L-Glutamate Enhances Barrier and Antioxidative Functions in Intestinal Porcine Epithelial Cells¹,²

Ning Jiao,³ Zhenlong Wu,³* Yun Ji,³ Bin Wang,³ Zhaolai Dai,³ and Guoyao Wu³,⁴
³State Key Laboratory of Animal Nutrition, China Agricultural University, Beijing, China; and ⁴Department of Animal Science, Texas A&M University, College Station, TX

Abstract

Background: L-Glutamate (Glu) is a major amino acid in milk and postweaning diets for mammals (including pigs and human infants). However, effects of Glu on intestinal mucosal barrier and antioxidative functions are unknown.

Objective: This study tested the hypothesis that Glu may enhance the barrier function of intestinal porcine epithelial cell line 1 (IPEC-1) cells by upregulating the expression of tight junction proteins.

Methods: IPEC-1 cells were cultured with or without Glu in the presence or absence of 1 mmol/L diquat (an oxidant) for indicated time points. Cell numbers, transepithelial electrical resistance (TEER), mRNA, and protein abundance of glutamate transporter, the release of lactate dehydrogenase (LDH), and the abundance of tight junction proteins were determined.

Results: Compared with 0 mmol/L Glu, 0.5-, 1-, and 2 mmol/L Glu stimulated (P < 0.05) cell growth by 13–37% at 24 h and 12–34% at 48 h, respectively. In addition, 0.5 mmol/L Glu increased (P < 0.05) TEER (by 58% at 24 h and by 98% at 48 h, respectively). These effects of Glu were associated with increased mRNA abundance of Glu transporter solute carrier family 1 member 1 (SLC1A1) by 30–130% and protein abundance of excitatory amino acid transporter 3 (encoded by SLC1A1) by 19–34%, respectively. In a cell model of oxidative stress induced by 1 mmol/L diquat, 0.5 mmol/L Glu enhanced cell viability, TEER, and membrane integrity (as indicated by the reduced release of LDH) in IPEC-1 cells by increasing the abundance of the tight junction proteins occludin, claudin-3, zonula occludens (ZO)-2, and ZO-3.

Conclusion: These findings indicate that Glu plays an important role in mucosal barrier function by enhancing cell growth and maintaining membrane integrity in response to oxidative stress. J Nutr 2015;145:2258–64.

Keywords: glutamate, intestinal epithelial cell, intestinal–mucosal barrier, oxidative stress, antioxidative function

Introduction

L-Glutamate (Glu) is an abundant amino acid in milk and postweaning diets for mammals (including pigs and human infants) and in the intestinal mucosa. Enteral Glu is a nutritionally and physiologically important precursor for the synthesis of bioactive molecules, such as polyamines, glutathione (3), and amino acids [e.g., L-proline (4), L-aspartate (5) and L-citrulline (6)] in porcine and human enterocytes. Polyamines are essential for DNA and protein synthesis, thereby promoting proliferation and migration of intestinal epithelial cells along the crypt-villus axis (7). Glutathione is required for regulating cell redox state and detoxification in all cell types (8). Furthermore, studies in rats and pigs indicate that Glu is a major metabolic fuel for enterocytes and that ~96% of enteral Glu is metabolized in the small intestine during the first pass (9–13). Furthermore, Glu can activate chemical sensors in the gastrointestinal tract, leading to the generation of serotonin (a neurotransmitter) by enteroendocrine cells (14). Emerging evidence also shows that Glu plays important roles in intestinal nutrition (15), cell signaling, the modulation of gene expression (16), anti-inflammatory responses (17,18), and immunity (19,20). Transport of Glu primarily by the excitatory amino acid transporter 3 (EAAT3) is the first step in the utilization of this amino acid by enteroocytes (21,22).

Tight junction between intestinal epithelial cells is the major portion of intestinal barrier that prevents the passage of bacteria from the intestinal lumen into blood circulation (23). This mucosal barrier function is impaired under conditions of oxidative...
stress brought about by excess reactive oxygen species (24). Diquat is widely used as an oxidant in in vitro or in vivo experiments to induce oxidative stress and apoptosis (25, 26). Glutamate supplementation ameliorates endotoxin-induced damage to the jejunal epithelium and intestinal paracellular hyperpermeability (27, 28), suggesting a critical role for Glu in intestinal–mucosal barrier function.

Although Glu is known to be essential for maintaining intestinal mucosa integrity and antioxidative responses (29), the underlying cellular and molecular mechanisms remain largely unknown. We hypothesized that Glu can maintain intestinal barrier function in diquat-challenged enterocytes by upregulating expression of tight junction protein. This hypothesis was tested in the present study with the use of intestinal porcine epithelial cell line 1 (IPEC-1) cells, which metabolize Glu in a way similar to that of enterocytes freshly isolated from neonatal pigs (30).

Methods

Reagents. DMEM F12 Ham medium, FBS, PBS, and Tytspin/EDTA were procured from Gibco. Epidermal growth factor and insulin–transferrin–selenium were obtained from BD Biosciences. Plastic culture plates were manufactured by Corning. Unless indicated, all other chemicals were purchased from Sigma–Aldrich.

Cell culture. The IPEC-1 cells were cultured as previously described (31). Cells were cultured with serum- and Glu-free custom-made DMEM (modified DMEM no. 08–5009EF, Gibco). The basal medium contained 5 mmol/L d-glucose, no Glu, and physiologic concentrations of all other amino acids (including 0.5 mmol/L l-glutamine) found in the plasma of neonatal pigs (31). The medium was supplemented with 5% FBS and 5 μg/mL insulin in diquat-free experiments. Cells were treated with 0-, 0.5-, 1-, or 2 mmol/L Glu for 24 or 48 h. These concentrations of Glu were chosen on the basis of those in the jejunal lumen (jejunal luminal fluid samples) of neonatal pigs (31). The medium was supplemented with 5% FBS and 5 μg/mL insulin in diquat-free experiments. Cells were treated with 0-, 0.5-, 1-, or 2 mmol/L Glu for 24 h. These concentrations of Glu were chosen on the basis of those in the jejunal lumen (jejunal luminal fluid samples) of neonatal pigs, which were 0.029 ± 0.006, 0.48 ± 0.04, 1.03 ± 0.07, and 2.15 ± 0.18 mmol/L (mean ± SEM, n = 6), respectively, in newborn unsuckled pigs, and 6-, 12-, and 24-h-old suckling piglets (N Jiao, Z Wu, Y Ji, B Wang, Z Dai, and G Wu, unpublished results, 2009). The medium was changed every 2 d. In diquat-challenged experiments, cells were cultured with 0-, 0.5-, or 1 mmol/L Glu for 24 h and then treated with 1 mmol/L diquat for indicated time periods.

Determination of cell growth. To determine the effect of Glu on IPEC-1 cell growth, cells were seeded in 96-well cell culture plates with ~5 × 10^4 cells per well. Cells were grown to 70% confluence in DMEM F12 Ham medium and then changed to serum- and Glu-free DMEM to be starved for 6 h for each treatment. Then cells were treated with 0-, 0.5-, 1- or 2 mmol/L Glu. Cell numbers were determined as previously described (31) and data are expressed as the percent of control cells.

Determination of transepithelial electrical resistance. Cells were seeded in cell-culture transwells with ~5 × 10^4 cells per well (the membrane area was 0.33 cm², pore size was 0.4 μm), which were placed in 24-well cell culture plates. When resistance approached 500 Ω·cm², cells were treated with Glu (0-, 0.5-, 1-, or 2 mmol/L) for 24 or 48 h. Transepithelial electrical resistance (TEER) was determined every 24 h with the use of an EVOM volt-ohmometer connected to a 12 mm Endohm unit (World Precision Instruments). In diquat-challenged experiments, cells were pretreated with Glu (0.5 mmol/L) for 24 h and then treated with diquat for another 6 h. TEER was determined every 1 h for 8 h. All data are expressed as the values relative to those for the 0 mmol/L Glu group.

PCR analysis. Total RNA was extracted from cells treated with various concentrations of Glu for 24 h. RT-PCR was carried out with the use of the TaKaRa One Step PCR Kit (AMV) (TaKaRa) according to the manufacturer’s instructions. β-Actin was used as the endogenous control. All data were expressed as the values relative to those for the 0 mmol/L Glu group. The primers used for solute carrier family 1 member 1 (SLC1A1) and β-actin were as follows: SLC1A1, 5′- GCCAGCGCTCTACCTGGAAGCA-3′ and 5′- GGCACCGCCACTTACCAAGCA-3′; β-actin, 5′- TCTGCGACCAACCTTCTACA-3′ and 5′- ATCTGCTGTCATCCTTCTACGG-3′, respectively.

Determination of cell membrane integrity. Lactate dehydrogenase (LDH) in IPEC-1 cell culture medium was determined as previously reported (32). Briefly, cells were cultured with or without Glu as indicated in the presence or absence of 1 mmol/L diquat for 6 h. Thereafter, the medium was collected and LDH activity was measured with the use of an ultraviolet-visible spectrophotometer (450 nm) and a kit from Jiancheng Bioengineering. All data are expressed as the values relative to those for the 0 mmol/L Glu group.

Western blot analysis. Cells were incubated with or without Glu in the presence or absence of diquat for indicated time points. Cells were then harvested for analysis of abundance of proteins through the use of Western blotting as previously described (33). Membranes were incubated with a primary antibody [occludin, claudin-1, claudin-3, claudin-4, zonula occludens (ZO)-1, ZO-2, or ZO-3 (Santa Cruz Biotechnology)] at 4°C overnight and then washed 3 times with tris-buffer saline solution with 0.1% Tween-20 for 15 min. The membranes were then incubated with an HRP-conjugated secondary antibody (Applygen Technologies) at room temperature for 1 h. The signal was detected with the use of the Super Enhanced Chemiluminescence Kit (Amersham Biosciences). Qualification of band density was determined with the use of Quantity One software (Bio-Rad Laboratories). All results were normalized to β-actin and data are expressed as the values relative to those for the 0 mmol/L Glu group.

Statistical analysis. Results are expressed as means ± SEMs. Data were analyzed by 1-factor ANOVA with the use of SAS, version 9.0. The Student–Newman–Keuls multiple comparison test was applied to determine differences between treatment means. P < 0.05 was taken to indicate statistical significance.

Results

Effects of Glu on the growth of IPEC-1 cells. The growth of IPEC-1 cells treated with different concentrations of Glu is
Effects of Glu on TEER in IPEC-1 cells. Cells were pretreated with different concentrations of Glu for 24 h and then exposed to diquat for 6 h. The abundance of tight junction proteins in IPEC-1 cells was determined. Compared with control, diquat treatment led to decreased (P < 0.05) protein abundance of claudin-1, claudin-3, claudin-4, ZO-1, ZO-2, and ZO-3 (Figure 7) in the absence of Glu. In the presence of diquat, cells cultured with 0.5 and 1 mmol/L Glu exhibited higher protein abundance of claudin-3, claudin-4, ZO-2, and ZO-3 compared with the 0 mmol/L Glu group (P < 0.05). In contrast, diquat treatment resulted in decreased (P < 0.05) protein abundance of claudin-1 and ZO-1 (Figures 6 and 7), regardless of Glu addition. In the absence of Glu, diquat exposure led to enhanced protein abundance of occludin (P < 0.05), which was not affected by Glu supplementation (Figure 6).

Discussion

Glu in the enteral diet is almost completely catabolized by the small intestine in the first pass, and only 5% of enteral glutamate enters the portal circulation in young pigs (11, 13). Enterocytes are major cell types for intestinal utilization of Glu in mammals, including pigs (9, 11, 12). Of note, as a building block of protein, Glu is required for the proliferation and differentiation of these cells.

![Figure 2](image-url) Effects of Glu on TEER in IPEC-1 cells. Cells were cultured with Glu (0.5- or 1 mmol/L) or without Glu for the indicated time points. Data are means ± SEMs, n = 3. Labeled means at a time point without a common letter differ, P < 0.05. Glu, L-glutamate; IPEC-1, intestinal porcine epithelial cell line 1; TEER, transepithelial electrical resistance.

![Figure 3](image-url) mRNA and protein abundance of Glu transporter in IPEC-1 cells cultured with or without Glu for 24 h. The mRNA expression of SLC1A1 (A) and protein abundance of EAAT3 (B) were analyzed. Data are means ± SEMs, n = 3. Means without a common letter differ, P < 0.05. SLC1A1, solute carrier family 1; EAAT3, excitatory amino acid transporter 3; Glu, L-glutamate; IPEC-1, intestinal porcine epithelial cell line 1; SLC1A1, solute carrier family 1, member 1.
cells (2). The intestinal epithelial barrier plays a critical role in preventing the translocation of bacteria and high-molecular-weight substances from the intestinal lumen into the systemic circulation (34). Many factors, such as weaning, illness, and toxins, can reduce the intestinal mucosal barrier function and, therefore, cause infection and growth depression in animals (35). We and others have reported that dietary supplementation with Glu enhances intestinal villus height, mucosal glutathione concentration, antioxidative capacity, and growth in early-weaned piglets (15, 36). However, the mechanisms responsible for the beneficial effects of Glu are largely unknown. Using the diquat-treated IPEC-1 cell model, we determined effects of physiologic concentrations of Glu on expression of tight junction protein concentrations, as well as cell membrane integrity and TEER. Our novel findings are expected to provide a hitherto unrecognized biochemical basis for dietary supplementation with Glu as a functional amino acid (19) to improve intestinal health and growth in neonatal mammals, including piglets and human infants.

Glutamate displays metabolic versatility in the small intestine (37). For example, Glu increases the expression of proliferating cell nuclear antigen in the jejunum of weanling piglets (38). In enterocytes, Glu undergoes transamination with oxaloacetate and pyruvate to produce L-aspartate and L-alanine (5), respectively. The carbon skeleton of Glu is oxidized to produce ATP in enterocytes via the mitochondrial tricarboxylic acid cycle (39). Furthermore, through the formation of N-acetylglutamate (an allosteric activator of carbamylphosphate synthetase-I) and L-aspartate (a substrate for argininosuccinate synthase), Glu stimulates the intestinal synthesis of citrulline and arginine (40). These Glu-dependent biochemical reactions may explain our finding that Glu enhanced the proliferation of IPEC-1 cells regardless of the presence or absence of diquat (Figure 2).

As noted previously, the intestinal epithelium is a physical structure separating the internal from the external environment in animals (34) while allowing for rapid uptake of water, electrolytes, and other nutrients from the intestinal lumen into the circulation (41). The intestinal barrier function is supported by the intestinal epithelial cell membrane and tight junction between the cells (34). Intracellular or extracellular stress leads to impaired mucosal barrier function and increased release of LDH from cells (as indicated by the amount of LDH in the culture medium) (32). In our study, we found that diquat induced oxidative stress in IPEC-1 cells and augmented LDH release from these cells. Of note, the addition of Glu to the culture medium markedly decreased LDH release from diquat-treated cells. These results indicate a novel and important role for Glu in maintaining the integrity of the IPEC-1 cell membrane.

Another indicator of enterocyte integrity is TEER, which refers to the permeability of intestinal epithelium. High TEER means low intercellular permeability of the epithelium. Some factors, such as inflammation, stress and injury, result in increased permeability of the intestinal epithelium, leading to dysfunction of intestinal mucosal barrier (42). Studies have shown that treatment with some probiotic bacteria may prevent or reverse the increase of permeability of the intestinal epithelium (43, 44). We showed that the presence of Glu in culture medium led to increased TEER regardless of oxidative stress in intestinal epithelial cells (Figures 2 and 5), suggesting a beneficial role for Glu in maintaining mucosal barrier function in animals. It should be noted that Glu is a major metabolite of the normal intestinal bacterial metabolism (17) that plays an important role in reducing the intercellular permeability of the intestinal epithelium. Thus, Glu may mediate in part the action of probiotics on enterocytes, suggesting close relationships between amino acids, gut microbes, and the host.

As the first step in identifying mechanisms for Glu to ameliorate diquat-induced oxidative stress and apoptosis in IPEC-1 cells, we determined the expression of EAAT3, which is the major transporter for transporting Glu into the enterocyte. Loss of function mutations in SLC1A1 causes dicarboxylic aminoaciduria in humans (45). In agreement with a previous study (46), we observed that both mRNA and protein abundances of EAAT3 (encoded by SLC1A1) were enhanced by Glu, which might facilitate Glu absorption and exert beneficial effects on...
intestinal epithelial cells. This effect of Glu on the expression of its major transporter is of nutritional and physiologic importance, considering that the concentrations of free Glu in sow milk collected and protein abundance was analyzed. Data are means ± SEMs, n = 3. Means without a common letter differ, P < 0.05. DQ, diquat; Glu, L-glutamate; IPEC-1, intestinal porcine epithelial cell line 1; ZO, zonula occludens.

FIGURE 6 Abundance of the tight junction proteins occludin (A), claudin-1 (B), claudin-3 (C), and claudin-4 (D) in diquat-challenged cells. IPEC-1 cells were cultured with Glu (0.5 or 1 mmol/L) or without Glu for 24 h, and then treated with 1 mmol/L diquat for 6 h. Cells were collected and protein abundance was analyzed. Data are means ± SEMs, n = 3. Means without a common letter differ, P < 0.05. DQ, diquat; Glu, L-glutamate; IPEC-1, intestinal porcine epithelial cell line 1.

FIGURE 7 Protein abundance of ZO-1 (A), ZO-2 (B), and ZO-3 (C) in diquat-challenged cells. IPEC-1 cells were cultured with Glu (0.5 or 1 mmol/L) or without Glu for 24 h, and then treated with 1 mmol/L diquat for 6 h. Cells were collected and protein abundance was analyzed. Data are means ± SEMs, n = 3. Means without a common letter differ, P < 0.05. DQ, diquat; Glu, L-glutamate; IPEC-1, intestinal porcine epithelial cell line 1.
increases rapidly from 0.16 to 1.05 mmol/L between day 0 and day 7 of lactation (47).

The intercellular junctional complex between enterocytes is critical for the integrity of the intestinal mucosal barrier, which consists of tight junctions, adherent junctions, gap junctions, and desmosomes (48, 49). Tight junctions that limit paracellular flux are the apical intercellular junction (50, 51). The tight junction proteins, including occludin, claudins, and ZO, provide a functional structure in the small-intestinal epithelium. Expression of these proteins is regulated by various stimuli, such as mitogen-activated protein kinases, protein kinase C, and certain amino acids, including tryptophan (33, 52, 53). Although the beneficial effect of Glu on intestinal health has been studied (53, 54), a role for Glu in regulating the expression and amounts of tight junction proteins has not been reported. Glutamate augments the abundance of these proteins possibly by stimulating their synthesis at the transcriptional level and inhibiting their degradation. At present, little is known about the effects of Glu on proteolysis in enterocytes via the lysosomal pathway (e.g., autophagy) or the nonlysosomal pathway (e.g., ATP- and ubiquitin-dependent protease). Nonetheless, our results have important implications for neonatal gut nutrition. Specifically, Glu deprivation may lead to decreased expression of tight junction proteins, thereby impairing intestinal mucosal barrier function. Because the small intestine does not take up arterial Glu (4), enteral provision of this amino acid is crucial for the growth, development, and function of the neonatal gut. Thus, newborn piglets, whose small-intestinal lumen has little Glu (i.e., 0.03 mmol/L) at birth (12), must receive enteral provision of adequate Glu in the form of either milk or Glu-containing infant formula to ensure healthy growth and development of the gut, particularly under conditions of oxidative stress.

In summary, the addition of physiologic concentrations of Glu to culture medium enhances proliferation and membrane integrity of IPEC-1 cells. These effects of Glu are mediated by increased expression of tight junction proteins in the cells. Furthermore, Glu has a protective effect on diquat-induced oxidative stress in IPEC-1 cells. Collectively, these results provide a new biochemical basis for the beneficial role of Glu in promoting intestinal growth, development, and health. Our findings have important implications for nutritional support of neonatal mammals, including piglets and human infants.

Acknowledgments
ZW and GW designed the study; NJ, YJ, and BW conducted the research; ZW, ZD, and GW analyzed the data; NJ, ZW, and GW wrote the paper; and ZW and GW had responsibility for the final content. All authors read and approved the final manuscript.

References

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