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Glycine Regulates Expression and Distribution of Claudin-7 and ZO-3 Proteins in Intestinal Porcine Epithelial Cells[1,2]

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Abstract

Background: Glycine traditionally is classified as a nutritionally nonessential amino acid in humans and animals. Because of its abundance in the body and its extensive use via multiple pathways, requirements for glycine are particularly high in neonates. Our recent studies show that dietary glycine supplementation is needed for optimal intestinal development in piglets. Importantly, reduced concentrations of glycine in the lumen of the small intestine are associated with gut dysfunction in low-birth-weight piglets. However, the mechanisms responsible for the beneficial effects of glycine on the intestinal mucosal barrier are largely unknown.

Objective: This study tested the hypothesis that glycine may regulate the expression and distribution of tight junction (TJ) proteins, thereby contributing to intestinal mucosal barrier function.

Methods: Enterocytes isolated from the jejunum of a healthy newborn pig were propagated to establish a stable cell line. The cells were cultured with 0.05 mmol glycine/L (control; concentration in the small intestinal lumen of low-birth-weight piglets) or 0.25 or 1.0 mmol glycine/L for the indicated periods of time. Epithelial barrier integrity and expression and localization of TJ proteins were analyzed by using monolayer transepithelial electrical resistance (TEER) and paracellular permeability, Western blot, and immunofluorescence imaging.

Results: Compared with controls, cells cultured with 0.25 or 1.0 mmol glycine/L increased TEER ($P < 0.05$) by 46–53% and 80–111%, respectively, at 60–72 h. Correspondingly, paracellular permeability was reduced ($P < 0.05$) by 6–21% and 18–27% for 0.25 or 1.0 mmol glycine/L treatment, respectively, at 36–72 h. Compared with controls, protein abundances for claudin-3, claudin-7, and zonula occludens (ZO) 3 were enhanced (25–33%, $P < 0.05$) by 0.25 and 1.0 mmol glycine/L at 8 h, whereas those for occludin, claudin-1, claudin-4, and ZO-2 were not affected. Compared with controls, 1.0 mmol glycine/L reduced the protein abundance of ZO-1 by 20% at 8 h ($P < 0.05$), but 0.25 mmol glycine/L had no effect. A glycine concentration of 0.25 mmol/L sustained the localization of claudin-7 and ZO-3 to the interface between enterocytes. Interestingly, 1 mmol glycine/L promoted the distribution of claudin-4 and claudin-7 to the cytosol and nucleus, and the localization of ZO-3 to the plasma membranes, while decreasing the distribution of ZO-1 at cell–cell contact sites, compared with control cells.

Conclusion: Physiologic concentrations of glycine support intestinal mucosal barrier function by regulating the abundance and distribution of claudin-7 and ZO-3 in enterocytes. Supplementation with glycine may provide an effective nutritional strategy to improve intestinal integrity in piglets. *J Nutr* 2016;146:964–9.

Keywords: glycine, intestinal epithelial cells, barrier function, tight junction proteins, permeability

Introduction

Glycine is the most abundant amino acid in the plasma of young pigs (1–3). Glycine traditionally has been categorized as a nutritionally nonessential amino acid because it is synthesized in the body (4–6). In addition to serving as a building block for protein synthesis, glycine is used for the biosynthesis of glutathione, heme, creatine, nucleic acids, and uric acid (4). Furthermore, glycine promotes the digestion and absorption of fats and long-chain FAs by conjugating bile acids in the lumen of

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the small intestine (4, 7, 8). Thus, glycine plays an important role in regulating DNA synthesis, cell proliferation, animal behavior, food intake, immune response, and whole-body homeostasis (7, 9–11). Based on the dietary intake of glycine and the accretion of glycine in the whole body, we have proposed that the amount of glycine synthesized by neonates is not sufficient to meet their maximal metabolic and growth requirements (12–14). Therefore, glycine must be included in the diet for the young, particularly low-birth-weight neonates with reduced concentrations of glycine in the plasma and small intestine (15).

Results of our recent study show that dietary supplementation of glycine enhances growth performance and intestinal development in milk-fed piglets (16). This effect was associated with a greater rate of protein synthesis and maintenance of intracellular redox states in enterocytes, which are required for intestinal mucosal barrier function (17, 18). In addition, reduced concentrations of glycine in the lumen of the small intestine are associated with gut dysfunction in low-birth-weight neonates (16). However, the underlying mechanisms remain largely unknown. We hypothesized that glycine may upregulate expression and distribution of tight junction (TJ)[6] proteins in intestinal epithelial cells, thus contributing to intestinal integrity in neonates.

Methods

Reagents. DMEM/Ham's F-12 and FBS were purchased from Invitrogen. DMEM was custom-made by Gibco. Epidermal growth factor was a product of BD Biosciences. Trypsin/EDTA was procured from Gibco. Antibodies against occludin, claudin-1, claudin-3, claudin-4, claudin-7, zonula occludens (ZO) 1, ZO-2, and ZO-3 were products of Invitrogen. Culture plates were purchased from Corning. Unless indicated, all other chemicals were purchased from Sigma-Aldrich.

Cell culture. Intestinal porcine epithelial cell 1 (IPEC-1) cells were isolated from the jejunum of a healthy newborn pig that did not have access to milk or any food (19). These cells were propagated to establish a stable cell line for our study. The cells were cultured in DMEM/Ham's F-12 as previously described (17). To evaluate the effect of glycine on intestinal integrity and TJ protein abundance, adhered cells were starved for 6 h in serum- and glycine-free custom-made DMEM (no. 08–5009EF; Gibco). The basal medium contained 5 mmol D-glucose/L, no glycine, and physiologic concentrations of all other amino acids found in the plasma of neonatal pigs (17). In our preliminary studies, we found that the concentrations (means ± SEMs; n = 6) of free glycine in the lumen of the jejunum were 0.06 ± 0.01 mmol/L in low-birth-weight (<0.7 kg birth weight) piglets not nursed by sows, 0.26 ± 0.02 mmol/L in normal-birth-weight piglets (~1.4 kg) not nursed by sows (KJ Sun, Z Wu, G Wu, unpublished data, 2015), and 1.05 ± 0.12 mmol/L in 7-d-old normal-birth-weight piglets nursed by sows (17). In the present study, IPEC-1 cells attached to the culture dish were treated with 0.05 (serving as control), 0.25, or 1.0 mmol glycine/L as previously described (17) for the indicated periods of time before respective analysis. This made our results physiologically more relevant to piglets with intrauterine growth restriction, a major health problem in both human medicine and animal production (18).

Measurement of transepithelial electrical resistance. Cells were incubated with 0.05 (serving as control), 0.25, or 1.0 mmol glycine/L for the indicated periods of time. Transepithelial electrical resistance (TEER) was determined every 12 h with the use of a Millicell ERS-2 Voltohmmeter (World Precision Instruments) equipped with an STX01 electrode as described (20). TEER was calculated by subtracting the resistance value of the filter and fluids, and was normalized to initial values. Six independent experiments were performed with IPEC-1 cells. All data are expressed as the relative values to those for the controls.

Measurement of paracellular permeability. Cells were treated as described for TEER determination. Fluorescein isothiocyanate (FITC)–labeled dextran (20 kDa) was added to the apical side of the monolayer at the final concentration of 1 g/L. Aliquots of the medium were removed from the basolateral chamber, and the concentration of FITC-dextran was measured with the use of the SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices) at excitation and emission wavelengths of 490 and 520 nm, respectively. The permeability of monolayer cells was defined as the amount of FITC-dextran that was transported from the apical side into the basolateral chamber. FITC-dextran concentration was calculated by subtracting the fluorescence value of FITC-free medium.

Determination of cell membrane integrity. Release of lactate dehydrogenase (LDH) from IPEC-1 cells into the cell culture medium was determined, as described previously by us (20). Briefly, cells were cultured with 0.05, 0.25, or 1 mmol glycine/L for 24 h. Thereafter, the medium was collected for determination of LDH activity with the use of an ultraviolet-visible spectrophotometer (450 nm) and an assay kit from Jiancheng Bioengineering.

Western blot analysis. IPEC-1 cells treated with various concentrations of glycine for 8 h were harvested for the analysis of abundance of TJ proteins, as previously described (20). Equal amounts of protein (25 μg) were separated on SDS-PAGE gels and then transferred to polyvinylidene difluoride membranes (Millipore). Blots were incubated with a primary antibody (1:2000) overnight at 4°C and then with an appropriate secondary antibody (1:2000) at 25°C for 1 h. The blots were detected with the ImageQuant LAS 4000 mini system (GE Healthcare Bio-Sciences) after reactions with ECL Plus detection reagents (Amersham Biosciences). The chemiluminescence signal was determined and band density was quantified with the use of Quantity One software (Bio-Rad Laboratories) (20). All results were normalized to β-actin and expressed as the relative values to those for the control group.

Immunofluorescence imaging. Cells were fixed with 4% paraformaldehyde at 37°C for 20 min and then incubated with a specific primary antibody against occludin, claudin-1, claudin-3, claudin-4, claudin-7, ZO-1, ZO-2, or ZO-3 for 16 h at 4°C. Cells were washed 3 times with PBS and then incubated with an appropriate secondary antibody (1:100) for 1 h at 25°C. Nuclei were stained with the use of Hoechst 33258 (1 μg/mL) for 10 min at 25°C. The distribution of TJ proteins was visualized under a fluorescence microscope (Axio Vert.A1; Zeiss).

Statistical analysis. Values are expressed as means ± SEMs. Data were analyzed by the use of 1-factor ANOVA and a Student-Newman-Keuls multiple comparison test at each time point separately with the use of SPSS statistical software for Windows (version 17.0). In statistical analysis, the number of observations for each glycine concentration group refers to the number of independent experiments with IPEC-1 cells. P values < 0.05 were taken to indicate statistical significance.

Results

Effects of glycine on barrier function in the IPEC-1 cell monolayer. Compared with control cells, 0.25–1.0 mmol glycine/L resulted in greater TEER (P < 0.05) at 48–72 h, (**Figure 1**A). Cells treated with 1.0 mmol glycine/L also had greater TEER (P < 0.05) than did those treated with 0.25 mmol glycine/L at 48–72 h. In contrast, no difference was observed between the cells treated with 0.25 or 1.0 mmol glycine/L and control cells at 12–36 h. Correspondingly, cells incubated with 0.25 or 1.0 mmol glycine/L had reduced paracellular permeability (P < 0.05), as indicated by FITC-dextran flux at 36–72 h (Figure 1B), compared with controls. Cells treated with 1.0 mmol

[6] Abbreviations used: FITC, fluorescein isothiocyanate; IPEC-1, intestinal porcine epithelial cell 1; LDH, lactate dehydrogenase; TEER, transepithelial electrical resistance; TJ, tight junction; ZO, zonula occludens.

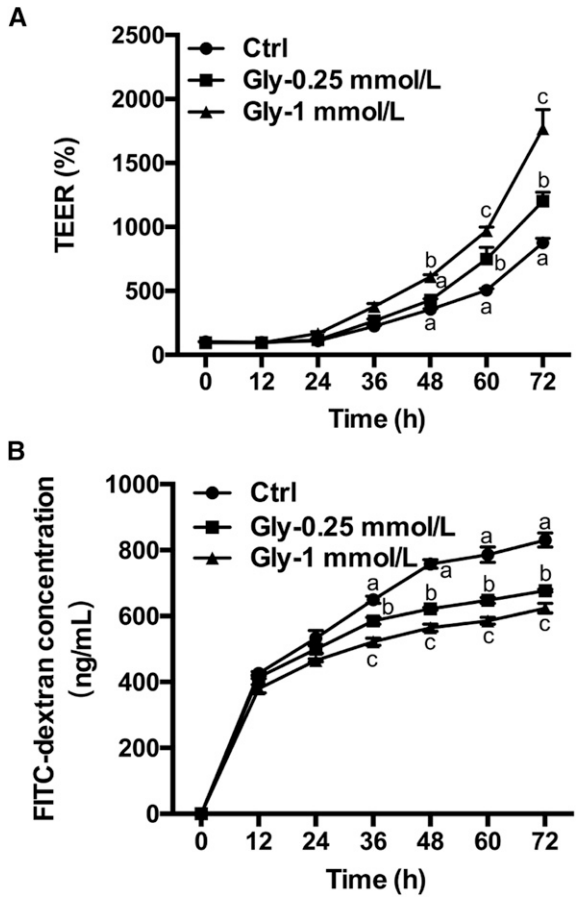

FIGURE 1 Effects of Gly on intestinal barrier function in IPEC-1 cells. Cells were cultured for the indicated time points in the presence of 0.05 (Ctrl), 0.25, or 1 mmol Gly/L. TEER (A) and paracellular permeability (B) were determined. Values are means ± SEMs; $n = 6$. Labeled means at a time without a common letter differ, $P < 0.05$. Ctrl, control (cells incubated with 0.05 mmol Gly/L); FITC, fluorescein isothiocyanate; IPEC-1, intestinal porcine epithelial cell 1; TEER, transepithelial electrical resistance.

glycine/L had lower permeability ($P < 0.05$) than did those treated with 0.25 mmol glycine/L at 36–72 h.

Effects of glycine on LDH release from IPEC-1 cells. The amount of LDH released into the medium of IPEC-1 cells did not differ between cells treated with 0.25 and 1.0 mmol glycine/L (240 ± 8.6 and 243 ± 11 units/L, respectively) and control cells (241 ± 7.9 units/L).

Effects of glycine on expression of TJ proteins in IPEC-1 cells. Compared with the control treatment, treatment with 0.25 and 1.0 mmol glycine/L enhanced the abundance of proteins ($P < 0.05$) for claudin-3, claudin-7 (**Figure 2**B and D), and ZO-3 (**Figure 3**C), without affecting that for claudin-1, claudin-4, occludin, or ZO-2 (Figure 2A and C and Figure 3B). The protein abundance of ZO-1 was reduced ($P < 0.05$) by 1.0 mmol glycine/L, but was not affected by 0.25 mmol glycine/L (Figure 3A).

Effects of glycine on the intracellular distribution of TJ proteins in IPEC-1 cells. The cellular distribution of TJ proteins was assessed by the use of an immunofluorescence microscope. Treatment with 0.25 mmol glycine/L promoted the localization of claudin-7 (**Figure 4**D) to the plasma membrane without affecting the localization of other TJ proteins, including claudin-1,

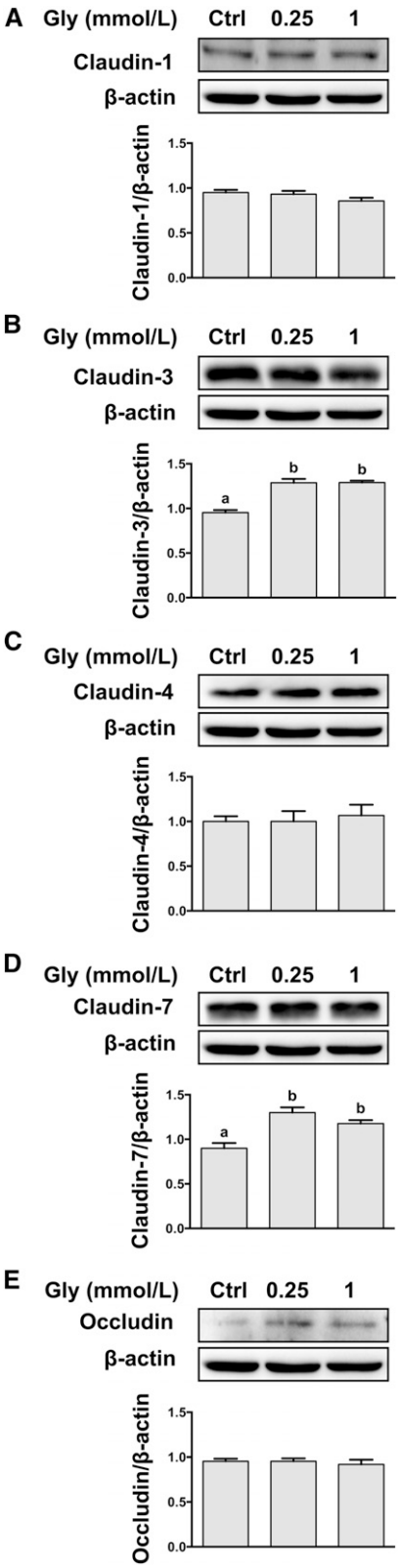

FIGURE 2 Protein abundances for claudin-1 (A), claudin-3 (B), claudin-4 (C), claudin-7 (D), and occludin (E) in IPEC-1 cells. IPEC-1 cells were cultured in the presence of 0.05 (Ctrl), 0.25, or 1 mmol Gly/L for 8 h. Cells were collected and protein abundances were analyzed. Values are means ± SEMs; $n = 6$. Means without a common letter differ, $P < 0.05$. Ctrl, control (cells incubated with 0.05 mmol Gly/L); IPEC-1, intestinal porcine epithelial cell 1.

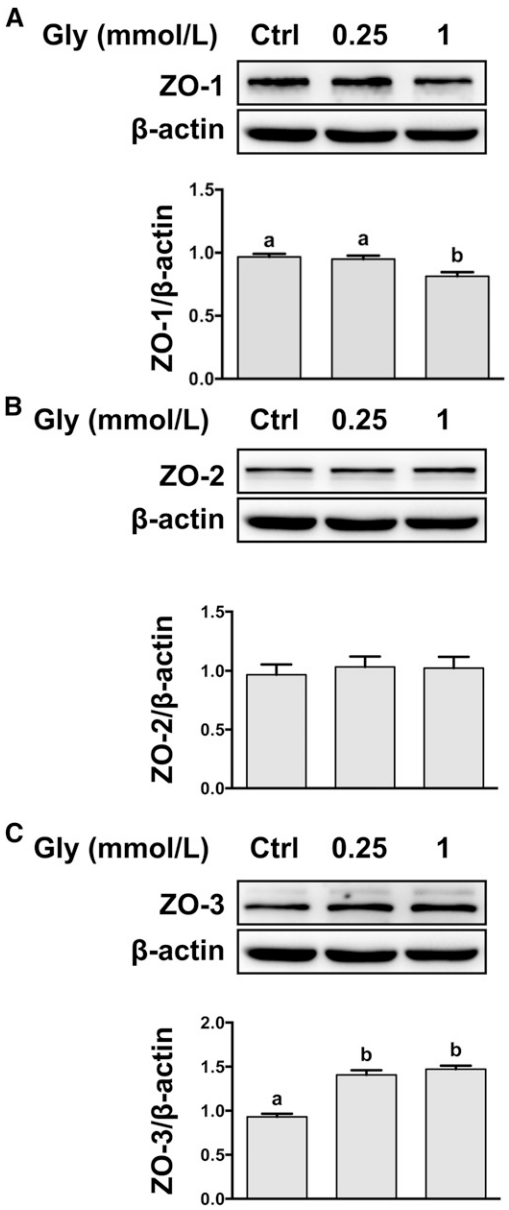

FIGURE 3 Protein abundances for ZO-1 (A), ZO-2 (B), and ZO-3 (C) in IPEC-1 cells. Cells were cultured in the presence of 0.05 (Ctrl), 0.25, or 1 mmol Gly/L for 8 h. Cells were collected and protein abundances were analyzed. Values are means ± SEMs; $n = 6$. Means without a common letter differ, $P < 0.05$. Ctrl, control (cells incubated with 0.05 mmol/L Gly); IPEC-1, intestinal porcine epithelial cell 1; ZO, zonula occludens.

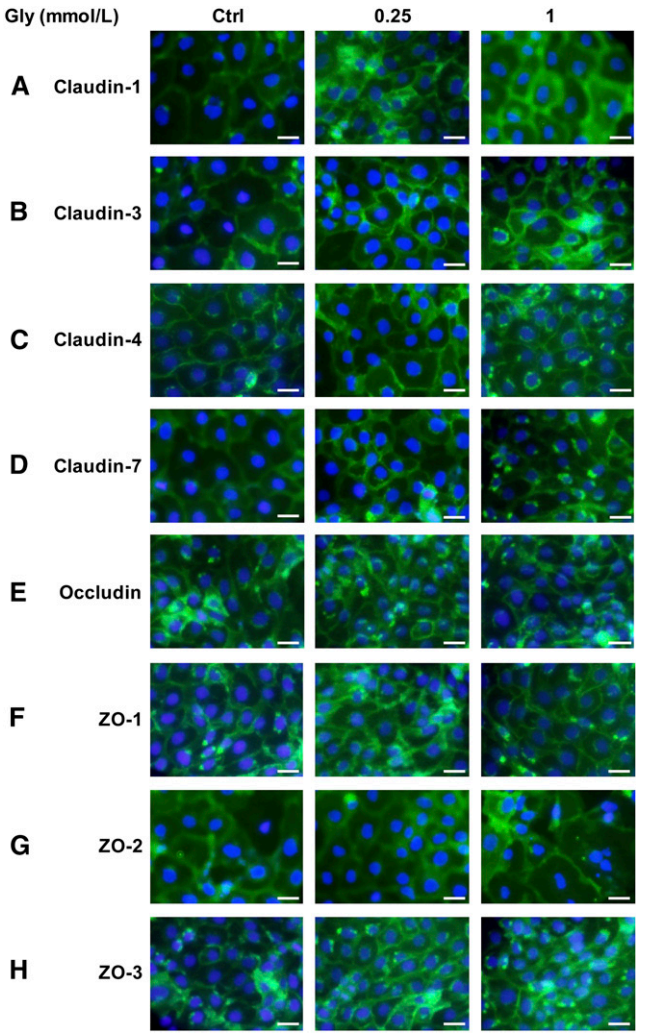

FIGURE 4 Effects of Gly on the distribution of the TJ proteins claudin-1 (A), claudin-3 (B), claudin-4 (C), claudin-7 (D), occludin (E), ZO-1 (F), ZO-2 (G), and ZO-3 (H) in IPEC-1 cells. Cells were treated as in Figure 3, and immunofluorescence staining was performed to identify the distribution of the TJ proteins. Scale bar, 50 μm. Ctrl, control (cells incubated with 0.05 mmol Gly/L); IPEC-1, intestinal porcine epithelial cell 1; TJ, tight junction; ZO, zonula occludens.

claudin-3, claudin-4, occludin, ZO-1, and ZO-2 (Figure 4). In contrast, 1.0 mmol glycine/L induced the localization of claudin-4 (Figure 4C) and claudin-7 (Figure 4D) from the cell membranes to the cytosol and the nucleus, and reduced the abundance of ZO-1 (Figure 4F) in the plasma membrane, compared with the controls. Cells treated with 0.25 or 1.0 mmol glycine/L had a greater abundance of ZO-3 at cell–cell contact sites than did control cells (Figure 4H).

Discussion

Intestinal mucosal barrier function is critical for nutrient transport, absorption, and intracellular homeostasis (21, 22). The epithelial barrier is formed by the apical plasma membrane and intercellular TJ, which provides physical and functional barriers to prevent bacteria, endotoxins, and other harmful substances from entering the blood circulation while allowing for the absorption of enteral nutrients (23). The intestinal mucosal barrier function can be regulated by diverse physiologic or pathologic stimuli (21, 24–26). Our recent studies showed that glycine supplementation enhanced growth performance and intestinal development in piglets (16) through the stimulation of global protein synthesis and maintenance of intracellular redox states (17). It is unknown whether glycine can regulate expression of TJ proteins and thereby contribute to intestinal mucosal barrier integrity.

To this aim, we first measured TEER, an indicator of intestinal epithelial integrity and permeability of intestinal epithelium. Multiple factors, such as inflammation, stress, and injury, result in increased permeability of the intestinal epithelium and dysfunction of the intestinal mucosal barrier (27). In the present study, we provided for the first time, to our knowledge, direct evidence that physiologic concentrations of glycine improve epithelial barrier integrity, as indicated by

enhanced TEER and reduced paracellular permeability (Figure 1).

Epithelial barrier function and paracellular permeability primarily are determined by epithelial TJs (28, 29). The TJs are multiprotein complexes composed of transmembrane proteins (e.g., the claudin family, occludin, and junction adhesion molecules) that interact with cytosolic peripheral proteins (e.g., ZO-1, ZO-2, and ZO-3) linking the transmembrane TJ proteins to the actin-based cytoskeleton (30, 31). Accumulating evidence shows that disruption of epithelial TJ integrity is associated with multiple intestinal disorders (28, 32). For example, it has been reported that enteric pathogens impair epithelial integrity in the intestine by either altering the cellular cytoskeleton or affecting the function of specific TJ proteins, such as integral membrane proteins (e.g., occludin), ZO-1, or members of the claudin family (31). We recently found that dietary supplementation with glutamine prevented weaning stress–induced intestinal barrier breakdown by augmenting TJ protein abundance (33), suggesting a functional role for amino acids in regulating mucosal barrier function.

Another important finding from the present study is that glycine regulates the cellular distribution of TJ proteins in intestinal cells. Specifically, we found that the presence of 0.25 mmol glycine/L in culture medium led to enhanced protein abundance in the cell membrane for claudin-7 and ZO-3, which is correlated well with augmented TEER values in IPEC-1 cells (Figure 1). The claudin family of proteins plays a crucial role in tightening cell–cell contacts and maintaining paracellular permeability (34, 35). Recent studies have demonstrated that the reduction of claudin-7 proteins is strongly associated with intestinal barrier disruption in rodents (36, 37); this was verified in our cell culture model. Interestingly, the exposure of IPEC-1 cells to glycine resulted in the translocation of claudin-7 from the cytosol to intracellular membranes. Thus, glycine regulates both the abundance and localization of claudin-7 in enterocytes. Considering the claudin-mediated barrier disruption in deoxynivalenol-treated pigs (38), supplementation with glycine may provide an effective nutritional strategy to attenuate mycotoxin-induced mucosal barrier dysfunction. It should be noted that 1 mmol glycine/L aided in the redistribution of claudin-4 and claudin-7 from intracellular membranes to the cytosol and the nuclear membrane compared with the control (Figure 4), suggesting a refined control of intestinal TJ function by glycine.

Another important group of TJ scaffolding molecules is the family of ZO proteins, including ZO-1, ZO-2, and ZO-3, as noted previously. It is generally believed that ZO-1 and ZO-2 can interact directly with occludin and claudins via their PSD95–DLG1–ZO1 domain, whereas their C-terminus can associate with actin, thus providing a direct link with the cytoskeleton (29). Such organization of ZO proteins is crucial for the assembly of TJ and epithelial barrier function (39). In contrast, in the present work and our previous in vivo studies (33), we observed that the abundance of proteins for ZO-2 or ZO-3, rather than ZO-1, was enhanced by certain amino acids. Although the existence of ZO-3 has been known for ~15 y, its function remains largely unknown (40, 41). In addition to providing a bridge that links occludin and claudins with the cytoskeleton, ZO proteins may also affect the expression of transmemberane proteins at transcriptional and post-translational levels (29). Moreover, even though numerous TJ proteins have been identified (30), their precise roles remain incompletely understood. More research is required to unravel the underlying molecular mechanisms responsible for the effects of glycine on the abundance and localization of TJ proteins in intestinal epithelial cells.

Low birth weight is a serious health problem in both human medicine and animal agriculture, and it affects 10–12% and 15–25% of newborn infants and piglets, respectively (42). Concentrations of glycine in the plasma of low-birth-weight neonates are reduced in comparison with their normal-birth-weight counterparts (15). Intestinal dysfunction is a major factor contributing to high rates of morbidity and mortality in both low-birth-weight infants (43) and piglets (42). Interestingly, protein abundances for claudin-7 and ZO-3, as well as the integrity of intestinal epithelial cells, were enhanced by 0.25 mmol glycine/L (found in the small-intestinal lumen of normal-birth-weight piglets) compared with 0.05 mmol glycine/L (found in the small-intestinal lumen of low-birth-weight piglets). We suggest that a deficiency of glycine in the gut contributes to its dysfunction in low-birth-weight piglets. This provides a new biochemical basis for the use of glycine to prevent intestinal abnormality, thereby improving neonatal survival and growth.

In summary, studies with porcine enterocytes revealed that physiologic concentrations of glycine improved intestinal epithelial barrier integrity, as indicated by increased TEER. This beneficial effect of glycine is accompanied by enhanced abundance and distribution of claudin-7 and the scaffolding protein ZO-3 in intestinal epithelial cells. Based on these findings, which, to our knowledge, are novel, we propose that supplementation with glycine may provide an effective nutritional strategy to improve intestinal mucosal barrier function in neonatal mammals, including piglets and human infants.

Acknowledgments

ZW and GW designed the research and had primary responsibility for the final content; WL, KS, YJ, and WW conducted the research; WL, KS, YJ, ZW, ZD, and GW analyzed the data; and WL, KS, ZW, and GW wrote the paper. All authors read and approved the final manuscript.

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