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ORIGINAL ARTICLE

Escherichia coli aggravates endoplasmic reticulum stress and triggers CHOP-dependent apoptosis in weaned pigs

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Abstract Intestinal cells can sense the presence of pathogens and trigger many important signaling pathways to maintain tissue homeostasis and normal function. *Escherichia coli* and lipopolysaccharides (LPS) are the main pathogenic factors of intestinal disease in pigs. However, the roles of endoplasmic reticulum stress (ERS) and its mediated apoptosis in intestinal malfunction induced by *E. coli* or LPS remain unclear. In the present study, we aimed to evaluate whether ERS could be activated by *E. coli* fed to piglets and whether the underlying mechanisms of this disease process could be exploited. Eighteen weaned pigs (21 days old) were randomly assigned to one of two treatment groups ($n = 9$ per group). After pre-feeding for 1 week, the diets of the piglets in one group were supplemented with *E. coli* (W25 K, 10^9 cells kg^{-1} diet) for 7 days. At the end of the experiment, all piglets were slaughtered to collect jejunum and ileum samples. Western blotting and immunofluorescence experiments were used to determine the expression levels and histological locations of ERS and its downstream signaling proteins. The intestinal porcine epithelial cell line J2 (IPEC-J2) was used as in vitro model to investigate the possible mechanism. The results showed that *E. coli* supplementation in the diet increased the GRP78 expression in the jejunum and ileum, especially in the jejunal epithelium and ileac germinal center, and elevated the expression levels of CHOP (in both the jejunum and ileum) and caspase-11 (in the ileum), indicating that ERS and CHOP–caspase-11 dependent apoptosis were activated in the porcine small intestine. Moreover, as demonstrated by in vitro experiments, the CHOP inhibitor 4-phenylbutyrate alleviated the damage to IPEC-J2 cells induced by LPS derived from *E. coli*. Taken together, these data strongly suggest that ERS can be triggered in the small intestine by dietary supplementation with *E. coli* and that CHOP–caspase-11 dependent apoptosis may play a key role in maintaining normal homeostasis of the intestine in response to pathogenic factors.

Keywords Endoplasmic reticulum stress · Lipopolysaccharide · GRP78 · Intestine · Pigs

Abbreviations

LPS	Lipopolysaccharide
ERS	Endoplasmic reticulum stress
CHOP	C/EBP homologous protein
GRP78	Glucose-regulated protein78
E. coli	*Escherichia coli*
IPEC-J2	Intestinal porcine epithelial cell line J2
4-PBA	4-phenylbutyrate

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Introduction

The intestines play key roles in the processes of digestion (Kondo et al. 2017), absorption (Ajakaiye et al. 2004), and immunity (Ren et al. 2014b). Maintaining intestinal tissue homeostasis and normal function is closely linked to animal health (Bloemendaal et al. 2016), growth (Yoda et al. 2014), and development (D'Alessio et al. 2014). However, weaning piglets are challenged by many pathogenic factors (Pluske 2013), including the malignant proliferation and colonization of the gastrointestinal tract by *Escherichia coli* (Zhang et al. 2015). These factors lead to gastrointestinal disorders (Mroz 2001), increased disease susceptibility (Prapasarakul et al. 2010), and pathological diarrhea (Yang et al. 2014a), as a result of the undeveloped intestinal and immune systems of the weaning piglets.

The endoplasmic reticulum (ER) is the primary intracellular organelle responsible for protein and lipid biosynthesis (Fryer et al. 2014), protein folding and trafficking (Sriburi et al. 2004), calcium homeostasis (He et al. 1997), and several other vital processes in cell physiology. There is growing interest in endoplasmic reticulum stress (ERS) and its signaling pathways, which mediate cell survival (Senft and Ronai 2015) or apoptosis (Fernandez et al. 2015). Disturbance in ER function results in ERS, and many downstream signaling pathways of ERS are triggered (Sovolyova et al. 2014). For example, moderate ERS can activate IRE-1α and XBP-1 signaling and protect cells from injury (Ling et al. 2012), whereas prolonged or excessive ERS may lead to cell death via the C/EBP homologous protein (CHOP), c-Jun N-terminal kinase (JNK), and other signaling pathways (McGuckin et al. 2010). Specifically, CHOP can mediate the mRNA expression of caspase-11 and activation of its effector, molecular caspase-1 (Fradejas et al. 2009). The cleavage of caspase-1 controls the maturation of proinflammatory cytokine IL-1 family members and induces cell apoptosis (Yang et al. 2014b). A previous report showed that lipopolysaccharide (LPS) failed to stimulate caspase-11 expression and activity in the lung tissue and macrophages of CHOP knockout mice (Endo et al. 2006). The connection between XBP-1 signaling and inflammatory bowel disease in mice has also been revealed (Kaser et al. 2008). However, the roles of ERS and its downstream signaling CHOP-dependent apoptosis in intestinal malfunction induced by *E. coli* still remain unclear. Therefore, we investigated the alteration of ERS-related protein expression both in vivo in the intestines of weaning piglets challenged with *E. coli* and in vitro in intestinal epithelium cells challenged with *E. coli* derived LPS. The roles of ERS and CHOP-mediated apoptosis in intestines infected with *E. coli* were verified by treating intestinal cells with an ERS inducer (tunicamycin, positive control) and a CHOP inhibitor (4-phenylbutyrate, 4-PBA). Here we report, for the first time, that ERS is a normal event in the developing intestine and that *E. coli* infection aggravates this stress and cell apoptosis via the CHOP–caspase-11 signaling pathway.

Materials and methods

Experimental diets and procedure

This study was conducted with the approval of the Animal Welfare Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences. Eighteen crossbred pigs (Duroc × Landrace × Yorkshire) were randomly assigned to two groups, of which one group was fed a diet supplemented with *E. coli* (*E. coli* group, $n = 9$) and the other received a normal diet (control group, $n = 9$). The diets were formulated according to the National Research Council (NRC 2012) to meet the nutrient requirements for growing and finishing pigs. The *E. coli* strain W25 K, the draft genome sequence of which was reported previously (Ren et al. 2014a), was mixed with the experimental diet at a concentration of 10^9 cells kg^{-1}. A 7-day acclimatization period was allowed prior to the commencement of each experiment. After pre-feeding for 1 week, the experimental piglets were administered diets with or without *E. coli* (10^9 cells kg^{-1} diet) supplementation for 7 days. The pigs had free access to feed and drinking water throughout the experimental period. The experiment lasted 14 days. The composition of the basic diet used here was described in our previous study (He et al. 2013).

Sample preparation

At the end of the experiment, the pigs were anesthetized with an intravenous injection of sodium pentobarbital (50 mg/kg body weight) and then euthanized. The entire intestines and viscera for each pig were rapidly removed. After washing the enteric cavity with phosphate buffer solution (PBS), the jejunum and ileum samples were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent analysis of protein expression by western blotting. The tissue samples for immunofluorescence were fixed with 4% paraformaldehyde for 24 h, and then embedded in paraffin for the subsequent analysis. Each sample was sliced into 5 μm sections and all of the experiments were performed in triplicate.

Cell culture

Intestinal porcine epithelial cell line J2 (IPEC-J2) cells were cultured by serial passage in uncoated plastic culture flasks (25 cm^2) in DMEM-H medium containing 10% fetal bovine serum (FBS), 5 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. At confluence, the cells

were trypsinized and seeded in six-well culture plates with approximately 8×10^3 cells per well and then maintained at 37 °C in a 5% CO_2 humidified incubator. After overnight incubation, the cells were cultured in basal medium (blank control), basal medium + tunicamycin (0.5 μg mL^{-1}), basal medium + LPS (2.0 μg mL^{-1}), or basal medium + LPS (2.0 μg mL^{-1}) + 4-PBA (1.0 μg mL^{-1}) for 24 h. All of the treatments were performed in triplicate. Further analysis was performed after the treatments described above.

Cell viability assay

The cell viability was assessed using a cell counting kit-8 (CCK-8, Dojindo, Osaka, Japan). After the treatments described previously, the culture medium was replaced with 100 μL of fresh medium containing 10 μL of reagent from the kit. After incubation for 45 min at 37 °C, the absorbance at 450 nm was measured for each well using an ELISA plate reader (Bio-Tek, Winooski, VT, USA). The results were expressed as optical density (OD450) values.

Immunofluorescence

After antigen retrieval using a citric acid working solution, the samples were incubated overnight at 4 °C with rabbit anti-GRP78 and mouse anti-CHOP polyclonal primary antibodies. After three washing cycles, the samples were incubated for 1 h with Alexa Fluor 488-conjugated goat anti-rabbit and Alexa Fluor 647-conjugated goat anti-mouse secondary antibodies (Life Technologies, NY, USA). After washing three times, the nucleus was stained with the 4′,6-diamidino-2-phenylindole (DAPI) working solution for 5 min according to the instructions. After a further three washing cycles, anti-fluorescence quenching solution was used as the mounting medium. The images were captured using a laser confocal microscope. The antibodies against GRP78 and CHOP/GADD153 used in this experiment were obtained from Abcam (Cambridge, USA).

Western blotting

After lysing the samples for 10 min in ice-cold buffer with a complete protease inhibitor cocktail, immunoblotting assays were performed as described previously (Jiang et al. 2016). The protein concentration in each sample was determined by the bicinchoninic acid (BCA) method, as described in the previous study. The blots were examined using the ECL Plus detection system (Thermo, Waltham, USA) under conditions recommended by the manufacturer, before visualizing the signals on Fujifilm (LAS-3000, Fuji, Tokyo, Japan). The protein band densities were normalized to the β-actin signal and quantified using the Quantity One software (Bio-Rad, California, USA). The antibodies against p-PERK, PERK, ATF6, IRE-1α, GRP78, XBP-1, CHOP/GADD153, caspase-11, and β-actin were obtained from Abcam (Cambridge, USA).

Statistical analysis

The data shown represent the mean ± SD of a minimum of three independent experiments. Two-tailed paired and unpaired Student's *t* tests were performed by comparing the data to the corresponding reference point or as indicated, and *p* values of less than 0.05 were considered statistically significant; in the figures, $P < 0.05$ is denoted by "*" and $P < 0.01$ is denoted by "**".

Results

Expression of ERS marker proteins in the intestine

The protein levels of GRP78, CHOP, caspase-11, and β-actin were determined by western blotting. Representative bands of the matched proteins in the jejunum and ileum are shown in Fig. 1a, c, respectively. Figure 1b, d shows the relative abundance of the proteins, using β-actin as the internal control. Compared to the control group, the piglets fed a diet supplemented with *E. coli* exhibited higher ($p < 0.05$) expression levels of GRP78 (3.1-fold in the jejunum and 3.3-fold in the ileum) and CHOP (2.1-fold in the jejunum and 4.2-fold in the ileum) in both of the intestinal regions tested. The level of caspase-11 protein was significantly ($p < 0.05$) increased by 2.2-fold in the ileum by the dietary supplementation with *E. coli*, although it was not significantly ($p = 0.75$) altered in the jejunum.

To verify the western blotting results, immunofluorescence experiments were performed on the jejunum and ileum tissues. Representative photographs captured with the laser scanning confocal microscope are shown in Fig. 2a, c for the jejunum and ileum, respectively. In this experiment, the CHOP protein, GRP78 protein, and nucleus (DNA) are indicated by red fluorescence, green fluorescence, and blue fluorescence, respectively. The fluorescence intensity of each color was considered to represent the color-matched protein level. Figure 2b, d shows the relative fluorescence intensities of each protein, using the intensity in the control group as the reference. Consistent with the western blotting results, the dietary supplementation with *E. coli* significantly elevated the protein levels of CHOP and GRP78 in both the jejunum and ileum.

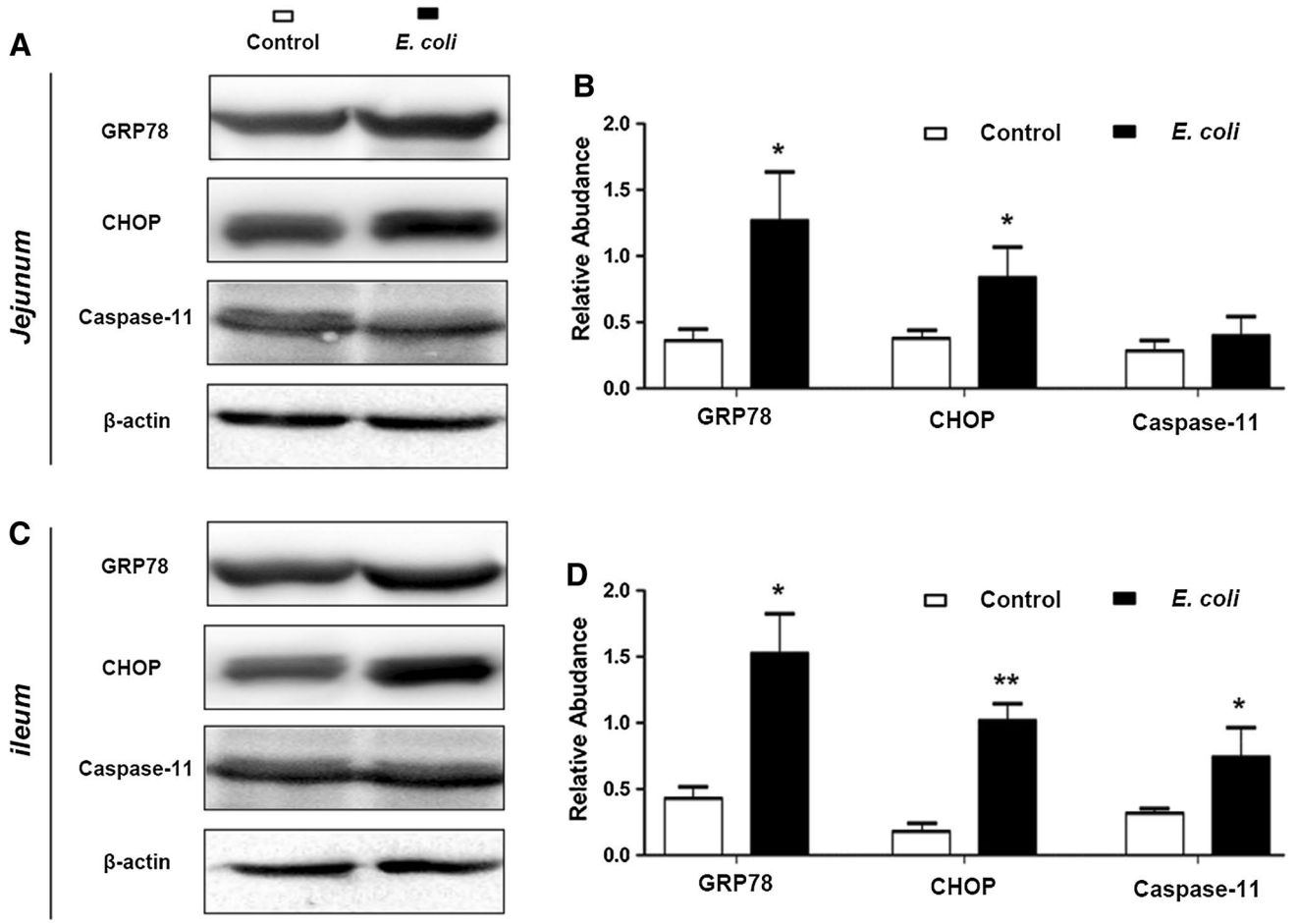

Fig. 1 Western blot analysis of ERS marker proteins in the jejunum and ileum with or without *E. coli* infection. **a** Representative bands of GRP78, CHOP, and caspase-11 proteins in the jejunum. **b** Relative abundance of GRP78, CHOP, and caspase-11 in the jejunum. **c** Representative bands of GRP78, CHOP, and caspase-11 in the ileum. **d** Relative abundance of GRP78, CHOP, and caspase-11 in the ileum. β-Actin was used as the internal control. Data are the mean ± SD of a minimum of three independent experiments. "*" and "**" indicate p values of $p < 0.05$ and $p < 0.01$, respectively, in the t test

Histological location of ERS marker proteins in the intestine

The immunofluorescence results were also used to determine the specific histological location of ERS in the intestines. As shown in Fig. 2a, the GRP78 protein was uniformly distributed (expressed) in the jejunum within the normal milieu, but distributed (expressed) at higher concentrations in the epithelial tissue in response to the *E. coli* infection. The CHOP protein was not observed in the jejunum within the normal milieu, whereas the *E. coli* infection triggered its expression mainly in the epithelial tissue.

As shown in Fig. 2c, similar to the results for the jejunum, the GRP78 protein was also uniformly distributed (expressed) in the ileum within the normal milieu. However, it was distributed (expressed) at higher concentrations in the germinal center of the ileum in response to the *E. coli* infection. The CHOP protein was not observed in the ileum within the normal milieu, whereas the *E. coli* infection also triggered its expression mainly in the germinal center of the ileum. A specific amplification of this germinal center is shown in Fig. 2e.

Expression of ERS downstream signaling proteins in the intestine

The previously discussed results have revealed that ERS was triggered by the *E. coli* infection, and we next determined the expression of its downstream signaling proteins to investigate the regulatory mechanisms. The proteins p-PERK, PERK, ATF6, IRE-1α, XBP-1, and β-actin were determined by western blotting. Representative bands of the matched proteins in the jejunum and ileum are shown in Fig. 3a. Figure 3b, d–f shows the relative abundances of the proteins using β-actin as the internal control. Compared to the control, the piglets fed diets supplemented with *E. coli*

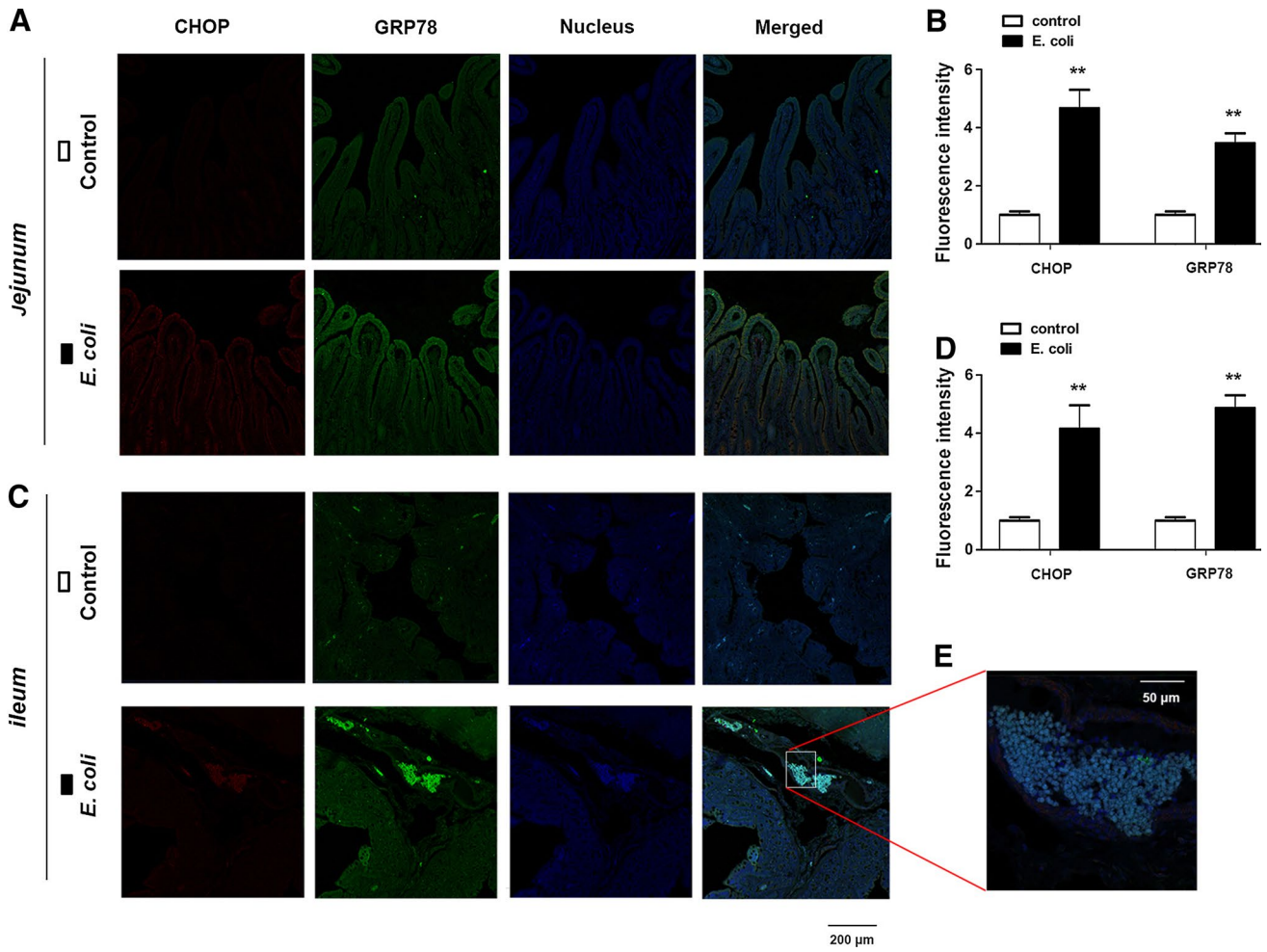

Fig. 2 Immunofluorescence analysis of GRP78 and CHOP proteins in the jejunum and ileum tissues with or without *E. coli* infection. **a** Representative immunofluorescence photographs of the jejunum with or without *E. coli* infection. **b** Relative fluorescence intensities of CHOP and GRP78 in the jejunum with or without *E. coli* infection. **c** Immunofluorescence photographs of the ileum with or without *E. coli* infection. **d** Relative fluorescence intensities of CHOP and GRP78 in the ileum with or without *E. coli* infection. The CHOP protein, GRP78 protein, and nucleus (DNA) are indicated by red fluorescence, green fluorescence, and blue fluorescence, respectively. The intensity of the control group was considered to be 1. **e** Specific amplification of the "germinal center". Data are the mean ± SD of a minimum of three independent experiments. "*" and "**" indicate p values of $p < 0.05$ and $p < 0.01$, respectively, in the t test

exhibited higher ($p < 0.05$) expression levels of PERK in both the jejunum (1.4-fold) and ileum (1.5-fold). The level of IRE-1α protein was significantly ($p < 0.05$) decreased in the ileum but not affected in the jejunum by the dietary supplementation with *E. coli*. Apart from these two proteins, no significant differences in the protein levels of p-PERK, ATF6, or XBP-1 were observed in the intestines infected with *E. coli* compared to the healthy intestines.

Cell death induced by LPS from *E. coli* was attenuated by a CHOP inhibitor

All of the data from the animal experiments indicated that CHOP-mediated apoptosis by activating caspase-11 may play a vital role in the intestines during the infection process with *E. coli*. To verify this specific role of CHOP in the response of the intestines to *E. coli*, an in vitro experiment using IPEC-J2 was performed. In this experiment, 4-PBA was used as a CHOP inhibitor, tunicamycin was used as an ERS inducer (ERS positive control), and the effect of the presence of *E. coli* in animal feed was replaced by LPS derived from *E. coli*. Figure 4a shows representative photographs of different samples captured by optical microscopy. The cell vitality analysis determined using a CCK-8 kit is shown in Fig. 4b. Compared with the tunicamycin-induced ERS milieu group, the addition of 4-PBA improved the cell vitality. A similar effect of 4-PBA in attenuating cell death induced by LPS derived from *E. coli* was also observed. Figure 4c shows the representative bands of matched proteins in the treated cells. Figure 4d–f show the relative abundances

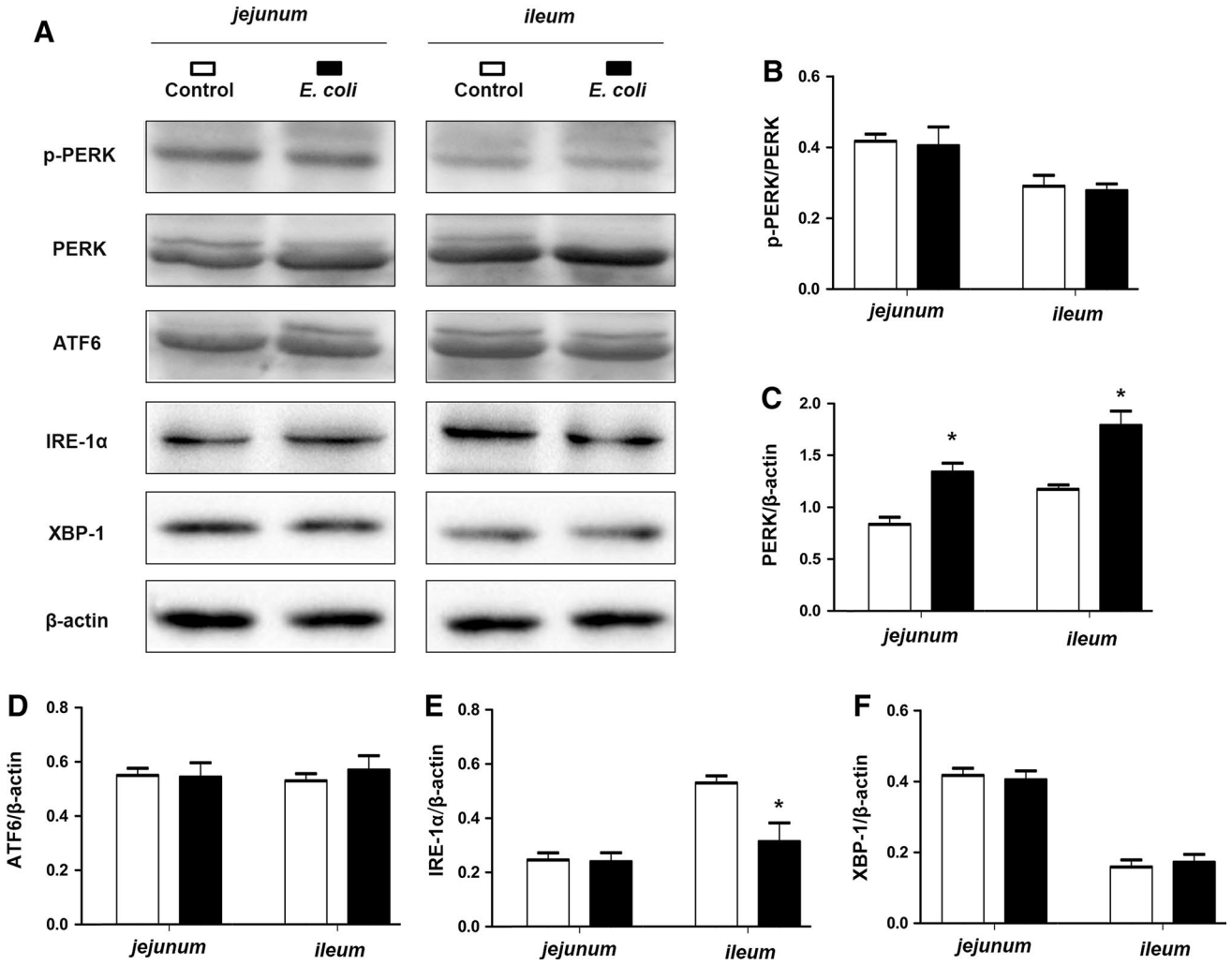

Fig. 3 Western blot analysis of ERS downstream signaling proteins in the jejunum and ileum with or without *E. coli* infection. **a** Representative bands of p-PERK, PERK, ATF-6, IRE-1α, XBP-1, and β-actin proteins in the jejunum and ileum with or without *E. coli* infection. **b** Relative abundance of phosphorylation level of PERK in the jejunum and ileum with or without *E. coli* infection. **c**–**f** Relative abundances of PERK, ATF6, IRE-1α, and XBP-1 proteins in the jejunum and ileum with or without *E. coli* infection. β-Actin was used as the internal control. Data are the mean ± SD of a minimum of three independent experiments. "*" and "**" indicate p values of $p < 0.05$ and $p < 0.01$, respectively, in the t test

of the proteins, using β-actin as an internal control. The addition of 4-PBA to the culture medium decreased the expression of CHOP and caspase-11 in IPEC-J2 within both the tunicamycin-induced ERS milieu and the *E. coli* LPS-induced infectious milieu, as shown in Fig. 4e, f.

Discussion

E. coli infection is the main cause of intestinal malfunction and diarrhea in weaning piglets (Deitmer and Parra 2017). Previous studies have shown that inflammation (Bucker et al. 2014) and cell apoptosis (Pietzak and Chakraborty 2004) are induced by this infection, and many pathways (Wu et al. 2016; Kim et al. 2003) are involved during the anti-disease process. ERS is thought to underlie many pathogenic progressions of the intestine (Kaser et al. 2008; Ringseis et al. 2016). However, the precise regulatory mechanisms of ERS signaling in response to intestinal infection are poorly understood. Here, for the first time, we have shown that *E. coli* infection can induce overloaded ERS and trigger CHOP–caspase-11 mediated apoptosis in the jejunum and ileum of weaning piglets. Moreover, the overloaded ERS was mainly triggered in the epithelial tissue of the jejunum and the germinal center of the ileum. Furthermore, the results suggest that overactivated ERS followed by activation of the CHOP–caspase-11 signaling pathway may play key roles in maintaining intestinal homeostasis.

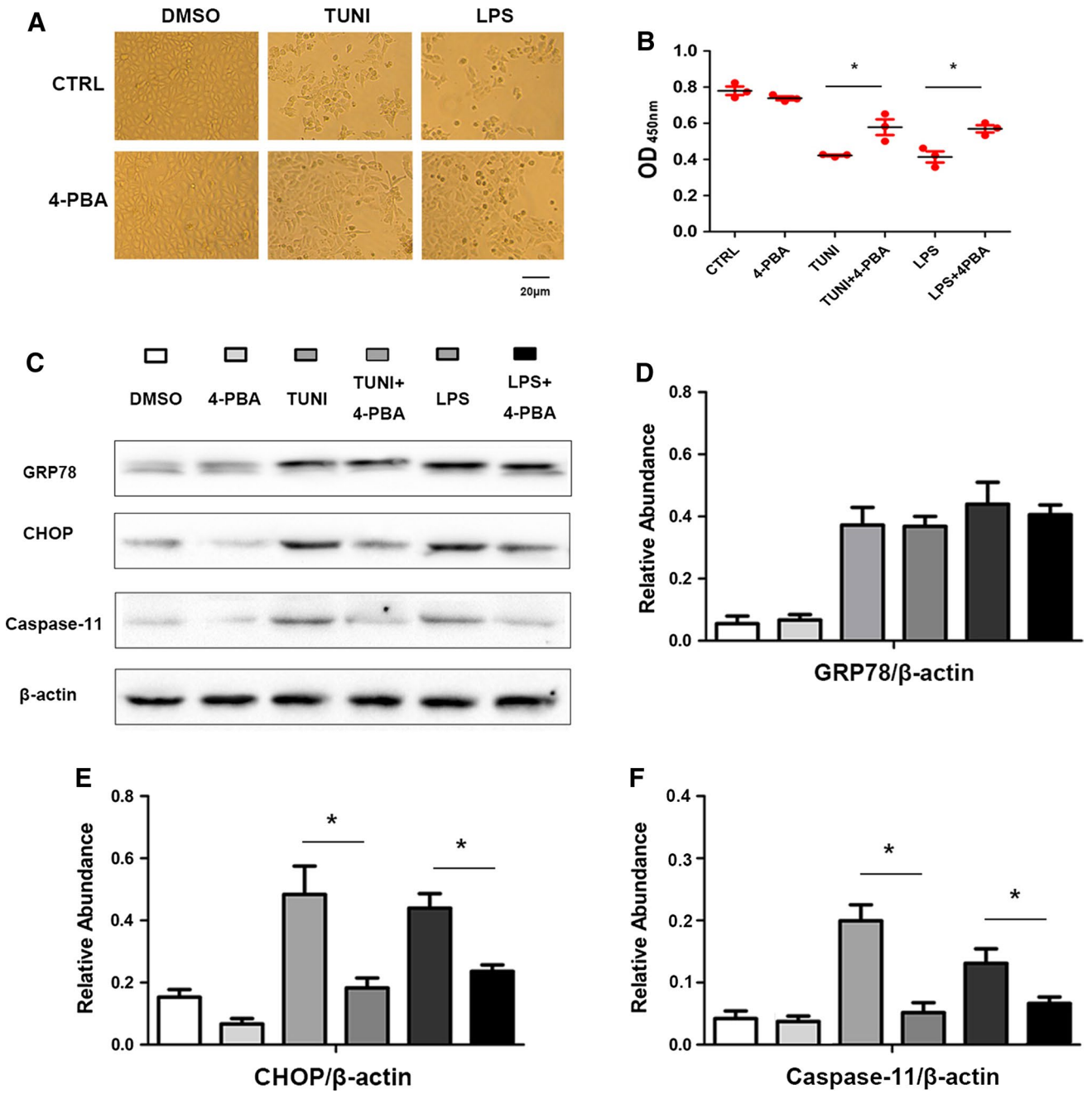

Fig. 4 4-PBA attenuated LPS-induced cell death by inhibiting CHOP and caspase-11. IPEC-J2 cells were treated with basal medium (blank control), basal medium + tunicamycin (0.5 μg mL^{-1}), basal medium + LPS (2.0 μg mL^{-1}), or basal medium + LPS (2.0 μg mL^{-1}) + 4-PBA (1.0 μg mL^{-1}) for 24 h. **a** Representative photographs of cells in the different groups captured using an optical microscope. **b** Cell vitality analysis determined using a CCK-8 kit. **c** Representative bands of the GRP78, CHOP, and caspase-11 proteins in cells from the different groups. **d**–**f** Relative abundances of GRP78, CHOP, and caspase-11 proteins in cells from the different groups. β-Actin was used as the internal control. Data are the mean ± SD of a minimum of three independent experiments. "*" and "**" indicate p values of $p < 0.05$ and $p < 0.01$, respectively, in the t test

The GRP78 protein is a central regulator of the unfolded protein response (UPR) and is considered a representative ERS marker (Ron and Hubbard 2008). When this stress occurs, GRP78 is released from UPR branches of the PERK, IRE-1α, and ATF6 and binds misfolded proteins, thereby activating the UPR (Kaira et al. 2016). In addition, CHOP is a major stress-inducible pro-apoptotic gene that participates in ERS-induced apoptosis (Wali et al. 2009; Guo et al. 2015). The activation of CHOP can be regulated by all three branches. Based on these considerations, we used GRP78 as

an ERS marker and CHOP as an ERS-mediated cell-death marker, and analyzed their expression levels and distributions in the jejunum and ileum in the presence or absence of *E. coli* infection. An increase of GRP78 in the jejunum and ileum was found to be induced by *E. coli* infection. Increased GRP78 expression has also been reported in human inflammatory bowel disease patients (Kaser et al. 2008), where the specific impairment of the ERS response was suggested to induce inflammation in the gut. In contrast, our data pointed to overloaded ERS and ERS–CHOP signaling mediated cell death being the result of infection, rather than a reason for *E. coli* induced inflammation. The recent studies on ERS have mainly focused on the mammary gland (Zhong et al. 2017) and colon tissue (Ma 2008), aiming to explore the regulatory mechanisms of ERS signaling in controlling the apoptosis of cancer cells (Verfaillie et al. 2013; Zou et al. 2015). One recent report concerning ERS in pigs revealed that ERS can be triggered in the porcine duodenal mucosa by supplementation of the diet with frying fat (Ringseis et al. 2016). Moreover, the present study has supplied extra data on the ERS signaling activation in the jejunum and ileum. Of particular interest, we found that the intestinal locations of ERS signaling activation in response to *E. coli* infection are different for the jejunum and ileum, that is, high levels of GRP78 and CHOP were mainly expressed in the epithelial tissue of the jejunum and the germinal center of the ileum. Here, we surmised that the different locations of ERS activation are attributable to the different functions and *E. coli* colonization of the jejunum and ileum. In support of this hypothesis, recent studies have shown that the ileum has a larger accommodation for the microbiota and a higher relative abundance of *E. coli* than the jejunum of the weaning piglets (Li et al. 2017; Liu et al. 2016). As reported previously (Gitlin et al. 2014), the germinal centers of the ileum mainly consist of T cells and B cells and are responsible for the proliferation of lymphatic tissue and maturation of B cells. In this regard, we surmised that the ERS and CHOP-mediated pathway may participate in the initial activation of the immune response in the ileum, and our further work will address this interesting finding.

When cells respond to damage, the inactive pre-caspase-1 will be recruited into the cytoplasm (Jin et al. 2017), followed by formation of the activated caspase-1. Caspase-11 is an upstream regulator of caspase-1 and mediates its activity by direct cleavage of the cysteine protease-1 (Broz et al. 2012). Recent studies (Man et al. 2017) have shown that caspase-11 can activate caspase-1 under the coordination of NLRP3 inflammatory cells and induce an independent pathogenic role of caspase-1. As a key transcription factor in ERS, CHOP also regulates caspase-11 (Fradejas et al. 2009). A previous report showed that LPS failed to stimulate caspase-11 expression and activity in the lung tissue and macrophages of CHOP knockout mice (Endo et al. 2006). In addition, cell apoptosis in lung epithelial cells was reduced in the CHOP-deficient mice, and the inflammatory response of the lung tissue was alleviated (Endo et al. 2006). Along these lines, the same tendency of CHOP and caspase-11 alteration was observed in the ileum after *E. coli* infection. Of particular interest, this caspase-11 in the jejunum tissue and IPEC-J2 line was differently altered by *E. coli* and LPS; we propose that this phenomenon may be attributable to the different cell types and compositions between intestinal epithelial cell lines and intestinal tissues. To verify the exact mechanism of CHOP and caspase 11 in regulating the intestinal response to *E. coli*, we treated IPEC-J2 cells with 4-PBA (a CHOP inhibitor). To verify the results of the in vivo experiment, in the in vitro experiment, the IPEC-J2 cells were treated with LPS derived from *E. coli*. Consistent with the in vivo data, the altered tendency of CHOP expression was similar with the levels of caspase-11. The GRP78 level in IPEC-J2 cells was not altered by this inhibitor, which is in accordance with a previous report in human glioblastoma stem cells (Liu et al. 2015). Moreover, the inhibition of CHOP by 4-PBA significantly suppressed the protein expression of caspase-11 as well as mediated cell death in IPEC-J2 cells subjected to treatment with LPS from *E. coli*. These data strongly suggest that the CHOP–caspase-11 signaling pathway may play a key role in determining the final fate of intestinal epithelial cells infected with *E. coli*.

In summary, our results suggest that *E. coli* infection triggers ERS and CHOP–caspase-11 mediated apoptosis in both the jejunum and ileum, indicating that the activation of the CHOP–caspase-11 axis by *E. coli* or LPS is crucial in the infection process. These novel findings have important implications for the development of new interventions to ameliorate diseases induced by *E. coli*. Further research is warranted to investigate whether CHOP/caspase-11 inhibition or conditional knockdowns can help the cells in the small intestine of weaning pigs survive *E. coli* infection without any malfunction.

Author contributions statement Q.J. G.L. and K.Y. conceived the experiment(s), Q.J. S.C. W.R. and G.L. conducted the experiments, Q.J. G.W. and Y.Y. analyzed the results. Q.J. G.L. and K.Y. prepared the manuscript. All authors reviewed the manuscript.

Compliance with ethical standards

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

Ethical standards The protocol for this study was approved by the Committee on the Ethics of Animal Experiments of Institute of Subtropical Agriculture, Chinese Academy of Sciences (Permit Number: 201509-03) and it was conducted out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Institute of Subtropical Agriculture, Chinese Academy of Sciences.

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