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ORIGINAL ARTICLE

Endoplasmic Reticulum Stress May Not Be Involved in Intestinal Epithelial Cell Apoptosis in Experimental Acute Pancreatitis

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Abstract
Objectives To investigate whether endoplasmic reticulum (ER) stress is activated in the intestinal epithelium of acute pancreatitis (AP), and whether it is one of the inducing factors of the intestinal epithelial cell apoptosis in AP.
Methods Twenty-four rats were randomly divided into two groups. AP was induced via retrograde injection of 3 % sodium taurocholate into the pancreatic duct. As a control group, rats received a sham operation. Forty-eight hours after the operation, the ultrastructural changes of ileal epithelial cells were investigated by transmission electron microscope. The protein expressions of GRP78, CHOP, caspase-12, and JNK in the ileal epithelium were determined by immunohistochemistry, and apoptosis was determined by TdT-mediated dUTP nick end labeling. The mRNA expressions of GRP78, CHOP, caspase-12, and JNK in the ileal epithelium were determined using quantitative RT-PCR.
Results The ileal epithelium in rats with AP had significantly higher apoptotic cells compared with that of the control rats ($P < 0.05$). ER stress was activated in the ileal epithelium, which was characterized by dilated, irregular ER and upregulated expressions of GRP78 mRNA and protein. The mRNA and protein expressions of CHOP, caspase-12, and JNK in rats with AP were similar to that in the control rats ($P > 0.05$).
Conclusions ER stress is induced in intestinal epithelium during AP; however, ER stress is not likely to be involved in the apoptosis of the intestinal epithelium during AP.

Keywords Acute pancreatitis · Intestinal epithelium · Apoptosis · Endoplasmic reticulum stress

Introduction

Intestinal barrier functional disturbance plays a pivotal role in the development of infectious complications, inducing and aggravating systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) in severe acute pancreatitis (SAP) [1, 2]. The exact mechanisms underlying intestinal barrier dysfunction during AP are not fully clear. Intestinal barrier dysfunction is related to excessive release of inflammatory mediators, microcirculatory disorders, ischemia–reperfusion (IR) injury, gut hypo-motility, long-term fasting, and apoptosis [3]. The stability of intestinal mucosa barrier depends on the balance between proliferation and apoptosis of intestinal epithelial cells. Intestinal epithelial cell apoptosis is increased in SAP, and inhibition of apoptosis improves the disorder of intestinal mucosa and reduces the endotoxemia in SAP [4, 5].

The mitochondrial pathway of apoptosis obtained a particular attention that serves as a major mechanism in the process of apoptosis. The critical contributions of the organelle endoplasmic reticulum (ER) to apoptosis are now becoming more apparent. The ER is possibly a major point

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of integration of pro-apoptotic signaling or damage sensing [6]; it regulates apoptosis both by sensitizing mitochondria to a variety of extrinsic and intrinsic death stimuli and by initiating cell death signals of its own [7, 8]. Furthermore, ER stress-induced apoptosis is possibly an important factor contributing to a variety of diseases [9]. ER stress signaling is also activated during AP and appears to be involved in pancreatic pathophysiology [10–12]. However, it is of interest to determine whether ER stress is activated in the intestinal epithelium, and whether it is one of the inducing factors of the intestinal epithelial cell apoptosis in AP.

The ER is highly sensitive to a variety of different stimuli and to alterations in its homeostasis. A disruption of its homeostasis leads to the accumulation of unfolded proteins in the ER lumen; these proteins bind to the ER chaperone BiP/Grp78, which serves as a marker for ER stress. However, if ER stress continues and a restoring response fails, proapoptotic signals are triggered. The molecular mechanisms of ER stress-induced cell death are not completely understood, but at least three apoptosis pathways are known to be involved in this apoptotic event. One of the components of the ER stress-mediated apoptosis pathway is the transcription factor CCAAT/enhancer-binding protein homologous protein (CHOP) [13, 14], also known as growth arrest and DNA damage-inducible gene 153 (GADD153); it decreases the expression of Bcl-2 and plays a role in apoptosis during ER stress [15]. Another major factor in local apoptosis regulation for ER stress is caspase-12, which is a relatively new member of the caspase family and the only member localized to the cytoplasmic side of the ER. Caspase-12 is believed to play a central role in the initiation of ER stress-induced cell death and is dispensable for the induction of the mitochondrial pathway of death [16, 17]. Misfolded protein in the ER could also activate c-Jun *N*-terminal kinase (JNK). The JNK signaling pathway is involved in regulation of many cellular events, including apoptosis. JNK may have proapoptotic, antiapoptotic, or no role in cell death depending on the context of the cell, the nature of the death stimulus, and the duration of its activation [18]. Recently, it was noted that activation of the JNK pathway for many cell types was responsible for cell apoptosis under ER stress conditions [19].

The relationship between ER stress and apoptosis of intestinal epithelial cells during AP has not been reported previously. In this study, using a rat model of AP, we examined the ultrastructural change of the ER and the expression of GRP78 mRNA and protein in intestinal epithelial cells to determine the activation of ER stress. Furthermore, three major mediators of ER stress-induced apoptotic pathways, CHOP, caspase-12, and JNK, were investigated to explore whether ER stress is implicated in the intestinal epithelial cell apoptosis during AP and, more importantly, to provide further information for understanding intestinal mucosal injury during AP.

Materials and Methods

Induction of AP Model

Male Wistar rats, weighing 150–200 g, were used in this study. The breeders were originally obtained from Luzhou Medical College Laboratories. All animal experiments were conducted according to the guidelines of the Local Animal Use and Care Committees of Luzhou and executed according to the National Animal Welfare Law of China. These animals were housed individually in cages and allowed to acclimate to our animal facilities 1 week prior to the experimental use. Twenty-four rats were randomly divided into two groups (12 for each group). A midline laparotomy was performed under general anesthesia using diethylether. Then, for the induction of AP, the biliopancreatic ducts of the rats were cannulated, and 3 % sodium taurocholate (2 mL/kg) was injected retrogradely under low pressure with the temporary clamp of common bile duct. As a control group, sham-operated rats received the same surgical procedure, but were injected with 0.9 % NaCl solution to the pancreatic duct. Afterward, the abdominal wall was closed in a double layer and the animals were returned to their cages.

Sample Preparation

Samples processed for histological and immunohistochemical examination were prepared at 48 h after operation. After the rats were killed, the abdomen was opened through an incision under strict aseptic conditions. A portion of pancreas was fixed in 4 % paraformaldehyde and embedded in paraffin. Slides were stained with hematoxylin and eosin (H&E) for light microscopy examination. Sections from the ileum were collected from each animal and were fixed in 4 % paraformaldehyde ready for immunohistochemistry (IHC) and TdT-mediated dUTP nick end labeling (TUNEL), respectively, fixed in 2.5 % glutaraldehyde in Sorensen's phosphate buffer, stored at 4 °C, and subsequently processed for routine transmission electron microscopy. Another segment of the ileum was opened and extensively washed in 100 mmol/L ice-cold PBS. The mucosa of the ileum was stripped under microscopic inspection and then frozen at −196 °C for RT-PCR.

Detection of Apoptosis

Apoptotic cells were detected by TUNEL assay (Roche Diagnostics, Bromma, Sweden). For the TUNEL reaction,

tissue sections from the ileum were dewaxed, rehydrated, and then incubated for 30 min at 37 °C with proteinase working solution. The slides were placed in a plastic jar containing 200 mL of 0.1 M citrate buffer (pH 6.0), and 350 W microwave irradiation was applied for 5 min. The slides were rinsed twice with PBS and incubated with a solution composed of the enzyme terminal deoxynucleotidyl transferase and nucleotide mixture (label solution) in a humidified box in the dark for 60 min at 37 °C. For the negative control, only 50 μL labeling solution was added. After incubation, the slides were rinsed three times with PBS. Then, slides were incubated after adding 50 μL converter-peroxidase. Diaminobenzidine was used as the substrate for peroxidase, yielding the characteristic brown color for nuclei. Then, the sections were washed, counterstained with hematoxylin, dehydrated, and sealed. For each test, negative controls were included. All cell counts were performed at 40× magnification. The apoptotic rate was determined as the average of the percentage of positive cells of 10 different, randomly selected fields.

Real-Time Reverse-Transcriptase (RT) PCR

Total RNA was extracted from stored ileum samples using an RNA simple total RNA kit (Tiangen biotech, Beijing, China) according to the manufacturer's protocol. Genomic cDNA eraser and cDNA synthesis (Primescript RT reagent kit with cDNA eraser, Takara, Dalian, China) were also performed following the manufacturers' protocols. The mRNA levels of GRP78, CHOP, caspase-12, and JNK were determined in a real-time quantitative RT-PCR using SYBR premix EX Taq (Takara Biotech, Dalian, China), iCycler thermal real-time PCR system (MJ research, MN, USA). Primers (Invitrogen, Carlsbad, CA, USA) for the amplification of the genes GRP78, CHOP, caspase-12, and JNK are presented in Table 1. Results were repeated in at least three independent RNA preparations. The expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal control gene and analyzed using the $2^{-\Delta\Delta C_t}$ method. mRNA fold induction values were calculated by the following equation:

$$\Delta C_t = C_{t_{\text{target}}} - C_{t_{\text{GAPDH}}},$$
$$\Delta\Delta C_t = \Delta C_{t_{\text{AP}}} - \Delta C_{t_{\text{control}}} \cdot \text{mRNA fold change} = 2^{-\Delta\Delta C_t}.$$

IHC

IHC was used to detect and locate the expression and distribution of GRP78, CHOP, caspase-12, and JNK proteins. Five-micrometer sections embedded in paraffin were dewaxed in xylene and taken through a graded series of ethanol. Antigen retrieval was performed by microwaving for 12 min in Tris–EDTA (pH 6.0) buffer. The tissues were washed with PBS (pH 7.4) for 10 min and 0.3 $\%H_2O_2$ for 30 min in the dark. Distilled water washing was followed by a blocking step with a blocking solution for 30 min. Then, the sections were incubated with 1:100 dilution of rabbit anti-rat GRP78, CHOP, caspase-12, or JNK antibody (Bioworld Technology Inc, MN, USA) overnight at 4 °C. Then, PBS washing (3 times) was followed by a dilution of biotinylated secondary antibody (Biosynthesis biotechnology CO, China) incubated for 30 min and a dilution of horseradish peroxidase-avidin complex incubated for 30 min. After washing with PBS for 3 times, the tissue was visualized by reacting in a solution containing diaminobenzidine. Then, the sections were washed, dehydrated, and sealed. For the negative control in the IHC procedures, PBS replaced the primary antibodies. The slides were examined by light microscopy, and the images were captured with a color video camera. The integrated optical density (IOD) value of each image was measured using IPP6.0 software. Semi-quantitative results of GRP78, CHOP, caspase-12, and JNK proteins were obtained by the average of the IOD values.

Statistical Analysis

All statistical tests were performed using SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean and standard deviation for continuous variables. Data were analyzed using a one-way ANOVA. Differences were considered significant if $P < 0.05$. All significance tests were two-tailed.

Table 1 Sequences of oligonucleotide primers used for RT-PCR

Gene	Forward primer	Reverse primer
GRP78	5′-TCAGCCCACCGTAACAAT-3′	5′-CAAACTTCTCGGCGTCAT-3′
CHOP	5′-CCAGCAGAGGTCACAAGCAC-3′	5′-CGCACTGACCACTCTGTTTC-3′
Caspase12	5′-GGAAGGTAGGCAAGAGT-3′	5′-GTAGAAGTAGCGTGTCATA-3′
JNK	5′-CCAAGAGAGCTTATCGGGAAC-3′	5′-TCCCAAGATGACTTCTGGAGC-3′
GAPDH	5′-AGTTCAACGGCACAGTCAAG-3′	5′-TACTCAGCACCAGCATCACC-3′

Results

Histological Examination

Induction of AP was documented by histological findings of the pancreas (Fig. 1). Histopathological sections stained with H&E showed a normal histological appearance of pancreas in the sham-operated rats. In contrast, all animals that developed AP were characterized by classical lesions, such as pancreatic hyperemia, edema, ascites, and subcapsular petechiaes. The glands were grossly enlarged with visible areas of fat necrosis. Histopathological sections stained with H&E in rats with AP revealed abnormal pancreatic lobules without typical architectures, marked interstitial edema with expansion of interlobular septum, damaged acini with leukocyte infiltration, and the reduction of pancreatic islets.

Furthermore, we investigated the ultrastructural changes, especially that of the ER of ileal epithelial cells under transmission electron microscope (TEM). The epithelial cell in rats with AP displayed extensively irregular, dilated ER (Fig. 2). The mitochondria swelled up and its cristae were disintegrated in parts; in contrast, no abnormal alterations were observed in the sham-operated rats.

Increased Apoptosis in Ileal Epithelium in Rats with AP

A small amount of labeling was detected in sham-operated rats; however, significantly increased labeling was observed on the nuclei of the epithelial cells and especially the tips of the villi in rats with AP. Compared with the sham-operated rats in the control group, the apoptotic rate of ileal epithelial cells was significantly increased in rats with AP (6.10 ± 1.51 vs 21.14 ± 3.86 %, $P < 0.05$) (Fig. 3).

Overexpression of GRP78 mRNA and Protein in Ileal Epithelium in Rats with AP

Compared with the sham-operated rats, the GRP78 mRNA expression was remarkably increased in rats with AP (Fig. 4a). By IHC, the expression of the GRP78 protein was mainly in the cytoplasm (Fig. 4b). Notably, the expression of the GRP78 protein in rats with AP (26.02 ± 2.94) was higher than that in the control rats (14.55 ± 1.25, $P < 0.05$). Taken together with the abnormal morphological ER changes, these results suggest that ER stress was initiated in the intestinal epithelium of rats with AP.

Expressions of CHOP, Caspase-12, JNK mRNA, and Protein in Rats with AP

To investigate whether the ER stress pathway is involved in ileal epithelial cell apoptosis in AP, we next examined the expressions of CHOP, caspase-12, and JNK mRNA and proteins in the ileal epithelium. Contrary to our expectation, the expression levels of CHOP, caspase-12, and JNK mRNA in rats with AP were similar to those of the sham-operated rats ($P > 0.05$, Fig. 5). Moreover, in agreement with the RT-PCR results, the protein levels of CHOP, caspase-12, and JNK were not significantly increased in the rats with AP compared to those of the control rats ($P > 0.05$, Fig. 6).

Discussion

The intestinal epithelium functions as a barrier to protect against invasion of potentially harmful antigens [20]. It is maintained largely by the epithelial lining of the gastrointestinal tract: gut epithelial cells joined at their apical

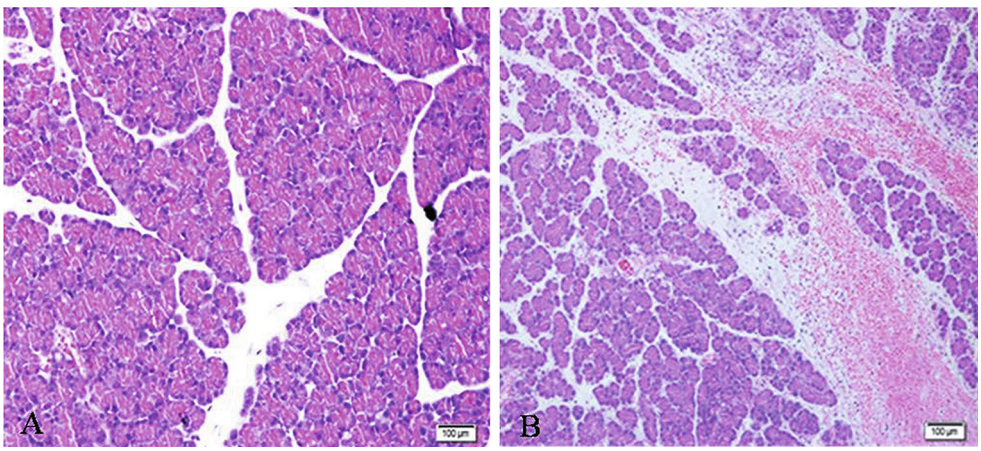


Fig. 1 Representative H&E-stained sections of the pancreas. Pancreas sections from the control rats (**a**) and the rats with AP (**b**). The original magnification is ×100

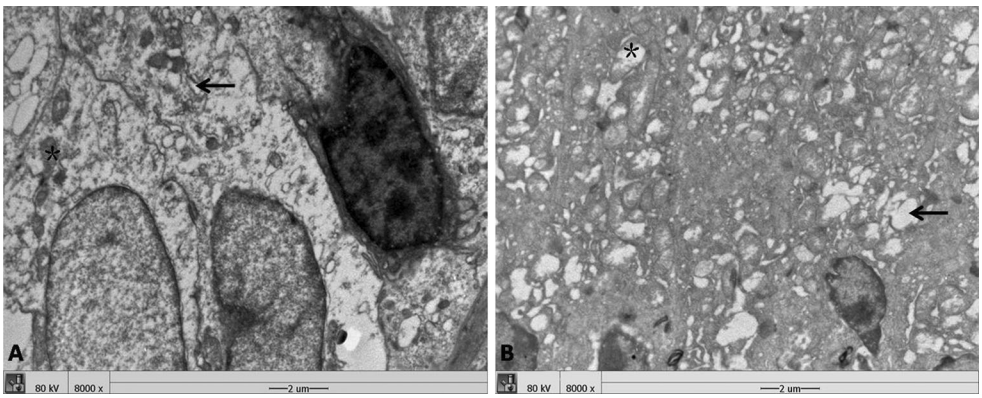


Fig. 2 Ileal epithelial cells by TEM, original magnification ×8,000. **a** Control group: ileal epithelial cell in sham-operated rat appear normal. **b** AP group: morphologic evidence of ER stress. Swollen, dilated ER with irregular ultrastructure in rats with AP (*: mitochondria, → : endoplasmic reticulum)

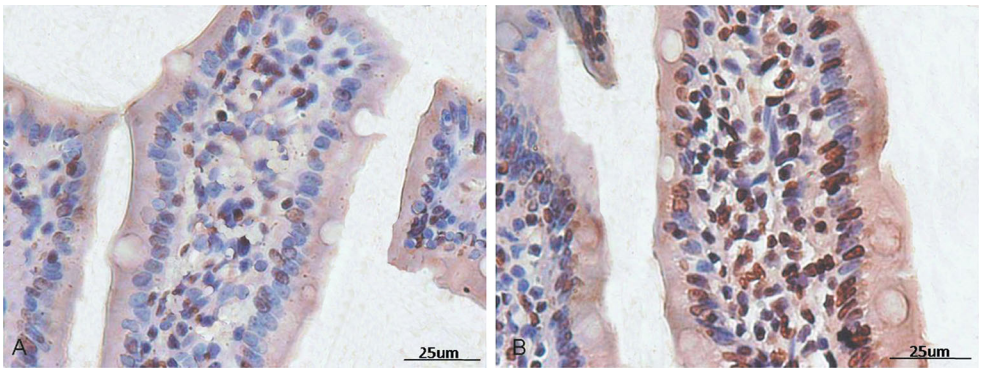


Fig. 3 Ileal epithelial cell apoptosis increased in rats with AP. Sections of ileum stained by TUNEL assay and counterstained with hematoxylin at 48 h after operation (DAB staining, light microscope, original magnification ×400). **a** Control group: small amount of labeling detected in sham-operated rats. **b** AP group: significant TUNEL-positive cells in intestinal epithelium of rats with AP

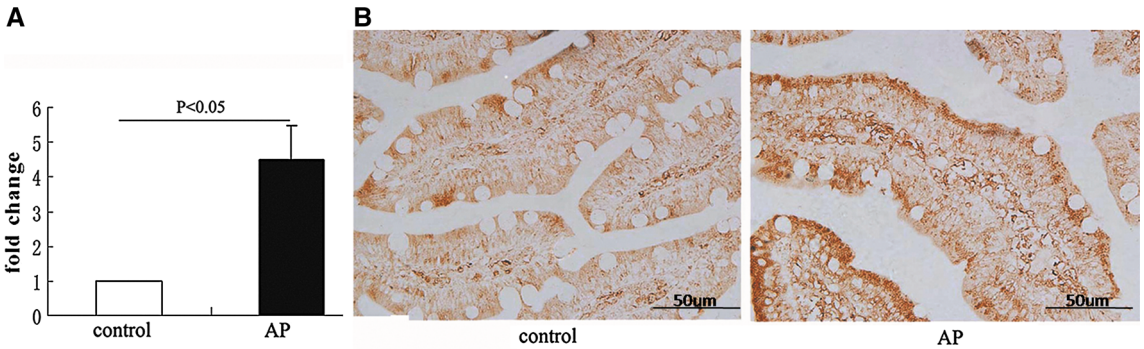


Fig. 4 GRP78 mRNA and protein levels remarkably upregulated in ileal epithelium of rats with AP. **a** GRP78 mRNA level assessed by semi-quantitative real-time RT-PCR. GAPDH was used as a housekeeping gene. Relative expression differences of mRNA were normalized to endogenous GAPDH expression and calculated using the $2^{-\Delta\Delta CT}$ method. The data of relative quantification from three independent experiments were expressed as mean ± SD. $P < 0.05$ denotes a significant difference compared with the sham-operated rats. **b** IHC staining of the GRP78 protein in ileal epithelium (DAB staining, light microscope, original magnification ×200). IHC staining showed the positive signal of GRP78 protein mainly intense in cytoplasm

poles by tight junctions, which seal the paracellular spaces and thereby establish the basic gastrointestinal barrier [21]. Thus, maintenance of an intact epithelium is critical to the integrity of the barrier. It is clear that a number of primary digestive diseases lead to disruption of the mucosal barrier, such as liver cirrhosis, inflammatory bowel disease, and

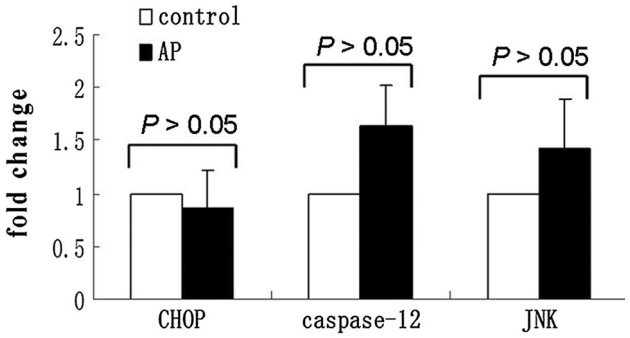


Fig. 5 CHOP, caspase-12, and JNK mRNA level assessed by semi-quantitative real-time RT-PCR. The data of relative quantification from three independent experiments were expressed as mean ± SD. $P > 0.05$; results not statistical significant

colitis. Injury to the intestinal mucosa is also a severe and common problem in SAP [22, 23]. Special attention has been given to the contribution of intestinal epithelial apoptosis to the barrier effect. Increased apoptosis in the intestinal epithelium was thought to be associated with intestinal injury, mucosal atrophy, bacterial translocation, and barrier dysfunction in experimental pancreatitis [24]. Inhibition of apoptosis was involved in the mechanism underlying the protective effects of vascular endothelial growth factor, growth hormone, glucagon-like peptide-2 on intestinal barrier function in acute pancreatitis [25–27]. In the present work, cellular apoptosis in the intestinal epithelium markedly increased in rats with AP, which is consistent with previous studies [25–27]. However, the possible apoptotic pathway of intestinal epithelial cells after induction of AP remains unclear.

Apoptosis can be induced by the ligation of plasma membrane death receptors or by the intrinsic mitochondrial pathway [6]. The ER is also a major point of integration of pro-apoptotic signaling or damage sensing. The ER is an elaborate cellular organelle essential for cell function and survival; ER stress-induced apoptosis is implicated in the pathophysiology of many human diseases. Here, for the first time we investigate whether the ER stress is involved in the AP-induced intestinal epithelial cell apoptosis.

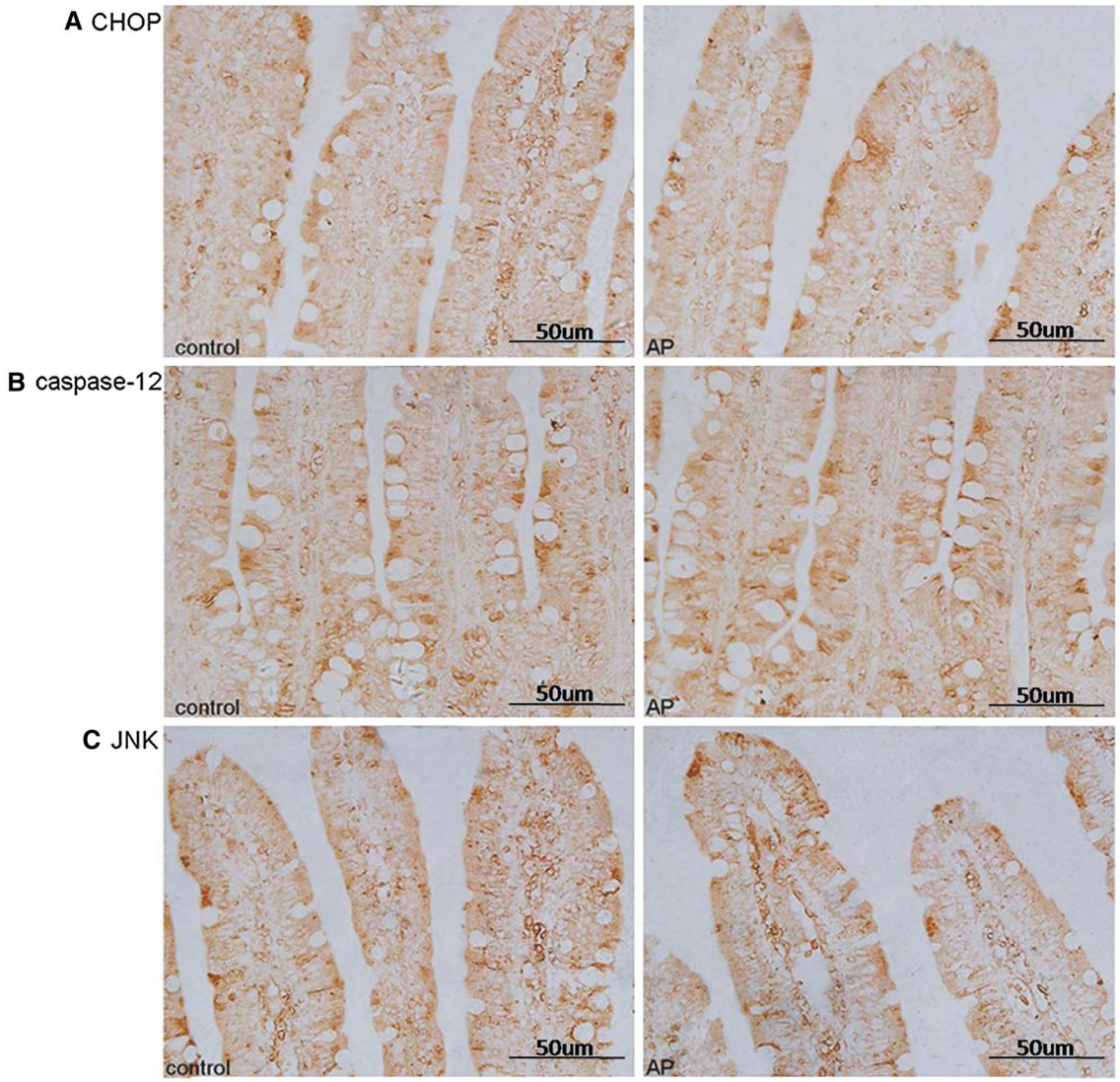
Upon ER stress, cells activate a series of complementary adaptive mechanisms to cope with protein-folding alterations, which together are known as the unfolded protein response (UPR). The UPR is regulated by three ER transmembrane receptors that mediate signal transduction: inositol requiring ER-to-nucleus signal kinase (IRE) 1, activating transcription factor (ATF) 6, and double-stranded RNA-activated kinase (PKR)-like ER kinase (PERK) [28]. When misfolded proteins accumulate in the ER lumen, resident chaperones, such as GRP78, dissociate from the luminal side of the ER stress transducers, allowing aggregation of these transmembrane signaling proteins and launching the UPR [29]. Overexpression of GRP78 either

Fig. 6 IHC staining of CHOP, caspase-12, and JNK protein in ileal epithelium (DAB staining, light microscope, original magnification ×200). IHC staining of CHOP, caspase-12, and JNK proteins could be investigated in AP and sham-operated rats. **a** CHOP; **b** caspase-12; **c** JNK. **d** Comparison of expression of CHOP, caspase-12, and JNK proteins in ileal epithelium. IOD value expressed as mean ± SD ($n = 12$ rats per group). In agreement with the RT-PCR data, these results did not show a significant difference between the two groups

alleviates the stress or is directly involved in signaling stress-induced expression of the glucose-regulated proteins [30]. Specific induction of GRP78 is indicative of ER stress; moreover, GRP78 is a key regulator of ER stress transducers [29]. In this report, we employed the upregulation of ER chaperone GRP78 as a marker of ER stress. In rats with AP induced by sodium taurocholate, we confirmed that GRP78 mRNA and protein levels were remarkably upregulated in the ileal epithelium. Furthermore, by TEM, the ultrastructural findings (including intact and dilated ER) also indicated that the ER was damaged in ileal epithelial cells of rats with AP. Therefore, ER stress is likely triggered in the intestinal epithelium in rats with AP.

The aim of the UPR is to alleviate ER stress, restore ER homeostasis, and prevent cell death. However, if the overload of misfolded proteins in the ER is not resolved, prolonged activation of UPR can lead to apoptosis. This apoptotic effect is mediated in part by the increased expression of CHOP, a downstream transcriptional target of ATF6 and PERK/eIF2α/ATF4. CHOP is a transcription factor that is activated at multiple levels during ER stress, leading to downregulation of bcl-2 mRNA, inducing apoptosis in a p53-independent manner [31]. CHOP is ubiquitously expressed at very low levels under physiological conditions, and its expression is increased in the presence of severe or persistent ER stress. $Chop^{-/-}$ mice exhibited significantly less programmed cell death; therefore, CHOP deficiency protects cells from ER stress-induced apoptosis [32]. Overexpression of GRP78 attenuates the induction of CHOP in ER stress [33]. We did not observe the induction of CHOP, rather a high expression of GRP78 in ileal epithelium of rats with AP. CHOP is likely not involved in the apoptosis of ileal epithelium in AP; however, it was not clear whether the overexpression of GRP78 inhibited the induction of CHOP in the present study.

A second proapoptotic pathway originating in the ER involves the ER initiator caspase-12, which is specifically localized on the cytoplasmic side of the ER and activated by cleavage under ER stress conditions; then, caspase-12 can activate caspase-9 and caspase-3, potentially eliminating the requirement of the mitochondria to carry out ER stress-induced apoptosis [34]. Caspase-12 mediates an ER-specific apoptosis pathway, not by death receptor-mediated or mitochondrial-targeted apoptotic signals [16]; specifically, mice deficient in caspase-12 are resistant to ER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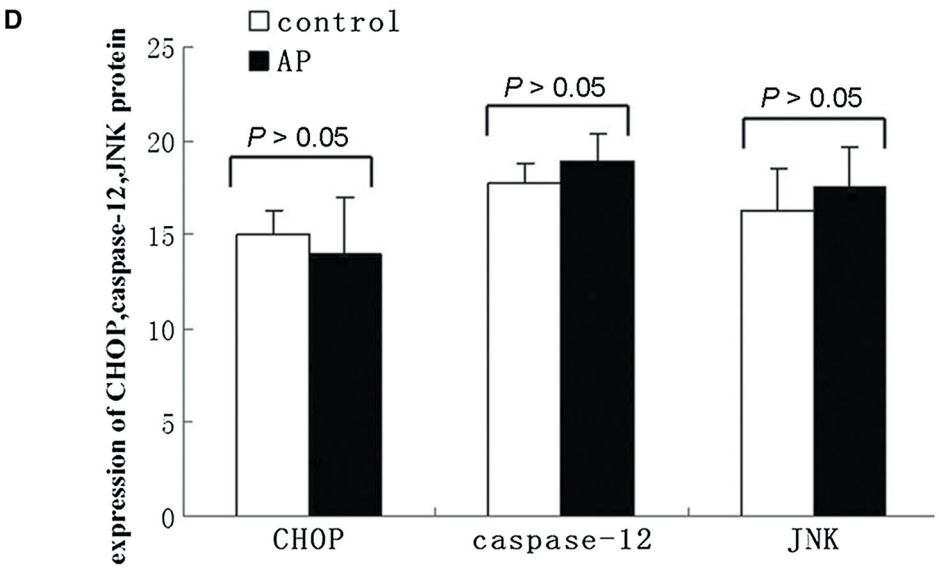
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stress-induced apoptosis. Here, the caspase-12 mRNA and protein levels were not elevated in the rat model of AP. Thus, the apoptosis of the ileal epithelium in rats with AP is unlikely to be associated with the activation of caspase-12.

During the UPR, unfolded proteins accumulating in the ER lumen bind to the ER chaperone BiP/Grp78, thereby competitively disrupting the interaction between GRP78 and Ire1-α. Ire1-α recruits tumor necrosis factor receptor-associated factor 2 (TRAF-2), which in turn participates in activation of the JNK pathway and activation of procaspase-12 [35, 36]. Here, we also examined whether the JNK pathway was activated in the ileal epithelium under condition of AP. We showed activation of GRP78, which is indicative of the occurrence of ER stress, but no increase of the expression of JNK mRNA and protein in rats with AP. Although previous reports proposed that JNK may be a modulator rather than an intrinsic component of the apoptotic machinery, recent studies demonstrate that the JNK pathway is activated during ER stress-induced apoptosis and its inhibition is sufficient to block or prolong death [36, 37]. However, we did not detect upregulation of JNK in ileal epithelium even in the setting of marked apoptosis. Therefore, the JNK pathway likely did not contribute to the ileal epithelial cell apoptosis.

In summary, we observed abnormal ultrastructural change of the ER and upregulated GRP78 mRNA and protein levels in the ileal epithelium in rats with AP and no activation of CHOP, caspase-12, and JNK. Thus, ER stress was induced in intestinal epithelium during AP, but no evidence demonstrated that the apoptosis in the intestinal epithelium was involved with ER stress. However, further studies are needed to fully understand the roles of ER stress in the intestinal epithelium during AP and the mechanisms involved with apoptosis of the intestinal epithelium.

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Conflict of interest The authors have no conflict of interest to disclose.

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