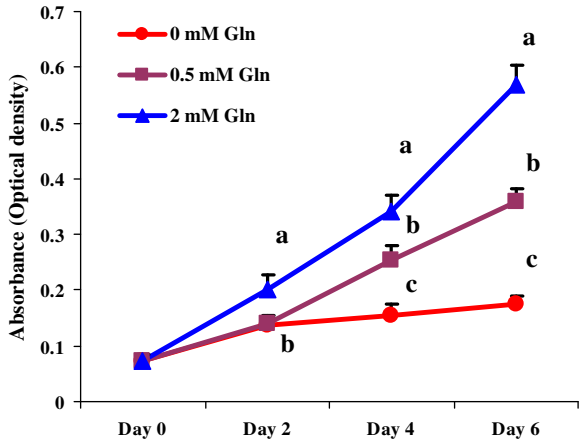


Fig. 1. Growth of IPEC-1 cells cultured with or without glutamine. The number of IPEC-1 cells cultured in Gln-free DMEM containing 0, 0.5 and 2 mM glutamine was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method on days 0, 2, 4 and 6. Values in the *y*-axis (absorbance) are means ± S.E.M.; $n$=12. Within a day, means sharing different superscript letters (a–c) differ ($P$<.01).

total S6K1 protein; 50 μg protein for phosphorylated mTOR, phosphorylated 4EBP1 and phosphorylated S6K1 proteins) were loaded onto Nupage 4%–12% Bris-tris gel (Invitrogen, Carlsbad, CA, USA). After separation on the gels using 20× MOPS SDS Running Buffer (Invitrogen, Carlsbad, CA, USA) under 200 V for 1 h, proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) in 20× MOPS SDS Transfer Buffer (Invitrogen, Carlsbad, CA, USA) under 30 V for 1 h using the Bio-Rad Transblot apparatus (Hercules, CA, USA). Membranes were blocked in 5% fat-free 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: total mTOR (Cell Signaling, 1:1000), phosphorylated mTOR (Ser2481) (Cell Signaling, 1:500), total S6K1 (Cell Signaling, 1:1000), phosphorylated S6K1 (Thr389) (Abcam, 1:750), total 4EBP1 (Cell Signaling, 1:1000), phosphorylated 4EBP1 (Thr70) (Cell Signaling, 1:500) or β-actin (Cell Signaling, 1:1000). After being washed three times with TTBS, the membranes were incubated at room temperature for 3 h with a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, Cell Signaling) at 1:50,000. Finally, the membranes were washed with TTBS, followed by development using the Supersignal West Dura Extended Duration Substrate according to the manufacturer's instructions (Pierce, Rockford, IL, USA). Exposure times to the Supersignal West Dura Extended Duration Substrate were 5 min for β-actin, total mTOR, total 4EBP1 and total S6K1 proteins and 10 min for phosphorylated mTOR, phosphorylated 4EBP1 and phosphorylated S6K1 proteins. The images were analyzed as arbitrary unit using the Molecular Imager ChemiDoc XRS+ System (Bio-Rad, Hercules, CA, USA). Images for β-actin were not overexposed and were used to normalize the abundance of total and phosphorylated mTOR, S6K1 and 4EBP1 proteins in cells. In our preliminary studies, we found that when the amounts of the samples loaded onto the gel were increased by 150% and 200%, the amounts of the proteins of interest increased by approximately 150% and 200%, respectively.

Although the calculated amount of total protein (e.g., 28 μg protein for total mTOR, total 4EBP1 and total S6K1 protein) used for Western blotting was the same on the basis of the analyzed content of protein in the cell supernatant fluid, variations among samples could occur in one or more of the following procedures: (a) heating of samples with the loading buffer in a 1.5-ml microcentrifuge, (b) loading of the samples onto Nupage 4%–12% Bris-tris gel, (c) running of the gel and (d) transfer of the gel onto the nitrocellulose membrane. Thus, it is a good laboratory practice to include in Western blot analysis a housekeeping protein whose expression is not altered by treatments ($P$=.852 for β-actin protein in this study).

## 5. Statistical analysis

Values are expressed as mean ± S.E.M. Data were statistically analyzed by one-way analysis of variance (ANOVA) using the Statistical Analysis System 8.0 (SAS Institute Inc., Cary, NC, USA). The experimental unit was the 96-well cell culture plate for each independent experiment. In one-way ANOVA, differences among treatment means were determined using the Student–Newman–Keuls multiple comparison test. In the experiment where cell numbers were determined at days 0, 2, 4 and 6 of culture in the presence of 0 to 2 mM L-glutamine, data were analyzed using ANOVA for repeated measurements (Procedure Mixed in SAS), with the inclusion of treatment×time interaction. Probability values≤0.5 were taken to indicate statistical significance.

## 6. Results

### 6.1. Proliferation of IPEC-1 cells

Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method, we assessed the number of IPEC-1 cells cultured in the presence of 0, 0.5 or 2 mM Gln for 2 to 6 days (Fig. 1). We determined that 0.1 unit of optical density in the UV/VIS spectrophotometry was equal to $1.46\times10^4$ IPEC-1 cells. When Gln was not added to culture medium, the number of IPEC-1 cells did not differ ($P$>.05) between days 0 and 6 of culture. On day 2 of culture, the number of IPEC-1 cells was greater ($P$<.01) in the presence of 2 mM Gln compared with the 0- and 0.5-mM Gln groups. On days 4 and 6, the number of IPEC-1 cells was dose-dependently enhanced ($P$<.01) with increasing concentrations of Gln from 0 to 2 mM. As compared to the 0-mM Gln group, the number of IPEC-1 cells cultured in the presence of 0.5 mM Gln was 65% and 105% greater on days 4 and 6, respectively, and cell numbers in the presence of 2 mM Gln were 119% and 223% greater on days 4 and 6, respectively.

Table 1
Concentrations of its metabolites in the medium of IPEC-1 cells cultured for 3 h in the presence of 0, 0.5 or 2 mM Gln

| [Gln] in medium (mM) | Glutamine and its metabolites in the medium at the end of 3-h culture (nmol/ml) | | | | |
|---|---|---|---|---|---|
| | Glutamine | Glutamate | Aspartate | Alanine | Ammonia |
| 0 | 16 ± 0.7$^c$ | 79 ± 1.2$^c$ | 21 ± 0.9$^c$ | 364 ± 5.3$^b$ | 5.2 ± 0.4$^c$ |
| 0.5 | 462 ± 6.5$^b$ | 86 ± 1.4$^b$ | 27 ± 1.3$^b$ | 373 ± 5.8$^b$ | 48 ± 3.4$^b$ |
| 2 | 1827 ± 17$^a$ | 112 ± 1.9$^a$ | 45 ± 1.5$^a$ | 412 ± 6.2$^a$ | 179 ± 12$^a$ |

Values are means ± S.E.M.; $n$=8. Cells were cultured for 3 h in Gln-free DMEM containing 0, 0.5 or 2 mM glutamine. Within a column, means sharing different superscript letters (a–c) differ ($P$<.01).

Table 2
Short-tem effects of Gln on protein turnover in IPEC-1 cells

| [Gln] (mM) | Protein synthesis, nmol Phe/mg protein/3 h | Protein degradation, %/3 h |
|---|---|---|
| 0 | 50.4 ± 2.8$^c$ | 7.72 ± 0.41$^a$ |
| 0.5 | 62.2 ± 3.1$^b$ | 6.19 ± 0.33$^b$ |
| 2 | 75.9 ± 4.4$^a$ | 5.04 ± 0.26$^c$ |

Values are means ± S.E.M.; $n$=8. Cells were cultured for 3 h in Gln-free DMEM containing 0, 0.5 or 2 mM glutamine. Within a column, means sharing different superscript letters (a–c) differ ($P$<.01).

Table 3
Long-term effects of Gln on protein turnover in IPEC-1 cells

| [Gln] (mM) | Protein synthesis, nmol Phe/mg protein/3 h | Protein degradation, %/3 h |
|---|---|---|
| 0 | 47.2 ± 2.3[c] | 7.56 ± 0.36[a] |
| 0.5 | 69.1 ± 4.0[b] | 5.60 ± 0.31[b] |
| 2 | 90.3 ± 6.2[a] | 4.76 ± 0.22[c] |

Values are means ± S.E.M.; $n$=8. Cells were cultured for 96 h in Gln-free DMEM containing 0, 0.5 or 2 mM glutamine. Within a column, means sharing different superscript letters (a–c) differ ($P$<.01).

### 6.2. Production of ammonia and amino acids from Gln in IPEC-1 cells

Catabolism of Gln resulted in the production of glutamate, aspartate, alanine and ammonia from Gln by IPEC-1 cells, as indicated by the disappearance of Gln from culture medium and the accumulation of glutamate, aspartate, alanine and ammonia in the medium at the end of 3-h culture (Table 1). Under the experimental conditions, there was no detectable formation of other amino acids from Gln in those cells (data not shown). Of particular note, concentrations of ammonia in the medium at the end of 3-h culture were 48 and 180 μM, respectively, in the presence of 0.5 and 2 mM Gln.

### 6.3. Short-term and long-term effects of Gln on protein turnover in IPEC-1 cells

Addition of 0.5 and 2 mM Gln to culture medium dose-dependently increased ($P$<.01) protein synthesis and reduced ($P$<.01) protein degradation in IPEC-1 cells in both short-term (Table 2) and long-term (Table 3) studies. In the short-term study (3-h culture), protein synthesis was 23.4% and 50.6% higher ($P$<.01), and protein degradation was 19.8% and 34.7% lower ($P$<.01), in the 0.5- and 2-mM Gln groups, respectively, compared with the 0-mM Gln group. In the long-term study (96-h culture), protein synthesis was 46.4% and 91.3% higher ($P$<.01), and protein degradation was 25.9% and 37.0% lower ($P$<.01), in the 0.5- and 2-mM Gln groups, respectively, compared with the 0-mM Gln group.

### 6.4. Effects of ammonia on protein turnover in IPEC-1 cells

Addition of 0.05 to 2 mM ammonia to culture medium had no effect ($P$>.05) on protein synthesis in IPEC-1 cells (Table 4). Rates of protein degradation in those cells did not differ ($P$>.05) between 0 and 0.2 mM Gln. However, increasing extracellular concentrations of ammonia to 0.5 and 2 mM decreased ($P$<.01) protein degradation in a dose-dependent manner when compared with the 0-mM Gln group.

### 6.5. Effects of Gln on the mTOR signaling pathway in IPEC-1 cells

Abundance of total and phosphorylated mTOR, 4EBP1 and S6K1 proteins in IPEC-1 cells is illustrated in Figs. 2, 3 and 4, respectively. As compared with the 0-mM Gln group, supplementation of 0.5 and 2 mM Gln to culture medium increased ($P$<.05) the amounts of phosphorylated 4EBP1 protein by 33% and 60%, as well as phosphorylated S6K1 protein by 39% and 53%, in the cells. However, addition of 0.5 and 2 mM Gln had no effect ($P$>.05) on abundance of total mTOR, 4EBP1 and S6K1 proteins, or phosphorylated mTOR protein.

Table 4
Short-term effects of ammonia on protein turnover in IPEC-1 cells

| [$NH_4Cl$] in medium (mM) | Protein synthesis, nmol Phe/mg protein/3 h | Protein degradation, %/3 h |
|---|---|---|
| 0 | 48.6 ± 3.1 | 7.91 ± 0.45[a] |
| 0.05 | 48.0 ± 3.5 | 7.74 ± 0.40[a] |
| 0.20 | 46.2 ± 3.7 | 7.58 ± 0.43[a] |
| 0.50 | 45.1 ± 2.8 | 6.64 ± 0.41[b] |
| 2 | 44.7 ± 3.5 | 5.23 ± 0.34[c] |

Values are means ± S.E.M.; $n$=8. Cells were cultured for 3 h in ammonia-free DMEM containing 0.5 mM glutamine and 0, 0.05, 0.20, 0.50 or 2 mM ammonia. Within a column, means sharing different superscript letters (a–c) differ ($P$<.01).

## 7. Discussion

L-Glutamine is a conditionally essential amino acid for infants and piglets [3,4]. The published work also shows that dietary supplementation with Gln effectively ameliorates intestinal atrophy, enhances the absorption of nutrients and improves growth performance in early-weaned piglets [7,16,29]. In addition, oral Gln or alanyl-glutamine dipeptide (Ala-Gln) enhances small-intestinal and whole-body growth performance and ameliorates intestinal injury in lipopolysaccharide-challenged piglet [15]. Consistent with these previous reports, we found that addition of physiological levels of Gln (0.5 and 2 mM) to culture medium promoted the growth of porcine intestinal cells (Fig. 1). Thus, under conditions associated with inadequate intake of Gln (e.g., weaning and infection), dietary supplementation of Gln is critical to maintain intestinal mass, integrity and function [2].

Cell growth depends on the balance between protein synthesis and degradation [30]. The continuous synthesis and degradation of proteins in cells are collectively termed intracellular protein turnover, which determines protein balance in tissues. Findings from a majority of clinical trials indicate that supplementation of appropriate doses of Gln in the form of free Gln or Ala-Gln *via* enteral and parenteral nutrition is beneficial for improving protein balance in animals and

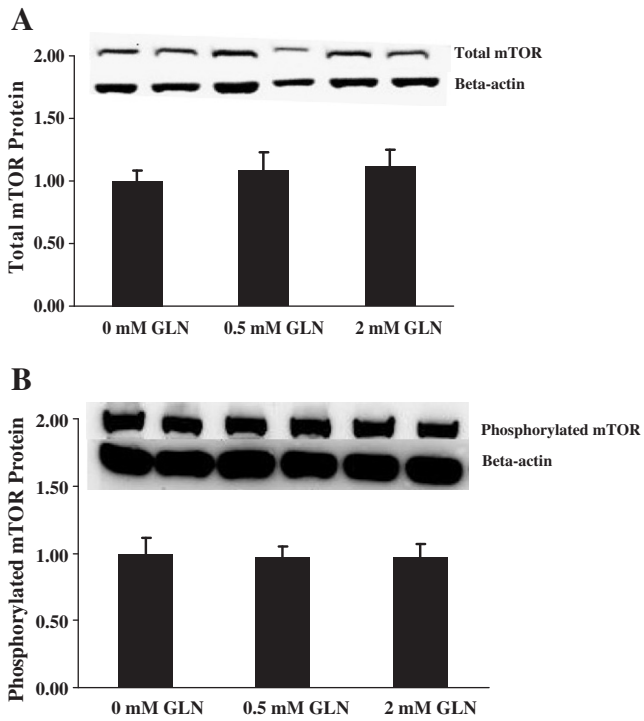


Fig. 2. Amounts of total (A) and phosphorylated (B) mTOR proteins in IPEC-1 cells. The IPEC-1 cells cultured for 96 h in Gln-free DMEM supplemented with 0, 0.5 and 2 mM Gln were used for analysis of both total and phosphorylated mTOR proteins. Data are expressed as means ± S.E.M.; $n$=8. No differences in total or phosphorylated mTOR proteins were detected among the three treatment groups ($P$>.05). Images for β-actin were used to normalize the abundance of total and phosphorylated mTOR proteins in cells.

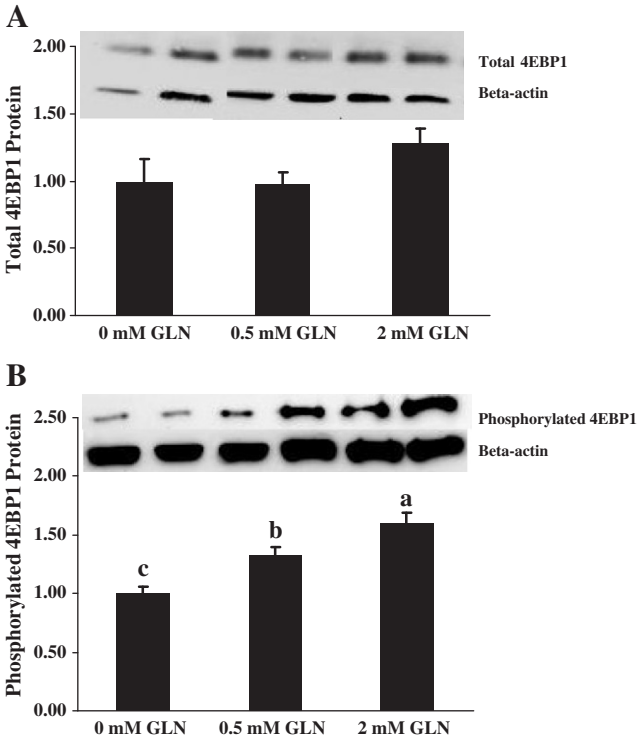


Fig. 3. Amounts of total (A) and phosphorylated (B) 4EBP1 proteins in IPEC-1 cells. The IPEC-1 cells cultured for 96 h in Gln-free DMEM supplemented with 0, 0.5 and 2 mM Gln were used for analysis of both total and phosphorylated 4EBP1 proteins. Data are expressed as means ± S.E.M.; $n$=8. No differences in total 4EBP1 protein were detected among the three treatment groups ($P$>.05). Increasing extracellular Gln concentrations from 0 to 2 mM enhanced ($P$<.05) the abundance of phosphorylated 4EBP1 protein in a dose-dependent manner (a–c). Images for β-actin were used to normalize the abundance of total and phosphorylated 4EBP1 proteins in cells.

humans with trauma, sepsis, severe burn and cancer [31]. These impacts are indicated by increases in protein synthesis and/or decreases in protein breakdown in skeletal muscle [28,32,33]. Additionally, enteral Gln could stimulate protein synthesis in the small-intestinal mucosa of humans [34] and rats [35] while reducing expression of ubiquitin-dependent proteases in the gut [29,34]. However, no direct measurement of proteolysis in the intestinal mucosa was made in humans or animals supplemented with Gln. At present, little is known about effects of Gln on protein turnover in enterocytes. In the current study, we observed that addition of 0.5 or 2 mM Gln to culture medium stimulated protein synthesis and inhibited protein degradation in IPEC cells (Tables 2 and 3). Interestingly, Gln had a greater effect on protein anabolism in intestinal cells exposed to this amino acid for 96 h compared with a 3-h treatment. These results indicate that changes in gene expression may be necessary to maximize beneficial effects of Gln on protein metabolism in IPEC cells.

L-Glutamine is degraded by enterocytes to produce ammonia [25]. Large amounts of ammonia (>0.5 mM) are known to inhibit hepatic proteolysis [36] and muscle protein synthesis [37]. To test whether the effects of Gln on protein turnover in IPEC-1 cells may result from ammonia productions, we determined rates of protein turnover in these cells cultured in the presence of 0 to 2 mM $NH_4Cl$. We found that addition of 0.5 and 2 mM ammonia to medium did reduce protein degradation but did not affect protein synthesis in IPEC-1 cells (Table 4). However, supplementing the medium with 0.05 and 0.20 mM ammonia, which mimicked its production by IPEC-1 cells cultured for 3 h in the presence of 0.5 and 2 mM Gln, respectively (Table 1), had no effect on protein synthesis or proteolysis in the cells (Table 4). These novel results indicate that the regulation of protein turnover by Gln in intestinal cells is not attributable to ammonia production. The findings prompted us to examine the mTOR signaling pathway, which is a master regulator of protein synthesis and possibly protein degradation in mammalian cells [19,38].

The mTOR, also known as FK506 binding protein 12-rapamycin associated protein 1, is a highly conserved serine/threonine protein kinase [19]. According to the current knowledge of the mTOR signaling, mTOR is activated when it is phosphorylated by an upstream kinase in response to stimulation by certain nutrients and growth factors [19,39]. Activated mTOR then phosphorylates 4EBP1 and S6K1, leading to the initiation of polypeptide formation [17]. Interestingly, we observed that phosphorylation of 4EBP1 (Fig. 2) and S6K1 (Fig. 3) was enhanced in IPEC-1 cells cultured with physiological levels of Gln (0.5 and 2 mM) when the abundance of phosphorylated mTOR protein was not altered (Fig. 3). Similar results were reported for skeletal muscle in pigs fed a diet containing 1.61% and 1.88% leucine [40]. Thus, in animal cells and tissues, activation of 4EBP1 and 6SK1 proteins can take place independent of phosphorylated levels of mTOR protein. This new notion may help modify the current model of the mTOR signaling and represent a paradigm shift in the research field. An important implication of our findings is that physiological concentrations of certain amino acids (e.g., Gln and L-leucine) may directly phosphorylate 4EBP1 and S6K1 proteins in cells. Future studies are warranted to test this novel and potentially significant hypothesis. Either 4EBP1 or S6K1 protein can be a target for activation or inhibition by biological metabolites or synthetic chemicals.

Our results are in contrast to the findings of Sakiyama et al. [41] that, compared with the absence of Gln from the culture medium, 0.7 mM

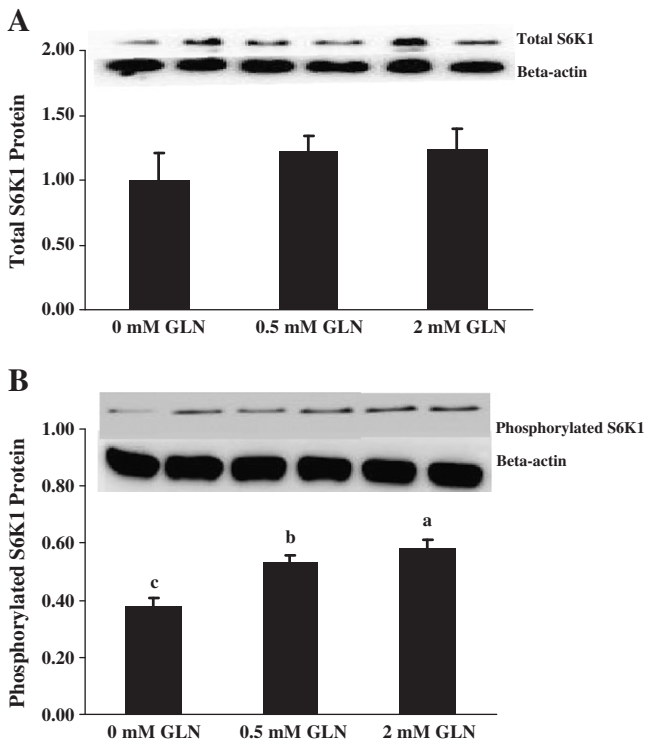


Fig. 4. Amounts of total (A) and phosphorylated (B) S6K1 proteins in IPEC-1 cells. The IPEC-1 cells cultured for 96 h in Gln-free DMEM supplemented with 0, 0.5 and 2 mM Gln were used for analysis of both total and phosphorylated S6K1 proteins. Data are expressed as means ± S.E.M.; $n$=8. No differences in total S6K1 protein were detected among the three treatment groups ($P$>.05). Increasing extracellular Gln concentrations from 0 to 2 mM enhanced ($P$<.05) the abundance of phosphorylated S6K1 protein in a dose-dependent manner (a–c). Images for β-actin were used to normalize the abundance of total and phosphorylated S6K1 proteins in cells.

Gln inactivated mTOR and increased the number of autophagosomes (a potential indicator of enhanced proteolysis) in the rat small intestinal epithelial IEC-18 cell line. The reasons for the discrepancy in the effects of Gln on the cellular mTOR signaling and protein turnover between our work and the study of Sakiyama et al. [41] are not clear at present and may include different experimental conditions (e.g., concentrations of glucose, L-arginine and other amino acids in culture media).

## 8. Conclusion

L-Glutamine increases protein synthesis and inhibits protein degradation in porcine intestinal cells, resulting in enhanced proliferation of cells. These results aid in explaining the previous finding that dietary supplementation of Gln promotes intestinal growth in young pigs. The beneficial effects of physiological levels of Gln on intestinal epithelial cells are not mediated by its metabolite ammonia. We suggest that Gln exerts its actions through activating two downstream proteins of the mTOR signaling pathway (4EBP1 and S6K1). Our observations have important implications for understanding the regulation of intestinal protein turnover in mammals (including pigs and humans).

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