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ORIGINAL ARTICLE

Glycine supplementation to breast-fed piglets attenuates post-weaning jejunal epithelial apoptosis: a functional role of CHOP signaling

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Abstract

This study was conducted to test the hypothesis that preweaning glycine supplementation to breast-fed piglets alleviated the post-weaning apoptosis of jejunal epithelium through CHOP signaling. Seven-day-old sow-reared piglets were orally administrated with 0, 50, 100, or 200% of glycine intake from sow's milk twice daily for 14 days and then were weaned at 21 days of age. Tissue samples were collected at 28 days of age for determining intestinal morphology, serum diamine oxidase (DAO) activity, abundances of proteins involved in ER stress and apoptosis. Glycine (100–200%) administration increased villus height, the ratio of villus height to crypt depth in the jejunum. Glycine supplementation (200%) enhanced average daily weight gain during the first 2 weeks post-weaning. Serum DAO activity and jejunal epithelium apoptosis were decreased, but the number of goblet cells in the jejunum was increased. Western blot analysis showed that 100–200% glycine enhanced the protein levels of occludin, claudin-1, and zonula occludens (ZO)-1 without affecting those of claudin-3, ZO-2, and ZO-3. Further studies showed that protein abundances of glucose-regulated protein 78 (BiP/GRP78) and p-IRE1α, instead of ATF6α, were reduced by glycine. Among the proteins related to apoptosis, abundances of CHOP and p53 were reduced, whereas those of Bcl-2 and Bcl-xL were enhanced in the jejunum of 100–200% glycine-supplemented piglets. Collectively, our results indicated that preweaning glycine supplementation improved the intestinal development of post-weaning piglets. The beneficial effect of glycine was associated with improved intestinal mucosal barrier and reduced apoptosis of enterocytes through CHOP signaling.

Keywords Glycine · Endoplasmic reticulum stress · Small intestine · Apoptosis · Piglets

Introduction

The small intestine is the site for the terminal digestion and absorption of nutrients (Wu 2018). Intestinal mucosal barrier is critical for preventing harmful antigens, pathogens and toxins in the intestinal lumen from entering the systemic circulation (Moens and Veldhoen 2012; Turner 2009). To maintain intracellular homeostasis, intestinal epithelial cells are tightly bound together by junctional complexes, which are essential for the epithelium to maintain its physiological function (Turner 2009; Marchiando et al. 2010). Disruption of tight junction proteins (TJPs) and increased intestinal permeability have been reported to be associated with the development of numerous gastrointestinal diseases, such as inflammatory bowel disease, infectious enterocolitis, irritable bowel syndrome, and celiac disease (Odenwald and Turner 2013; Arrieta et al. 2006). Weaning stress is one of the most stressful events that involved multiple factors

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(Wijtten et al. 2011). It is well known that weanling piglets have a decreased feed intake, reduced villous height, increased apoptosis of epithelial cells, and dysfunction of intestinal mucosal barrier during the first week post-weaning (Yang et al. 2016; Bauer et al. 2011; Wijtten et al. 2011; Wu et al. 1996).

The endoplasmic reticulum (ER) is the intracellular organelle in which proteins are synthesized, folded, and secreted (Ron and Walter 2007). A variety of conditions, such as hypoxia, starvation, and infections, cause the accumulation of misfolded proteins within the ER and result in the endoplasmic reticulum stress (Iurlaro and Munoz-Pinedo 2016). Eukaryotic cells have evolved a protective strategy, collectively known as the unfolded protein response (UPR) to restore ER homeostasis by reducing protein synthesis, promoting proper protein folding, or enhancing the degradation of unfolded proteins (Ron and Walter 2007; Hetz et al. 2015). The UPR is mainly mediated by three ER membrane-associated proteins, PKR-like eukaryotic initiation factor 2α kinase (PERK), inositol requiring enzyme 1 alpha (IRE1α), and activating transcription factor-6 alpha (ATF6α) (Hetz et al. 2015). Under normal conditions, the transmembrane proteins are bound to the chaperone BiP/GRP78 in the intralumenal domain and, therefore, are maintained in an inactive state (Hotamisligil 2010). In response to ER stress, the sensor proteins (including PERK, IRE1α, and ATF6α) are released from BiP, thus activating downstream signaling cascades to reduce the deleterious effect of unfolded protein accumulation (Bertolotti et al. 2000; Hotamisligil 2010). UPR can activate downstream pro-apoptotic proteins, such as CHOP (also known as growth arrest and DNA damage-inducible gene 153, GADD153), Noxa, Bim, or repress anti-apoptotic proteins such as Bcl-2 and Bcl-xL, ultimately leading to cell death (Iurlaro and Munoz-Pinedo 2016).

Glycine has traditionally been categorized as a nutritionally nonessential amino acid because it is synthesized in the body (Wang et al. 2013). However, glycine is severely deficient in milk and plant proteins (Birchenough et al. 2015; Wu et al. 2014). More and more evidence shows that the amount of glycine synthesized de novo is insufficient to meet maximal growth or optimal health of piglets (Melendez-Hevia et al. 2009; Wang et al. 2014a). Based on glycine content in sow's milk and its accretion in the whole body, it is estimated that sow's milk meets at most only 23% of daily glycine needs for protein synthesis in piglets (Wu 2010). In addition, dietary glycine supplementation improves the abundances of intestinal epithelial TJPs and improves intestinal health in piglets (Wang et al. 2014a). Moreover, glycine administration is associated with reduced apoptosis in cultured cells (Bhattacharyya et al. 2012; Weinberg et al. 2016). However, the underlying mechanisms are largely unknown.

Based on the foregoing, this study was conducted to test the hypothesis that preweaning glycine supplementation to suckling piglets during 7–21 days of age may prevent post-weaning intestinal epithelial apoptosis in the small intestine, in which ER stress signaling is involved.

Materials and methods

Piglets and experimental design

Experiment 1: Piglets were the offspring of Yorkshire × Landrace and maintained at the Yinfa Animal Husbandry Co. farm (Henan, China). The average birth weight of the piglets used for this study was 1.55 kg. Multiparous sows were fed a corn- and soybean meal-based diet (containing 18.1% crude protein and 3160 kcal metabolizable energy/kg diet) during lactation (Supplemental Table 1). At 7 days of age, 64 piglets from 8 litters (8 piglets per litter) with the body weight of 3.1 ± 0.07 kg were allotted randomly into one of the four groups based on body weight and litter origin. The piglets were orally administered with 0, 50, 100, or 200% of glycine intake from sow's milk daily until 21 days of age. L-alanine was used as the isonitrogenous control (Table 1). Supplemented glycine or L-alanine was dissolved in 10-mL saline and supplied twice daily at 0800 and 1400 h. The amount of glycine given to piglets was calculated based on milk intake as previously described (Kim and Wu 2004; Wu et al. 2004; Sun et al. 2015). All piglets had free access to sow's milk and water throughout days 1–21 of age. Piglets were weaned at 21 days of age to a corn- and soybean meal-based diet, and transferred to a nursery room. There were 8 pens (2 piglets/pen) per treatment group. All piglets had free access to drinking water and the post-weaning basal diet was formulated to meet nutritional requirements [National research council (NRC), 2012] of piglets (Table 2) throughout days 21–42 of age. The body weights of preweaning piglets, as well as the feed intake and body weights of post-weaning piglets, were recorded weekly.

Experiment 2: To investigate the molecular mechanisms for glycine to alleviate the weaning stress-induced apoptosis of the small intestine, Experiment 2 was conducted as in Experiment 1, except that each treatment group of Experiment 2 had 3 pens (2 piglets/pen). Blood samples

Table 1 The oral dose of glycine or L-alanine to preweaning piglets between 7 and 21 says of age

Treatment	Control	50% Glycine	100% Glycine	200% Glycine
Supplement	0%	50%	100%	200%
Glycine (g/kg BW)	0	0.18	0.35	0.70
Alanine (g/kg BW)	0.83	0.62	0.41	0

Table 2 Ingredient composition and chemical analysis of the basal diet for weanling piglets

Ingredients	Percentage (%; as-fed basis)
Corn	38.83
Extruded corn	20
Soybean meal (46% CP)	15.31
Extruded soybean	10
Fish meal (63% CP)	4
Whey powder	6.5
Soybean oil	2
L-Lysine (99.0%)	0.15
Limestone powder	0.61
Dicalcium phosphate	1.2
Salt	0.4
Vitamin–mineral premix[a]	1
Total	100
Nutrient levels %	
Digestible energy (Mcal/kg)	3.53
Crude protein	21.0
Total phosphorus	0.66
Calcium	0.76
Glycine	0.91
L-Lysine	1.18
Methionine + cysteine	0.68
Threonine	0.73
Isoleucine	0.75

[a]Supplied per kilogram of complete diet: retinyl acetate, 7709 IU; cholecalciferol, 2200 IU; DL-α-tocopheryl acetate, 60 IU; menadione sodium bisulfite complex, 9 mg; riboflavin, 7.7 mg; vitamin B12, 0.044 mg; D-calcium pantothenate, 33 mg; niacin, 33 mg; choline, 287 mg; D-biotin, 0.22 mg; Mn (as $MnSO_4 \cdot H_2O$), 20 mg; Fe (as $FeSO_4 \cdot H_2O$), 100 mg; Zn (as $ZnSO_4 \cdot H_2O$), 100 mg; Cu (as $CuSO_4 \cdot 5H_2O$), 15 mg; I (as KI), 0.60 mg; Se (as Na_2SeO_3), 0.10 mg

were collected 1 h after feeding at 28 days of age (7 days post-weaning). Thereafter, all piglets were euthanized by intravenous administration of sodium pentobarbital (50 mg/kg BW) (Schering-Plough, Canada). The jejunal tissues were collected and frozen in liquid nitrogen for later analysis.

Histologic analyses of the jejunum and goblet cell staining

The segments (1 cm) of the jejunal tissues fixed in 4% paraformaldehyde were embedded in paraffin and were sectioned (5 µm of thickness), stained with hematoxylin–eosin for histological analysis (Wu et al. 1996). The goblet cells were stained with Alcian Blue and periodic acid-Schiff reagent (Beijing ZSGB-BIO Co. Ltd), according to the protocol provided by the manufacturer. Briefly, the tissue sections were incubated with Alcian Blue solution for 30 min, and then were washed with water for 5 min. After incubation with periodic acid for 10 min, the sections were incubated with Schiff reagent for 10 min. The goblet cells were observed under a microscopy (Axio Vert.A1; Zeiss).

Analyses of diamine oxidase (DAO) activity in serum

The DAO activity in serum was determined by the enzymatic assay using glutamate dehydrogenase and NADH in the indicator system. The ammonia formation per min was determined by the decrease in extinction due to an equimolar oxidation of NADH, which was a measure of DAO activity as previously described (Kusche et al. 1974). The kit used for DAO analysis was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

TUNEL assay

Jejunal tissues fixed with optimal cutting media (O.C.T., Tissue-Tek) were sectioned (8 µm of thickness). Apoptosis was determined by the TUNEL assay (Beyotime Biotechnology, China) following the manufacturer's instruction. The nuclei were stained with the Hoechst 33258 (1 mg/mL) for 1 min at 25 °C and then were observed under a fluorescence microscope (Axio Vert.A1; Zeiss).

Western blot analysis

Jejunal tissues were homogenized and lysed in ice-cold 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, Indianapolis, IN). Equal amounts of protein (50 µg) were separated on SDS-PAGE gels and then transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked in a 5% skimmed-milk solution at 25 °C for 1 h. The blots were incubated with a primary antibody overnight at 4 °C and then incubated with an appropriate secondary antibody at 25 °C for 1 h. The blots were detected with the ImageQuant LAS 4000 mini system (GE Healthcare BioSciences) after reaction with ECL Plus detection reagents (Amersham Biosciences). The chemiluminescence signal was determined and band density was quantified with the One Quantity software (Bio-Rad Laboratories). Anti-occludin (1:2000, 40–4700), anti-claudin-1 (1:2000, 51–9000), anti-claudin-3 (1:2000, 34-1700), anti-ZO-1 (1:2000, 61–7300), anti-ZO-2 (1:2000, 38–9100), and anti-ZO-3 (1:2000, 36–4100) polyclonal antibodies were obtained from Invitrogen (California, USA). Anti-GRP78/BiP (1:2000, 3183, polyclonal antibody) and anti-p53 (1:2000, 2524, monoclonal antibody) were purchased from Cell Signaling Technology (Massachusetts, USA). Anti-p-IRE1α (1:2000, phosphorylated serine 724,

ab48187) polyclonal antibody was procured from Abcam (Cambridge, UK). Anti-ATF6α (1:1000, sc-22799), Anti-Bcl-2 (1:1000, sc-492), Anti-Bcl-xL (1:1000, sc-634), Anti-Bax (1:1000, sc-493), and Anti-CHOP (1:1000, sc-575) polyclonal antibodies were obtained from Santa Cruz (California, USA). All results were normalized to GAPDH (1:2000, sc-59540, Santa Cruz) and expressed as the relative values to the control group.

Statistical analysis

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

Results

Growth performance and intestinal morphology of piglets

As shown in Table 3, oral administration of glycine to preweaning piglets did not affect their growth performance before weaning ($P > 0.05$), as compared with the control group. Interestingly, preweaning supplementation of 200% glycine (days 7–21 of age) enhanced ($P < 0.05$) daily body weight gain and decreased feed/gain ratio in post-weaning piglets between 21 and 28 days of age (the first week post-weaning) when compared with alanine-supplemented piglets. The beneficial effect of preweaning glycine supplementation lasted until 35 days of age (2 weeks post-weaning). No effect on weight gain was observed ($P > 0.05$) between 35 and 42 days of age ($P > 0.05$). Compared with the control group, preweaning supplementation of 200% glycine enhanced ($P < 0.05$) the post-weaning daily BW gain between 21 and 42 days of age by 14%. Data on villus height, crypt depth, and villus height–crypt depth ratio in the jejunum of 28-day-old pigs (1 week post-weaning) are summarized in Table 4. Preweaning glycine supplementation increased villus height and reduced crypt depth ($P < 0.05$) in the jejunum of weanling piglets. The ratio of villus height to crypt depth in the jejunum was higher ($P < 0.05$) in glycine-supplemented piglets, compared with the controls.

Table 3 Effects of glycine or alanine (the isonitrogenous control) supplementation between 7 and 21 days of age on growth performance in weanling piglets

Items	Oral administration of glycine (% of milk glycine intake)			
	0	50	100	200
Body weight (kg)				
Day 7	3.1 ± 0.17	3.1 ± 0.16	3.1 ± 0.13	3.1 ± 0.16
Day 14	5.4 ± 0.22	5.3 ± 0.25	5.6 ± 0.21	5.4 ± 0.25
Day 21	7.4 ± 0.27	7.5 ± 0.31	7.8 ± 0.27	7.5 ± 0.34
Day 28	7.6 ± 0.25^{b}	7.8 ± 0.31^{ab}	8.1 ± 0.22^{ab}	8.4 ± 0.24^{a}
Day 35	9.5 ± 0.29^{b}	9.6 ± 0.31^{b}	10.0 ± 0.22^{ab}	10.6 ± 0.32^{a}
Day 42	11.9 ± 0.42	12.0 ± 0.43	12.4 ± 0.30	12.6 ± 0.45
Average daily weight gain (g/day)				
Days 7–14	323 ± 14	309 ± 15	355 ± 16	320 ± 17
Days 14–21	286 ± 16	307 ± 20	313 ± 14	307 ± 24
Days 21–28	23.9 ± 14^{b}	51.4 ± 10^{ab}	45.3 ± 13^{ab}	71.9 ± 19^{a}
Days 28–35	266 ± 13^{b}	261 ± 13^{b}	264 ± 12^{b}	304 ± 11^{a}
Days 35–42	358 ± 19	337 ± 23	351 ± 23	347 ± 21
Average daily feed intake (g/d)				
Days 21–28	100 ± 10	104 ± 11	102 ± 15	112 ± 17
Days 28–35	352 ± 21	334 ± 18	346 ± 14	355 ± 11
Days 35–42	584 ± 52	520 ± 34	597 ± 42	591 ± 25
Feed/gain ratio (g feed/g BW)				
Days 21–28	2.7 ± 0.33^{a}	1.9 ± 0.19^{ab}	2.0 ± 0.39^{ab}	1.7 ± 0.28^{b}
Days 28–35	1.3 ± 0.02	1.3 ± 0.04	1.3 ± 0.04	1.2 ± 0.06
Days 35–42	1.7 ± 0.09	1.7 ± 0.05	1.7 ± 0.04	1.7 ± 0.04

Values are mean ± SEM, $n = 16$ piglets for data on body weight and average daily weight gain; and $n = 8$ pens for data on average daily feed intake and feed/gain ratio. Within a row, means without a common letter differ, $P < 0.05$

Glycine intake from sow's milk was 0.35 g/kg BW/day

The activity of DAO in serum

An increase in the amount of DAO in serum is considered as an indicator of damage in intestinal mucosa integrity (Yi et al. 2017; Wu 2018). As shown in Fig. 1, preweaning glycine administration reduced serum DAO activity in a dose-dependent manner ($P < 0.05$), as compared to controls, indicating a beneficial effect of glycine on the integrity of intestinal mucosa.

Tight junction protein expression in jejunum

Western blot analysis revealed that preweaning supplementation of 100% and 200% glycine enhanced ($P < 0.05$) protein levels of occludin, claudin-1 and ZO-1 (Fig. 2a, b, d) in jejunal tissues of piglets at day 7 post-weaning, as compared with the controls. The protein abundances of jejunal ZO-2, ZO-3, and claudin-3 (Fig. 2c, e, f) were not affected ($P > 0.05$) by glycine supplementation.

Goblet cell morphology and apoptosis

Goblet cells can synthesize and secret mucins, which form a viscoelastic gel and protect the intestinal epithelium against external insult (Wu et al. 2018). To assess a functional role

Table 4 Effects of glycine or alanine (the isonitrogenous control) supplementation between 7 and 21 days of age on jejunal morphology in weanling piglets

Intestinal morphology of piglets at day 7 post-weaning	Oral administration of glycine (% of milk glycine intake)			
	0	50	100	200
Villus height (μm)	248 ± 10.7^{b}	272 ± 12.4^{ab}	288 ± 8.4^{a}	308 ± 15.8^{a}
Crypt depth (μm)	142 ± 10.9^{a}	138 ± 12.0^{a}	106 ± 8.2^{b}	102 ± 6.8^{b}
Villus height/crypt depth, V/C	1.8 ± 0.10^{b}	2.0 ± 0.16^{b}	2.8 ± 0.18^{a}	3.1 ± 0.26^{a}

Values are means ± SEM, $n = 6$. Means without a common letter differ, $P < 0.05$

Glycine intake from sow's milk was 0.35 g/kg BW/day

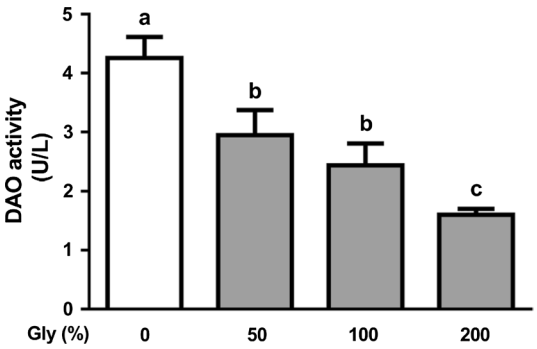

Fig. 1 Serum activity of DAO in 28-day-old piglets that were orally administered with glycine or alanine (the isonitrogenous control) between 7 and 21 days of age before weaning. Piglets were weaned at 21 days of age, and serum was obtained 1 week post-weaning. Values are mean ± SEM, $n = 6$ piglets/group. Means without a common letter differ, $P < 0.05$

of glycine on goblet cells and the production of mucins, alcian Blue and periodic acid-Schiff staining were performed. We found that preweaning supplementation of 100% glycine, instead of 50% and 200% glycine, increased ($P < 0.05$) the number of goblet cells in post-weaning piglets (Fig. 3), as compared with the control group. Apoptosis of enterocytes as showed by TUNEL-positive cells in the jejunal tissues was detected (Fig. 4). Interestingly, glycine administration markedly decreased ($P < 0.05$) the number of apoptotic cells in a dose-dependent manner in the jejunal epithelium of post-weaning piglets, indicating a inhibitory effect of glycine on cell death in the small intestine.

ER stress and apoptosis-related protein expression

As shown in Fig. 5, dietary supplementation of 200% glycine decreased ($P < 0.05$) the protein abundance of BiP in the jejunum of post-weaning piglets (Fig. 5a), and supplementation of 50–200% glycine decreased ($P < 0.05$) the abundances of p-IRE1α, CHOP, and p53 proteins (Fig. 5c–e) in the jejunum, as compared to the controls. In addition, supplementation of 50% glycine, instead of 100% and 200% glycine, decreased ($P < 0.05$) jejunal protein expression of Bax (Fig. 5f) as compared with the control group. The protein abundances of jejunal Bcl-2 and Bcl-xL were markedly augmented ($P < 0.05$) by preweaning supplementation of 100 and 200% glycine (Fig. 5g, h). However, glycine had no effect ($P > 0.05$) on the expression of jejunal ATF6α (Fig. 5b).

Discussion

In the present study, preweaning administration of glycine to sow-reared piglets improved intestinal mucosal barrier function at day 7 post-weaning, as shown by increases in jejunal villus height, goblet cells, protein abundances of TJPs, as well as decreases in serum DAO activity and jejunal epithelium apoptosis. This effect of glycine was associated with downregulation of the expression of proteins implicated in ER stress and apoptosis signaling.

The small intestine of weanling piglets is subjected to dramatic changes due to exposure to various factors, including pathogens and toxins present in the intestinal lumen (Campbell et al. 2013; Yi et al. 2018). Previous study has reported that weaning stress results in reduced growth performance, which is associated with decreased TJPs and increased intestinal permeability (Hu et al. 2013). Hence, nutritional strategies to alleviate the damaging effects of weaning stress on intestinal–mucosal barrier are of significance for the health, growth and development of neonates. Growing evidence indicates that the traditionally classified nutritionally non-essential amino acids, such as arginine, glycine, glutamine, and glutamate, promote intestinal development and regulate nutrient metabolism in intestinal tissues through various mechanisms (Li and Wu 2018; Rhoads and Wu 2009; Wu 2009).

Glycine is the simplest amino acid in nature and a major constituent in extracellular structural proteins in animals (Wu 2009). Extensive studies have shown that glycine plays an important role in modulating animal behavior, food intake, DNA synthesis, cell proliferation, immune response, and whole-body homeostasis (Zhong et al. 2003; Hall 1998; Amelio et al. 2014). We and others have reported that glycine supplementation enhances growth performance (Powell et al. 2011; Wang et al. 2014a), and regulates the expression of TJPs in the intestinal cells of pigs (Li et al. 2016). However, it is unknown whether glycine can alleviate weaning

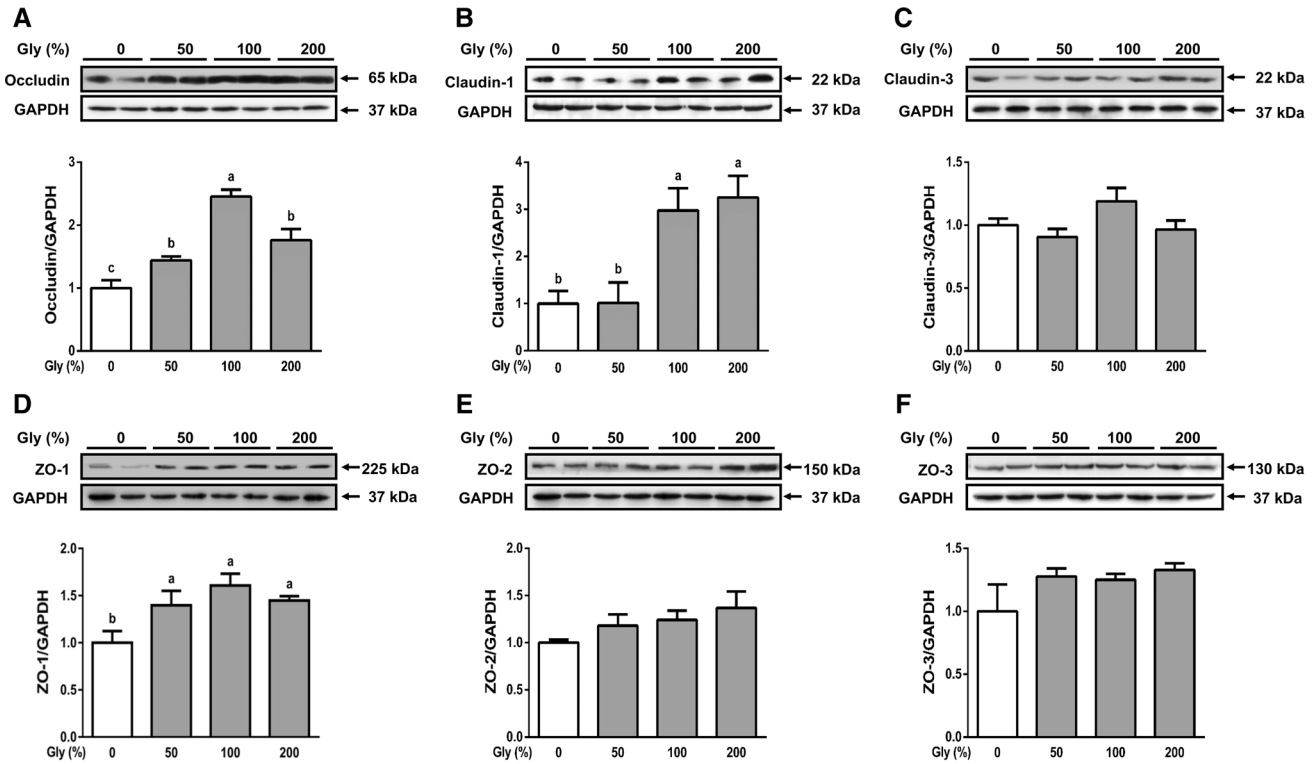

Fig. 2 Protein abundances of occludin (**a**), claudin-1 (**b**), claunin-3 (**c**), ZO-1 (**d**), ZO-2 (**e**), and ZO-3 (**f**) in the jejunum of 28-day-old piglets that were orally administered with glycine or alanine (the isonitrogenous control) between 7 and 21 days of age before weaning. Piglets were weaned at 21 days of age, and jejunum was obtained one week post-weaning. Representative results are protein abundance from two individual piglets. Values are mean ± SEM, $n = 6$ piglets/group. Means without a common letter differ, $P < 0.05$

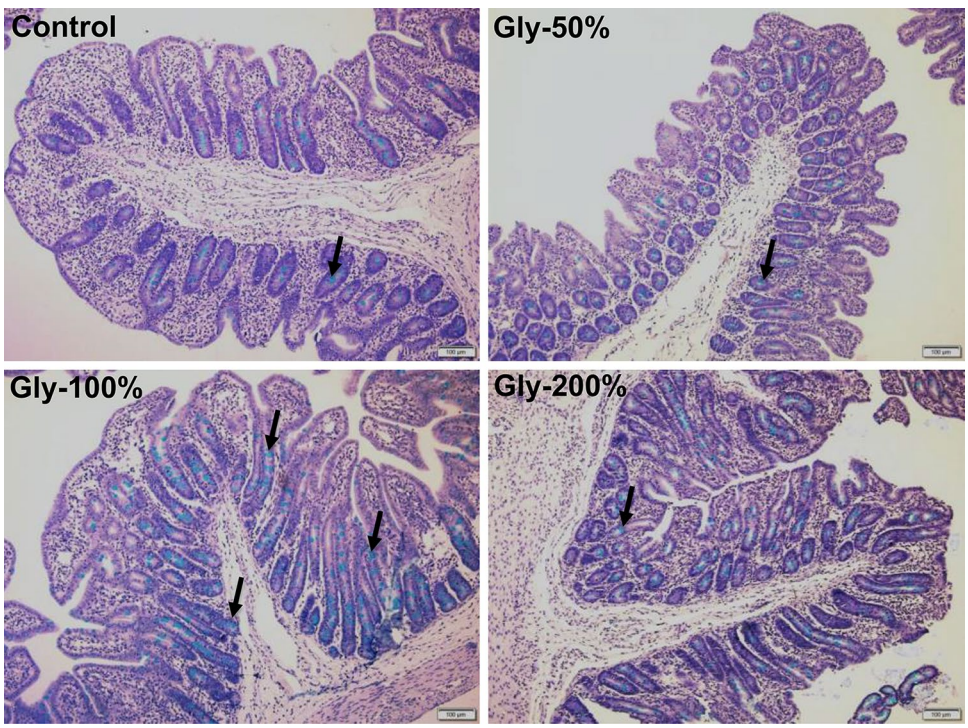

Fig. 3 Goblet cells staining with Alcian Blue and periodic acid-Schiff assay in jejunum of 28-day-old piglets that were orally administered with glycine or alanine (the isonitrogenous control) between 7 and 21 days of age before weaning. Piglets were weaned at 21 days of age, and jejunum was obtained one week post-weaning

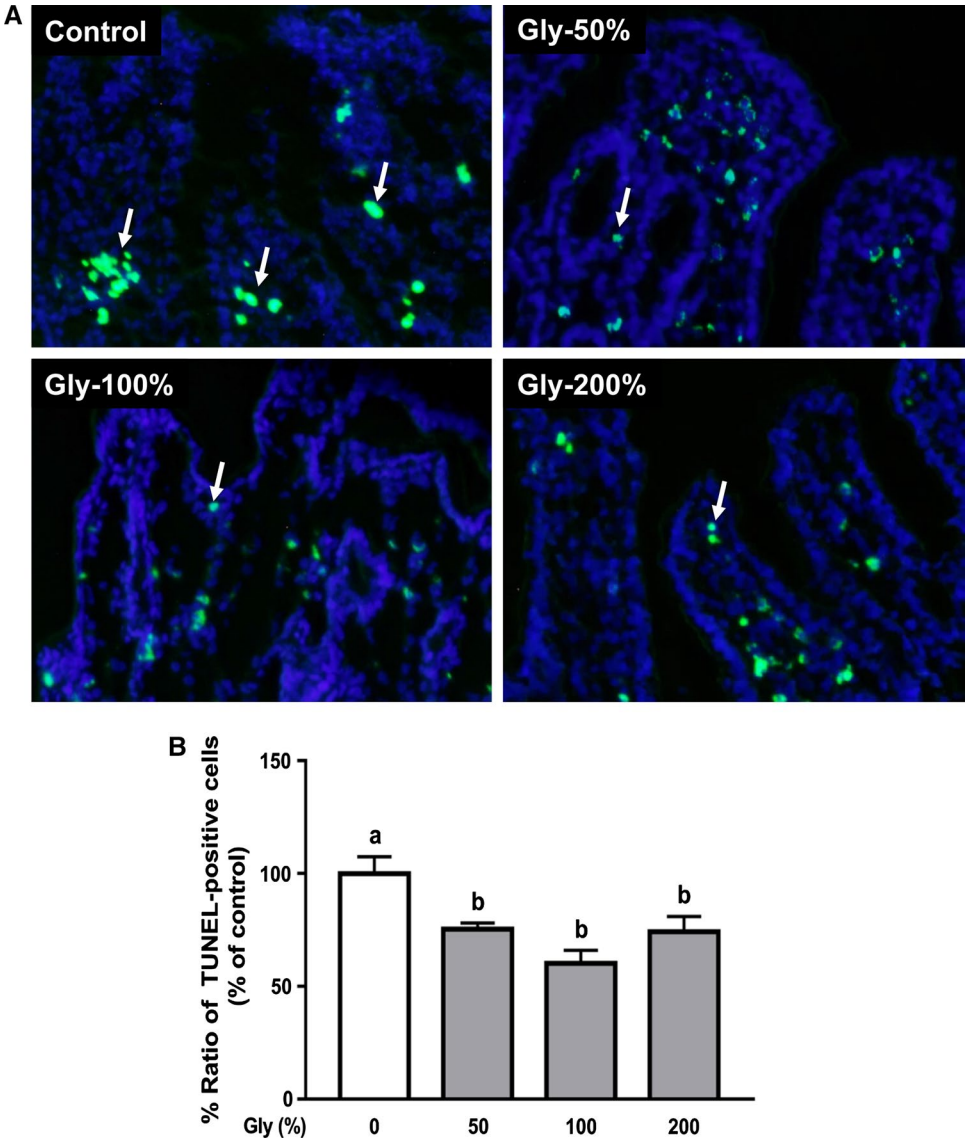

Fig. 4 Apoptotic cells in the jejunum of 28-day-old piglets that were orally administered with glycine or alanine (the isonitrogenous control) between 7 and 21 days of age before weaning. Piglets were weaned at 21 days of age, and jejunum was obtained one week post-weaning. **a** Apoptotic cells were stained with an in situ TUNEL staining reagent (green, arrow). Nuclei are stained with DAPI (blue). **b** The percentage of apoptosis was counted from three random areas of individual sections. Values are mean ± SEM, $n = 6$ piglets/group. Means without a common letter differ, $P < 0.05$

stress-induced intestinal mucosal barrier disruption, thereby improving the growth performance of post-weaning piglets. In the present study, we found that preweaning glycine supplementation during 7–21 days of age increased the growth of 21- to 35-day-old post-weaning pigs (2 weeks post-weaning).

DAO is an enzyme that is widely distributed in intestinal villus cells (Wu et al. 2018). Intestinal mucosal damage leads to an increase of serum DAO activity (Tossou et al. 2016; Yi et al. 2018). Therefore, it is regarded as an indicator of intestinal epithelial integrity and permeability (Miyoshi et al. 2015). A novel finding of the present study is that oral administration of glycine to piglets during the suckling period decreased serum DAO activity. The intestinal mucosal barrier is mainly formed by the epithelial cells and paracellular TJPs, which prevent bacteria, endotoxins, and other lumen substance from entering the blood circulation (Jacobi and Odle 2012). Breakdown of TJPs augments intestinal permeability and serum DAO activity, while decreasing the intestinal absorption of nutrients in humans and animals (Gilani et al. 2017). Interestingly, weaning stress impaired the expression of TJPs and promoted apoptosis in the small intestine, which was attenuated by preweaning supplementation of glycine to neonates. This indicates that milk-born glycine is insufficient for: (1) the preweaning development of the small intestine in piglets; and (2) the post-weaning health of the piglet intestine or the post-weaning growth performance of piglets.

Goblet cells are specialized for the synthesis and secretion of mucins, critical components of the mucus layer (Birchenough et al. 2015; Hou and Wu 2018). The gastrointestinal mucus systems separate the luminal content (especially bacteria) from direct contact with the epithelial cells. In our study, the jejunal mucins were more abundant

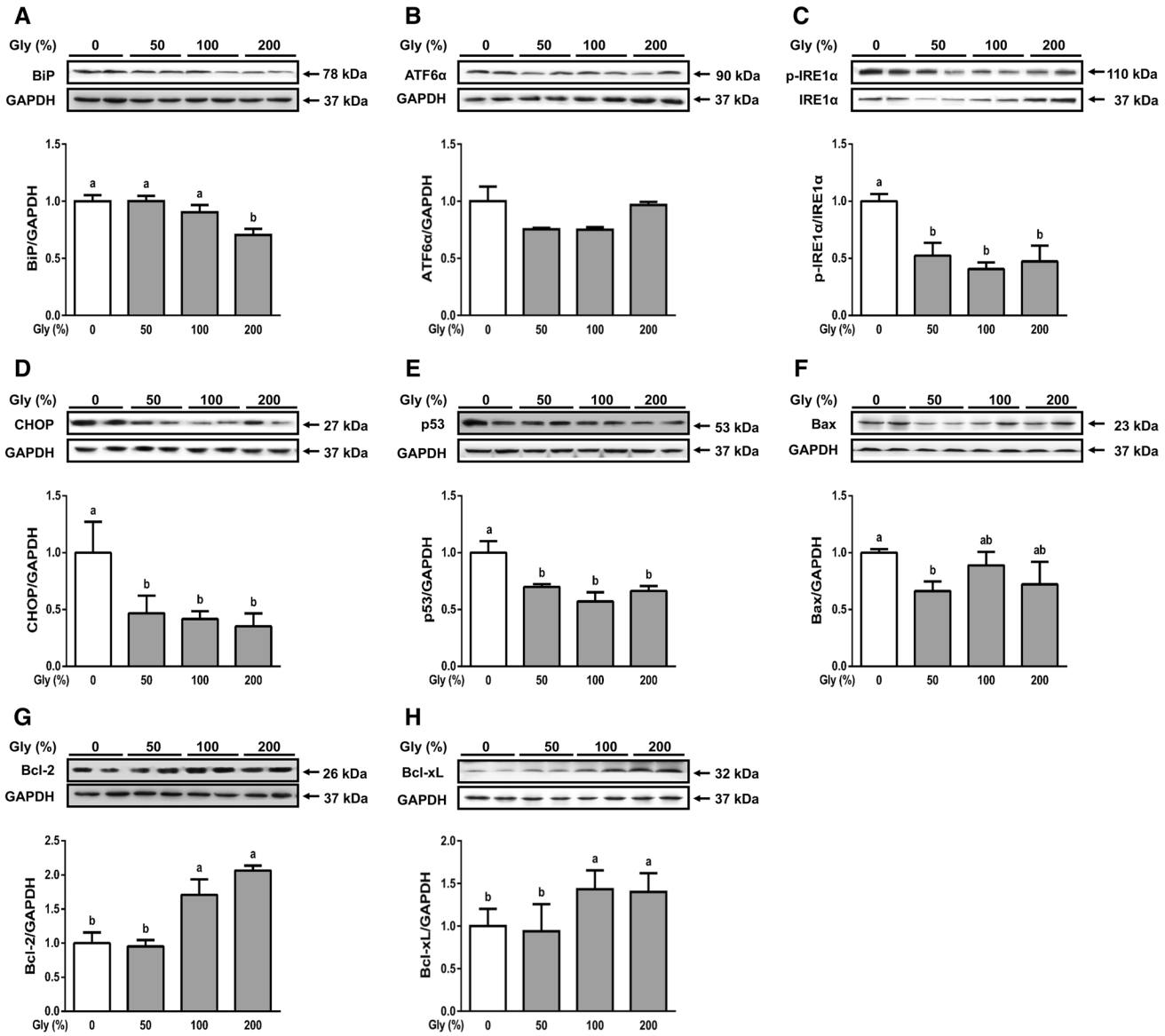

Fig. 5 Protein abundances for BiP (**a**), ATF6α (**b**), IRE1α (**c**), CHOP (**d**), p53 (**e**), Bax (**f**), Bcl-2 (**g**), and Bcl-xL (**h**) in the jejunum of 28-day-old piglets that were orally administered with glycine or alanine (the isonitrogenous control) between 7 and 21 days of age before weaning. Piglets were weaned at 21 days of age, and jejunum was obtained one week post-weaning. Representative results are protein abundance from 2 individual piglets. Values are mean ± SEM, $n = 6$ piglets/group. Means without a common letter differ, $P < 0.05$

in glycine-supplemented pigs compared to the control group, which is consistent with a previous report (Ospina-Rojas et al. 2013), indicating that glycine supplementation increases the mucus layer and protects the integrity of the intestinal epithelium. Oral administration of glycine may promote the biosynthesis of mucin, which is then secreted to protect epithelial cells from insults by various pathogens in the intestinal lumen. Moreover, it has been reported that the enterocyte apical glycocalyx (also known as the pericellular matrix) mainly consists of transmembrane mucins and the tight junctions (Sheng et al. 2013; Turner 2009). Enhanced production of mucins may improve intestinal permeability through an enhanced interaction between mucin and TJPs, ultimately contributing to intestinal integrity and growth performance.

Glycine exerts anti-inflammatory, immunomodulatory, and anti-apoptotic effects in various tissues (Fuchs et al. 2012; de Aguiar Picanco et al. 2011; Amin et al. 2003; Jacob et al. 2003). In our previous study, we found that glycine attenuated 4-hydroxynonenal (an end product of lipid oxidation)-induced apoptosis in enterocytes (Wang et al. 2014b). However, in vivo data on the anti-apoptotic effect are not available. Using the piglet as an animal model, we found that glycine supplementation prevented

cell death in the jejunum of weanling piglets (Fig. 4), validating an in vivo effect of glycine on apoptosis. Mechanistically, this effect of glycine is associated with up-regulation of anti-apoptotic proteins of Bcl-2 family, including Bcl-2 and Bcl-xL, which has been reported in various cells (Lu et al. 2012). It has also been demonstrated that Bax, a pro-apoptotic protein, can be induced by p53 and contribute to stress-induced cell death (Wawryk-Gawda et al. 2014). Results of the present study indicated that preweaning glycine supplementation reduced the protein levels of p53 and Bax in the small intestine of post-weaning pigs, indicating a functional role of p53 on the apoptosis of intestinal epithelial cells by regulating the expression of Bcl-2 family proteins. ER stress has been reported to stimulate p53 expression through NF-κB signaling, causing ER stress-related cell death (Lin et al. 2012). In a recent study, Yi et al. reported that phosphorylation of NF-κB was correlated with enhanced IL-6 expression in the jejunum of weanling piglets (Yi et al. 2016). It is possible that the elevation of p53 in the jejunum of weanling piglets might be due to ER stress and/or NF-κB activation.

Another novel and important finding from the present study is that preweaning glycine supplementation to piglets attenuated the expression of jejunal CHOP (an apoptotic protein) in response to ER stress signaling during the weaning period. Induction of CHOP has been reported to promote cell death via upregulating the expression of several apoptotic genes, including the death receptor 5 and Bcl-2 family proteins (Szegezdi et al. 2006). In our study, we observed significant downregulation of Bcl-2 in post-weanling piglets supplemented with glycine prior to weaning. Nevertheless, it remains unknown whether other pro-apoptotic proteins may also be implicated in the protective effect of glycine on the small intestine. More studies are required to address this question.

In response to ER stress, PERK, IRE1α, and ATF6α are released from BiP, thus activating downstream signaling cascades to restore homeostasis (Bertolotti et al. 2000). The enhanced protein abundances of ATF6α and phosphorylation of IRE1α in the small intestine of piglets indicated a disruption of cellular homeostasis (Petrat et al. 2011; Stoffels et al. 2011; Diestel et al. 2007). Both PERK and IRE1α activate CHOP and contribute to cell death. We found that the downregulation of CHOP by glycine supplementation was accompanied by a decrease in jejunal protein levels of IRE1α, suggesting an inhibition of IRE1α by glycine in the small intestine of weanling piglets. Considering that the CHOP can also be regulated via post-translational modification by p38 MAPK, which, in turn, interacts with the IRE1α-TRAF2 complex on ER (Szegezdi et al. 2006), further studies are needed to explore the underlying mechanisms responsible for CHOP repression in the jejunal tissues of piglets receiving preweaning administration of glycine.

It should be noted that we did not detect the protein of PERK in the piglet jejunum, due to no cross reaction between the antibody used in our study and porcine PERK protein. It was not conclusive whether PERK is involved in or regulates jejunal CHOP expression in young pigs. Milk is the main nutrient required for neonatal growth and intestinal health (Wu 2018). Considering a limited availability of glycine in the human milk and the sow's milk (Hou et al. 2016), as well its beneficial effect on the intestine, skeletal muscle cells, adipocytes, and whole body metabolism (Chen et al. 2018; Li and Wu 2018; Sun et al. 2016), dietary supplementation of glycine to formulas may improve the growth, development and health of infants, particularly in preterm or low-birth weight neonates nursed by women or other mammals (Wang et al. 2012; Dai et al. 2015; Wu 2013; Wu et al. 2014).

In conclusion, oral administration of glycine to suckling piglets improved intestinal mucosal barrier function during the post-weaning period. The protective effect of glycine was associated with reduced apoptosis and increased protein levels of tight junction proteins in the small intestine. We suggest that repression of ER stress-induced CHOP induction is critical for the anti-apoptotic effect of glycine in the small intestine of piglets. More studies are needed to understand how the intestine is reprogrammed by glycine during the suckling period for improved health, growth and development in later life.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics statement The studies were approved by China Agricultural University Institutional Animal Science and Technology College and conducted according to the Guidelines for Experimental Animal Research of the Ministry of Science and Technology (Beijing, China).

Informed consent All authors have read and approved the final manuscript.

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