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Glutamine Enhances Tight Junction Protein Expression and Modulates Corticotropin-Releasing Factor Signaling in the Jejunum of Weanling Piglets[1,2]

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Abstract

Background: Dysfunction of tight junction integrity is associated with decreased nutrient absorption and numerous gastrointestinal diseases in humans and piglets. Although L-glutamine has been reported to enhance intestinal-mucosal mass and barrier function under stressful conditions, in vivo data to support a functional role for L-glutamine on intestinal tight junction protein (TJP) expression in weanling mammals are limited.

Objective: This study tested the hypothesis that glutamine regulates expression of TJPs and stress-related corticotropin-releasing factor (CRF) signaling in the jejunum of weanling piglets.

Methods: Piglets were reared by sows or weaned at 21 d of age to a corn and soybean meal–based diet that was or was not supplemented with 1% L-glutamine for 7 d. Growth performance, intestinal permeability, TJP abundance, and CRF expression were examined.

Results: Weaning caused increases ($P < 0.05$) in intestinal permeability by 40% and in CRF concentrations by 4.7 times in association with villus atrophy ($P < 0.05$). Western blot analysis showed reductions ($P < 0.05$) in jejunal expression of occludin, claudin-1, zonula occludens (ZO) 2, and ZO-3, but no changes in the abundance of claudin-3, claudin-4, or ZO-1 in weanling piglets compared with age-matched suckling controls. Glutamine supplementation improved ($P < 0.05$) intestinal permeability and villus height, while reducing ($P < 0.05$) jejunal mRNA and protein levels for CRF and attenuating ($P < 0.05$) weanling-induced decreases in occludin, claudin-1, ZO-2, and ZO-3 protein abundances.

Conclusion: Collectively, our results support an important role for L-glutamine in regulating expression of TJPs and CRF in the jejunum of weanling piglets. *J Nutr* 2015;145:25–31.

Keywords: glutamine, tight junction, corticotropin-releasing factor, pigs, small intestine, weaning stress

Introduction

In addition to serving as a major organ for nutrient digestion and absorption, the single layer of epithelium lining the gastrointestinal tract forms a selective barrier to prevent the passing of toxins, allergens, and pathogens from the luminal content into subepithelial tissues and systemic blood circulation (1). To maintain intracellular homeostasis, intestinal epithelial cells are tightly bound together by junctional complexes, which are essential for the biological function of the epithelium (2, 3). Consistently, loss of tight junction (TJ)[6] integrity and increased intestinal permeability are associated with the pathogenesis of numerous gastrointestinal diseases, such as inflammatory bowel disease, irritable bowel syndrome, celiac disease, and infectious enterocolitis (1, 3–5). These in vitro and in vivo data indicate that TJ proteins (TJPs) play an important role in mucosal barrier function, and abnormal expression of these proteins may result in intestinal disease in humans and other animals.

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[6] Abbreviations used: CRF, corticotropin-releasing factor; PKC, protein kinase C; TJ, tight junction; TJP, tight junction protein; ZO, zonula occludens.

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Increasing evidence from epidemiologic and animal studies shows that environmental factors, including stress and diet, can disrupt the epithelial barrier and contribute to the pathogenesis of gastrointestinal disorders (1, 6–8). Early weaning is a stressful event commonly associated with reduced growth, structural and functional alterations in the gut mucosa, as well as increased occurrence of intestinal disease in mammalian neonates (6, 9). Studies in rodents also demonstrate that early separation of the mother from the offspring leads to increased intestinal permeability (10). The impaired gut function not only impairs the absorption of nutrients, postnatal growth, and gut development in weanling piglets (6, 9, 11), but it also increases the prevalence of diseases that manifest in later life, including food allergies, celiac enteropathy, and inflammatory bowel disease (12, 13). Results of several studies indicate that mucosal corticotropin-releasing factor (CRF) receptor signaling can be activated in response to weaning stress (6, 11, 14, 15). Interestingly, pretreatment of pigs with CRF receptor antagonist abrogated weaning-induced mucosal damage (11, 15), suggesting a critical role for stress-related hormone in the development of intestinal dysfunction.

Glutamine, a conditionally essential amino acid for pigs (16), was reported to enhance gut development and regulate intestinal barrier function in multiple animal models (17–19). The negative correlation between plasma concentrations of glutamine and mucosal barrier function (20) implicates glutamine as a potentially protective nutrient on mucosal integrity. In line with this viewpoint, deprivation of glutamine decreases the expression of claudin-1 (a TJP) and increases permeability in cultured Caco-2 cells (21–23). Of note, our studies in piglets demonstrated that dietary glutamine supplementation prevents jejunal atrophy in weaned piglets (18, 24). Using microarray analysis, Wang et al. (20) reported that glutamine increases the expression of genes that are crucial for intestinal growth and antioxidative capacity in weanling pigs. It is unknown how these transcriptional alterations translate into enhanced gut growth and function.

There is evidence that L-glutamine can prevent acetaldehyde-induced TJ disruption in cultured Caco-2 cells (25, 26). With consideration of the important role for mucosal CRF receptor signaling and TJPs on the intestinal barrier function, we hypothesized that dietary glutamine supplementation ameliorates weaning-induced TJ disruption and modulates CRF receptor signaling in the piglet small intestine, thereby contributing to improvements in mucosal-barrier function in vivo. In the present study, piglets were nursed by sows or weaned at 21 d of age to a corn and soybean meal–based diet supplemented with or without 1% (wt:wt) L-glutamine for 7 d. Growth performance, intestinal permeability, TJP abundance, and CRF concentrations were examined. Our results indicate a novel role for L-glutamine to regulate protein abundance of occludin, claudin-1, zonula occludens (ZO)-2, ZO-3, and CRF in the small intestine of weanling mammals.

Methods

Animals. The animal handling procedures were approved by the Institutional Animal Care and Use Committee of China Agricultural University. A total of 36 crossbred healthy barrows (Duroc × Landrace × Yorkshire), with similar a body weight at 21 d of age, were randomly assigned to 1 of the 3 groups (n = 12/group). Piglets in group 1 continued to be nursed by sows (suckling piglets), whereas piglets in groups 2 and 3 were weaned to a corn and soybean meal–based diet that was supplemented with 1.22% L-alanine (isonitrogenous control) or 1% L-glutamine, as previously described (20). The basal diet was formulated to meet nutrient requirements for the pigs according to the National Research Council (20). The diet contained the following (on an as-fed basis): 63.7% corn, 21.0% soybean meal, 4.3% dried whey, 0.7% soybean oil, 8% fish meal, 0.1% dicalcium phosphate, 0.36% limestone, 0.3% NaCl, 0.33% lysine, 0.09% methionine, 0.1% threonine, 0.02% tryptophan, and 1.0% vitamin-mineral mix (20). The basal diet contained 2.20% glutamine, 2.07% glutamate, 1.43% arginine, 1.58% lysine, and 22.1% true protein (based on amino acid composition), as analyzed by Dai et al. (27). Weanling piglets had free access to experimental diets and drinking water. At 28 d of age, the jejunum was obtained, processed for removal of intestinal content, and stored at −80°C for the later analysis.

Histologic analyses of the jejunal tissue. Villus height and crypt depth in the jejunum were determined with the aid of a microscope, as previously described (24). Briefly, the jejunum was harvested and fixed in neutral buffered formalin until processing. Paraffin-embedded intestinal samples were sectioned and stained with hematoxylin and eosin for histologic analysis (28). Measurements for villus height and crypt depth were taken by using the computer-aided video microscopy (DXM1200C; Nikon) and the NIS-Elements BR software (version 2.20; Nikon).

CRF determination. Jejunal CRF concentration was determined by using a commercial ELISA kit (Jian Cheng Biotech) according to the instructions provided by the manufacturer. The values are expressed as picograms of CRF per milligram of jejunal protein.

Determination of intestinal permeability. Intestinal permeability was determined according to the methods previously described (29, 30), with modifications. Briefly, piglets were intragastrically administered lactulose (500 mg/kg body weight; Sigma) and mannitol (50 mg/kg body weight; Sigma). Urine samples were collected over a 24-h period from individual pigs placed in metabolism cages, and pooled as 24-h urine samples. Urinary recoveries of lactulose and mannitol were determined by using enzymatic spectrophotometric methods (31, 32).

Quantitative real-time RT-PCR. Total RNA isolated by Trizol reagent from jejunal tissues was transcribed into cDNA in a volume of 20 μL with a high-capacity cDNA archive kit (Applied Biosystems) following the manufacturer's protocol. The cDNA was used to perform PCR for *CRF* gene expression according to the standard protocol (28). Primer sequences used for the *CRF* gene were previously reported (15).

Western blot analysis. Frozen jejunal tissues were homogenized and lysed in ice-cold radioimmunoprecipitation assay lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% nonidet P (NP)-40, 0.1% SDS, 1.0 mmol/L PMSF, 1.0 mmol/L Na_3VO_4, 1.0 mmol/L sodium fluoride (NaF), and protease inhibitor cocktail (Roche Applied Science). Cell lysate was centrifuged at 12,000 × g for 15 min at 4°C to remove cellular debris. Protein concentration was determined by using the bicinchoninic acid (BCA) protein assay kit (Applygen Technologies). Equal amounts of protein (20 μg) were separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked in 5% skimmed-milk solution at room temperature for 1 h and then were incubated with diluted primary antibodies. Antibodies against occludin, claudin-1, claudin-3, claudin-4, ZO-1, ZO-2, and ZO-3 were obtained from Invitrogen. Blots were stripped and reprobed with anti–β-actin antibody (Santa Cruz) to demonstrate equal loading. After incubation with HRP-conjugated secondary antibody, the chemiluminescence signal was detected by using the Super Enhanced Chemiluminescence Kit (Applygen Technologies). Quantification of band density was determined by using Quantity One software (Bio-Rad Laboratories).

Statistical analysis. All data are presented as means ± SEMs and were analyzed by using 1-factor ANOVA. Differences between means were determined by using the Duncan multiple comparison method. All statistical analyses were performed by using SPSS statistical software (SPSS for Windows, version 17.0). $P < 0.05$ was considered significant.

Results

Food intake, body weights, and intestinal morphology. Dry matter intake and daily body weight gain between 21 and 28 d of age were markedly reduced in weanling piglets ($P < 0.05$) compared with age-matched suckling piglets (**Table 1**). Supplementation with 1.0% L-glutamine did not affect food intake but enhanced ($P < 0.05$) daily body weight gain between 21 and 28 d of age when compared with alanine-supplemented control piglets. Data on villus height, crypt depth, and villus height–to–crypt depth ratio in jejunal tissues are summarized in **Table 2**. Weaning stress led to decreased villus height and increased crypt depth in the jejunum of weaned piglets compared with that in suckling piglets (Table 2). Glutamine supplementation partially restored villus height in the jejunum ($P < 0.05$) but did not affect crypt depth in the weaned piglets. The ratio of villus height to crypt depth in the jejunum was lower in weaned piglets ($P < 0.05$) compared with that in age-matched suckling piglets, and this alteration was partially reversed ($P < 0.05$) by glutamine supplementation.

Intestinal permeability. The urinary recovery of permeability markers over 24 h was determined to evaluate intestinal permeability. As shown in **Figure 1**, lactulose recovery was greater in weanling piglets ($P < 0.05$) than in age-matched suckling controls (Figure 1A). The weaning-induced increase in the appearance of lactulose in the urine was partial reversed ($P < 0.05$) by glutamine supplementation. In contrast, mannitol recovery was not affected by weaning stress or glutamine supplementation (Figure 1B).

Effects of glutamine supplementation on stress hormone CRF in the jejunum. Because the activity of the hypothalamic-pituitary-adrenal axis is affected by weaning stress (6, 11, 14, 15), we next explored whether glutamine supplementation affected intestinal CRF expression in weanling piglets. As shown in **Figure 2**A, jejunal CRF concentrations were greater ($P < 0.05$) in weanling piglets than in age-matched suckling piglets, indicating the activation of the hypothalamic-pituitary-adrenal axis. However, the weaning-induced increase in jejunal CRF concentrations was reduced ($P < 0.05$) by glutamine supplementation. Similar results were found for *CRF* mRNA levels in the jejunum (Figure 2B).

Effects of glutamine supplementation on the abundance of TJPs. Western blot analysis was performed to investigate the jejunal expression of TJPs in suckling or weanling piglets. The results showed that weaning stress led to a marked decrease ($P < 0.05$) in the occludin protein in the jejunum, and this reduction was partially reversed ($P < 0.05$) by glutamine supplementation (**Figure 3**). Similar results were observed for protein abundances of claudin-1 (**Figure 4**A) and ZO-2 and ZO-3 (**Figure 5**B, C). Weaning stress had no effect on the protein abundance of claudin-3 or claudin-4 (Figure 4B, C), but their abundances were enhanced ($P < 0.05$) by glutamine supplementation (Figure 4). The protein expression of ZO-1 in the jejunum was not affected by weaning or glutamine supplementation.

TABLE 1 Dry matter intakes and BWs of suckling piglets or weanling piglets fed diets that were or were not supplemented with 1% L-glutamine from days 21 through 28[1]

Variable	S	W	W+Gln
Dry matter intake, g · kg BW^{-1} · d^{-1}	38.2 ± 2.5[a]	18.3 ± 3.8[b]	19.8 ± 3.1[b]
Initial BW on day 21, kg	6.66 ± 0.37	6.71 ± 0.41	6.68 ± 0.36
Final BW on day 28, kg	8.56 ± 0.31[a]	6.90 ± 0.18[c]	7.28 ± 0.27[b]
Daily BW gain, g/d	271 ± 10.2[a]	27.7 ± 7.1[c]	87.2 ± 10.4[b]

[1] Values are means ± SEMs, $n = 12$. Means in a row without a common letter differ, $P < 0.05$. BW, body weight; S, suckling piglets; W, weaning piglets fed a diet not supplemented with 1% L-glutamine; W+Gln, weaning piglets fed a diet supplemented with 1% L-glutamine.

TABLE 2 Jejunal morphology in 28-d-old suckling piglets or weanling piglets fed diets that were or were not supplemented with 1% L-glutamine from days 21 through 28[1]

Variable	S	W	W+Gln
Villus height, μm	438 ± 11.2[a]	254 ± 6.8[c]	334 ± 9.4[b]
Crypt depth, μm	198 ± 11.3[b]	235 ± 7.9[a]	224 ± 9.4[a]
Villus height:crypt depth	2.21 ± 0.1[a]	1.08 ± 0.1[c]	1.49 ± 0.2[b]

[1] Values are means ± SEMs, $n = 12$. Means in a row without a common letter differ, $P < 0.05$. S, suckling piglets; W, weaning piglets fed a diet not supplemented with 1% L-glutamine; W+Gln, weaning piglets fed a diet supplemented with 1% L-glutamine.

Discussion

The structure and function of the small intestine in weanling piglets undergo dramatic changes due to exposure to various stress factors (9, 33). Strategies to alleviate the detrimental effects of weaning on intestinal-mucosal barrier integrity are of great significance for the health of neonates. Recent studies suggest that the previously classified nutritionally nonessential amino acids, such as arginine, glycine, glutamine, and glutamate, can enhance gut development and regulate nutrient metabolism in animals through various mechanisms (34–39). Results from in vivo (24, 40–43) and in vitro (22, 25, 44, 45)

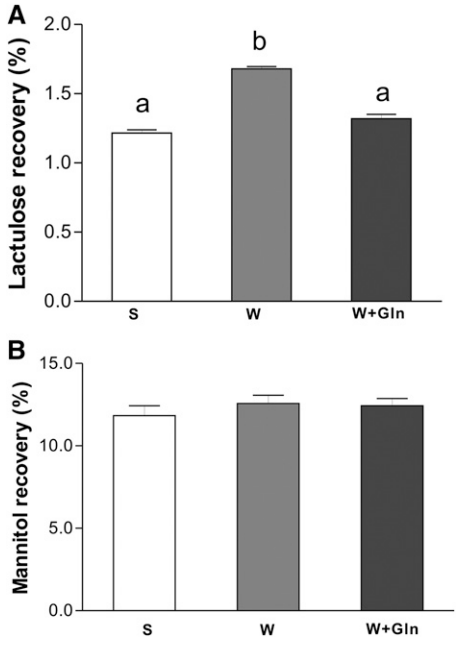

FIGURE 1 Urinary recovery of lactulose (A) and mannitol (B) after intragastric administration of lactulose and mannitol to suckling piglets or weanling pigs fed diets that were or were not supplemented with 1% L-glutamine from days 21–28. Values are means ± SEMs, $n = 6$. Means without a common letter differ, $P < 0.05$. S, suckling piglets; W, weaning piglets fed a diet not supplemented with 1% L-glutamine; W+Gln, weaning piglets fed a diet supplemented with 1% L-glutamine.

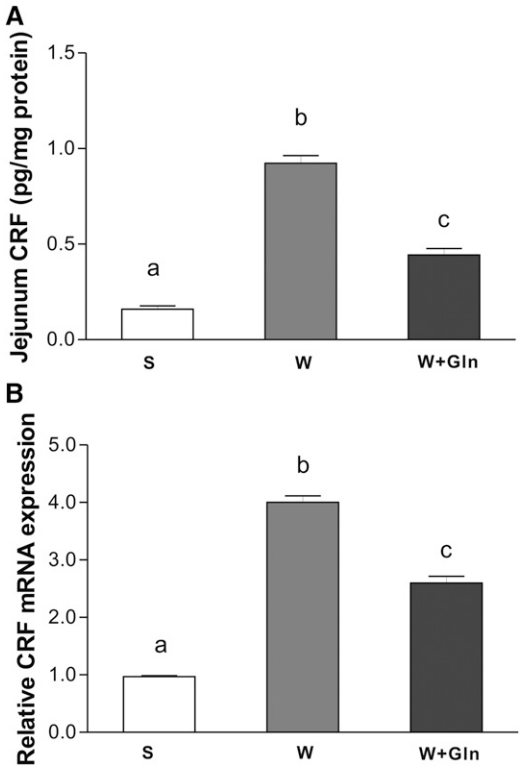

FIGURE 2 CRF concentrations (A) and mRNA abundance (B) in the jejunal tissue of 28-d-old suckling piglets or weanling piglets fed diets that were or were not supplemented with 1% L-glutamine from days 21 through 28. Values are means ± SEMs, $n = 6$. Means without a common letter differ, $P < 0.05$. CRF, corticotropin-releasing factor; S, suckling piglets; W, weaning piglets fed a diet not supplemented with 1% L-glutamine; W+Gln, weaning piglets fed a diet supplemented with 1% L-glutamine.

studies indicate that glutamine is critical for the maintenance of mucosal integrity under stress conditions. However, the regulatory effect of glutamine on TJP expression in weanling piglets has not been previously studied. The present study was a continuation of our previous work on intestinal gene expression and growth performance of glutamine-supplemented weanling piglets (18, 20, 24) and demonstrated, for the first time to our knowledge, that

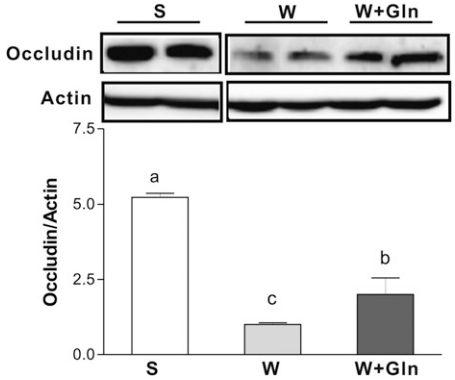

FIGURE 3 Immunoblot analysis of occludin protein abundance in the jejunal tissue of 28-d-old suckling piglets or weanling piglets fed diets that were or were not supplemented with 1% L-glutamine from days 21 through 28. Representative Western blots are shown. Values are means ± SEMs, $n = 6$. Means without a common letter differ, $P < 0.05$. S, suckling piglets; W, weaning piglets fed a diet not supplemented with 1% L-glutamine; W+Gln, weaning piglets fed a diet supplemented with 1% L-glutamine.

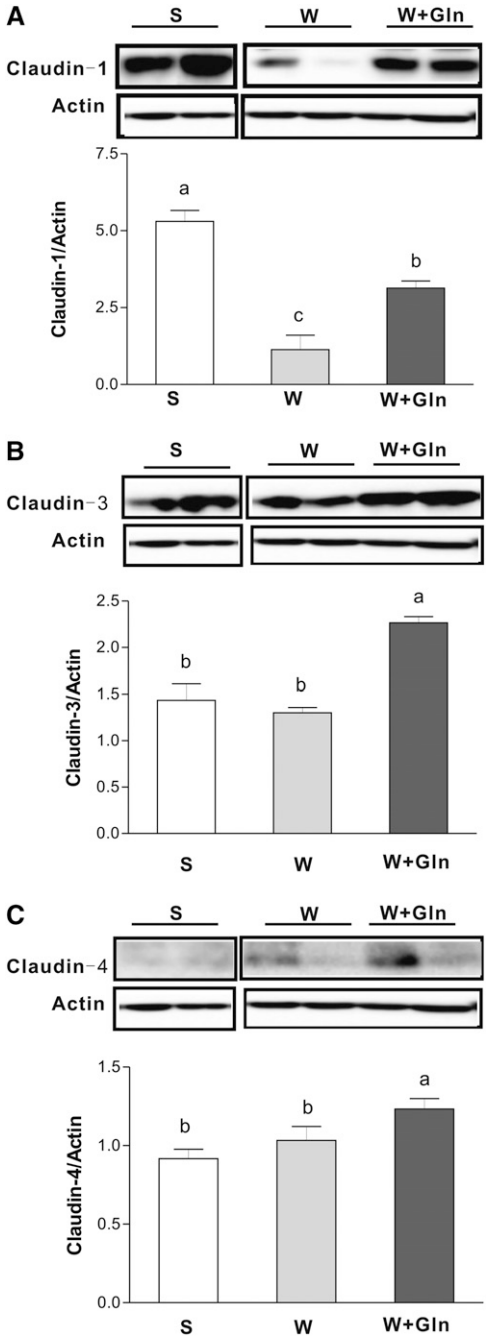

FIGURE 4 Immunoblot analysis of claudin-1 (A), claudin-3 (B), and claudin-4 (C) protein abundances in the jejunal tissue of 28-d-old suckling piglets or weanling piglets fed diets that were or were not supplemented with 1% L-glutamine from days 21 through 28. Representative Western blots are shown. Values are means ± SEMs, $n = 6$. Means without a common letter differ, $P < 0.05$. S, suckling piglets; W, weaning piglets fed a diet not supplemented with 1% L-glutamine; W+Gln, weaning piglets fed a diet supplemented with 1% L-glutamine.

dietary glutamine supplementation regulated intestinal protein abundance of occludin, claudin-1, ZO-2, ZO-3, and CRF in vivo.

We first validated that weaning stress resulted in reduced growth performance, as shown by decreased food intake and daily body weight gain, increased intestinal permeability, and reduced jejunal villus height. These findings indicate a stressful event occurring in weanling piglets (9, 15, 33). The abundance of glutamine in sow milk (46) and intestinal-mucosal barrier dysfunction after weaning indicate that glutamine is a critically

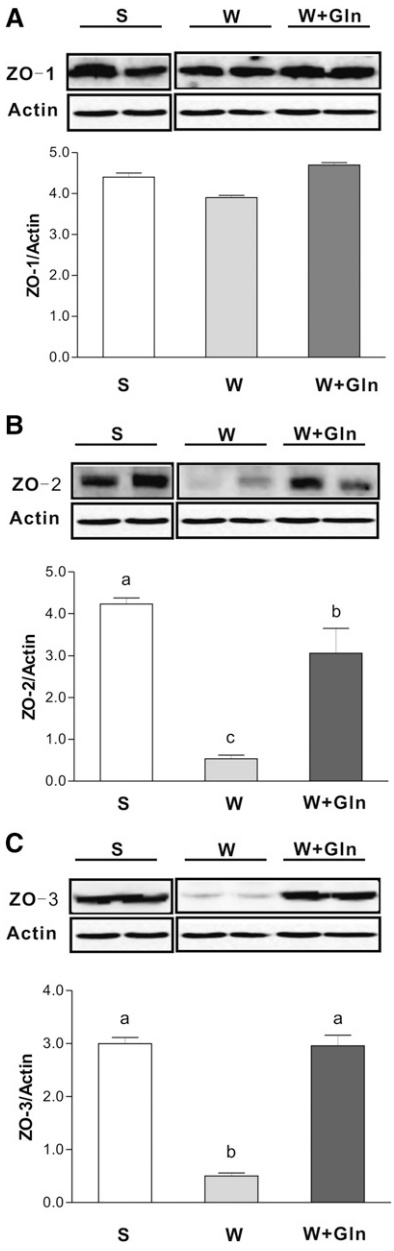

FIGURE 5 Immunoblot analysis of ZO-1 (A), ZO-2 (B), and ZO-3 (C) protein abundances in the jejunal tissue of 28-d-old suckling piglets or weanling piglets fed diets that were or were not supplemented with 1% L-glutamine from days 21 through 28. Representative Western blot are shown. Values are means ± SEMs, $n = 6$. Means without a common letter differ, $P < 0.05$. S, suckling piglets; W, weaning piglets fed a diet not supplemented with 1% L-glutamine; W+Gln, weaning piglets fed a diet supplemented with 1% L-glutamine; ZO, zonula occludens.

important nutrient for maintenance of TJ integrity and function under stressful conditions. This notion is further supported by improvements in mucosal-barrier function as evidenced by reduced permeability after dietary glutamine supplementation to weanling piglets (Figure 1). It should be noted that weaning stress led to increased recovery of lactulose in the urine but did not affect the recovery of mannitol. This result is consistent with the known mechanisms for their passage through the gut. Lactulose traverses the intestinal well by the paracellular pathway, whereas mannitol passes through the small intestine predominantly by the transcellular route (47). Weaning stress disrupted multiple TJPs (Figures 4 and 5), thereby leading to the elevated recovery of the orally administered lactulose in the urine due to the leaky gut.

A novel and important finding from this study is that weaning stress resulted in a marked alteration in the expression profile of TJPs. Specifically, the protein abundances of occludin (Figure 3), claudin-1 (Figure 4), and ZO-2 and ZO-3 (Figure 5) were decreased in comparison with those in suckling controls. Interestingly, the downregulation of occludin observed after weaning was abolished by glutamine supplementation (Figure 3). This result is consistent with the previous in vitro study with glutamine-deprived Caco-2 monolayers (21). In addition, we observed marked downregulation of scaffolding proteins ZO-2 and ZO-3, instead of ZO-1, at the protein level after weaning. This finding is not in agreement with previous studies that showed that glutamine depletion led to a reduced protein amount for ZO-1 in cultured Caco-2 monolayers (25, 45). The discrepancy might be explained by the following differences between these 2 studies. First, our study involved an in vivo animal model in which glutamine was always present in the extracellular fluid, whereas the previous work was conducted with cultured cells in which glutamine was presumably depleted by methionine sulfoximine (an inhibitor of glutamine synthetase) but was not verified by instrumental analysis. Second, the cell line used in the previous study was a colon cancer cell line, whereas our results were based on the jejunal tissue of young pigs. It is well known that there are major differences between normal cells and transformed cancer cells, including their genetic or epigenetic background, as well as their metabolism and cell signaling. Moreover, in the previous study, the authors examined the protein abundance of ZO-1 but did not analyze the protein abundances of other ZO family members, including ZO-2 and ZO-3 (45). In contrast, we determined all of the known proteins of the ZO family in our study.

ZO proteins, comprising ZO-1, ZO-2, and ZO-3, are important molecules that can interact directly with occludin, claudins, and actin, thus providing a scaffold for the assembly and the regulation of expression and distribution of the TJ complex (2, 48). Our results indicate that ZO-2, not ZO-1, is a critical factor that is sensitive to regulation by both weaning stress and glutamine in pigs. The role of ZO-2 in mucosal barrier integrity is supported by a recent study showing that ZO-1 and ZO-2 can independently determine the formation of the TJ strand (49). Although the existence of ZO-3 has been known for >15 y (50), its function remains largely unknown (51). In addition to providing a bridge that links occludin and claudins with the cytoskeleton (48), ZO proteins may also affect the expression of transmembrane proteins at the transcriptional or post-translational level (52). Thus, intestinal TJPs are likely modified by multiple mechanisms. At present, it is unknown how weaning stress and glutamine regulate the expression or function of the ZO protein. A recent study showed that ZO-2 can interact with several protein kinase C (PKC) isoforms, suggesting the possibility that ZO-2 regulates the TJ complex by post-translational modifications (53). Considering the dual function of PKC activation in the TJ assembly (54, 55) or disassembly (56, 57), more studies are needed to elucidate the underlying molecular mechanisms by using genetic manipulation technologies or specific pharmacologic inhibitors.

The activation of stress signaling pathways contributes to intestinal dysfunction in weanling mammals (6, 11, 14, 15). In line with previous studies (7, 11, 15), CRF concentrations in the jejunum of weanling piglets were increased when compared with controls. Importantly, the weaning-induced increase in the jejunal abundance of CRF was attenuated by glutamine supplementation. This result, along with the enhanced expression of

TJPs and intestinal permeability, suggests that CRF is a key stress hormone associated with intestinal-mucosal barrier function (15). Maillot et al. (58) reported that in vitro administration of CRF to monolayer cells increased intestinal permeability in colonic tissues. Smith et al. (11) found that weaning-induced mucosal barrier dysfunction could be prevented by a CRF receptor antagonist, providing direct evidence for the functional role of CRF in mucosal barrier integrity. In addition, a recent study found that this effect of CRF was mediated by enhanced release of proteases and TNF-α from mast cells (59). In our study, glutamine supplementation reversed the elevation of jejunal CRF, which might, in turn, block the release of proteases and TNF-α from mast cells in the jejunum, thereby conferring a protective function of the intestinal mucosa. Glutamine is a truly functional amino acid in nutrition (38), and weanling piglets have dietary requirements of this nutrient to support intestinal health and growth as well as whole-body homeostasis (1).

In summary, weaning stress led to marked alterations in the expression profile of TJPs and jejunal CRF concentrations. The abundances of occludin, claudin-1, ZO-2, and ZO-3 in the jejunum were decreased by weaning and were ameliorated by dietary glutamine supplementation. Glutamine treatment also attenuated weaning-induced increases in jejunal mRNA and protein abundance of CRF. Collectively, our results revealed a novel and important role for glutamine in regulating TJP expression and CRF signaling in the small intestine of weanling piglets, thereby conferring a beneficial effect on mucosal barrier function and health in neonates. Glutamine is a functional amino acid in piglet nutrition.

Acknowledgments

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