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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·2H_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 (1×10^5 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 3000g 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 3000g 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 3000g 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 3000g 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 16,000g 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