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C/EBP homologous protein is crucial for the acceleration of experimental pancreatitis

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Abstract

C/EBP homologous protein (CHOP) is one of the main mediating factors in the ER stress pathway. To elucidate the role of the ER stress-CHOP pathway in experimental pancreatitis, wild-type ($Chop^{+/+}$) and *Chop* deficient ($Chop^{-/-}$) mice were administered cerulein, a cholecystokinin analogue, or both cerulein and lipopolysaccharide (LPS). In cerulein-induced acute pancreatitis, ER stress, serum amylase elevation and histological interstitial edema were induced. However, there was no remarkable activation downstream of the CHOP pathway regardless of the presence or absence of CHOP. Whereas, in the cerulein and LPS model, inflammation-associated caspases (caspase-11, caspase-1) and IL-1β, but not apoptosis-associated caspases, were activated. In $Chop^{-/-}$ mice, the expression levels of these mediators returned to basal levels resulting in a milder pancreatitis and decreased serum amylase level. These results indicated that the ER stress-CHOP pathway has a pivotal role in the acceleration of pancreatitis through the induction of inflammation-associated caspases and IL-1β.

Keywords: Acute pancreatitis; ER stress; BiP; XBP-1; CHOP; IL-1β; LPS; Apoptosis; Caspase

One of the most important pathological states of the exocrine pancreas is acute pancreatitis, which initially arises in the pancreatic acinar cells by mechanisms that are incompletely understood [1]. After initiation, the disease spreads systemically through the development of an inflammatory response driven by proinflammatory cytokines [2]. Along with proinflammatory mechanisms, protective and restorative mechanisms are also activated in the stressed acinar cells. Thus, the ultimate severity of acute pancreatitis depends on the balance of these opposing forces.

Pancreatic acinar cells have the highest rate of protein synthesis among all human tissues [3]. Because of this prominent role in digestive enzyme synthesis, pancreatic acinar cells contain particularly abundant ER. Thus, pancreatic acinar cells appear susceptible to perturbations of ER homeostasis. Indeed, secretagogue treatment of isolated rat pancreatic acini leads to the activation of the ER stress response in pancreatic acinar cells [4]. As well, all major ER stress sensing and signaling mechanisms have been shown to be activated in the exocrine acini of the arginine model of acute pancreatitis [5].

ER performs several important functions, including posttranslational modification such as folding, and the assembly of newly synthesized secretory or cell membrane proteins. Its proper function is essential to ensure cell survival [6,7]. ER has an especially important function in the survival of hepatocyte and pancreatic acinar cells, as well as in the differentiation of plasma cells [8]. When the cells

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are subjected to ER stress, several pathways are activated to protect the cells [9,10]. These responses involve induction of ER stress-associated factors, including Ig H chain binding protein (BiP), X-bix binding protein (XBP1) and C/EBP homologous protein (CHOP). BiP and XBP1 are good markers of the activation of the ER stress pathway, function as intracellular stress sensors, and protect cells from various stresses. However, when the ER functions are severely impaired, CHOP is induced by ER stress, leading to apoptosis [11].

Recently, the significance of ER stress in various inflammatory diseases has come under discussion. Endo et al. reported that CHOP plays a crucial role in the pathogenesis of inflammation through the induction of caspase-11 [12,13]. They showed that LPS-induced inflammation in the lung, including IL-1β activity in bronchoalveolar lavage fluid, was attenuated in *Chop*$^{-/-}$ mice. Various cytokines, produced from macrophages and other cells, are involved in the pathogenesis of many inflammatory diseases. IL-1β is secreted at an early stage of the inflammatory response and plays a triggering role in inflammatory reactions [13]. In macrophages with activated CHOP as a result of ER stress, procaspase-11 is processed by autoproteolysis. The active form of caspase-11 proteolytically converts procaspase-1 to caspase-1, which then activates pro-IL-1β to IL-1β [14]. Because both caspase-11 and canspase-1 play a pivotal role in the initiation of inflammatory reactions, they are called inflammation-associated caspases [13]. IL-1β is secreted after activation from pro-IL-1β, and has an important function in the initial process of the inflammatory reaction. On the other hand, apoptosis-associated caspases, caspase-3 and caspase-9, were not apparently activated. These observations suggest that CHOP may have a critical role in the pathogenesis of inflammatory reactions.

In this study, we examined whether the ER stress-CHOP pathway is involved in the pathogenesis and acceleration of acute experimental pancreatitis, and whether inflammation-associated caspases and apoptosis-associated caspases are activated in acute pancreatitis.

Materials and methods

Generation and genotyping of animal models. All procedures were approved by the Animal Care and Use Committee of Kumamoto University. Mice lacking the *Chop* gene (C57BL/6J-Chop^{tm1}; *Chop*$^{-/-}$) were generated as previously described [15]. The genotype for *Chop* was determined by PCR. For detecting the wild allele, the following primers were used: 5′-CCTGGATTAAGCTTGGTAGT-3′ as the sense primer, and 5′-GGACGCAGGGTCAAGAGTAG-3′ (derived from the *Chop* gene) as the antisense primer. For detecting the knockout allele, the following primers were used: 5′-GAGAAAAAAAGAGTACAAATG GCCTGG-3′ (derived from the *Chop* gene), as the sense primer and 5′-A TCGCCTTCTATCGCCTTCTTGACGAG-3′ (derived from the neomycin resistant gene) as the antisense primer. Thermal cycle reaction was performed as follows: 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and 72 °C for 7 min. The wild-type and knockout alleles yielded 1.1 kb and 0.8 kb transcripts, respectively.

Cerulein-induced pancreatitis/cerulein-induced and LPS-accelerated pancreatitis (cerulein + LPS pancreatitis). In this experiment, wild-type C57BL/6J mice (*Chop*$^{+/+}$), *Chop*$^{-/-}$ mice were used. After overnight fasting, female mice (7–8 weeks old and weighing 19–23 g) were given hourly intraperitoneal injections of saline as control ($n = 3$) or a supramaximal stimulating concentration of cerulein dissolved in saline (50 μg/kg; $n = 3$) (Sigma–Aldrich Corp, Tokyo, Japan) for several hours (6–12 h). One hour after the last injection, mice were killed and the serum and pancreas rapidly prepared for study. The serum was used for measurement of amylase activity. The pancreas was used for Western blot and RT-PCR analysis. Lipopolysaccharides (LPS. *Escherichia coli* 0111, Sigma–Aldrich Corp.) was injected three times; at the beginning of overnight fasting, at the same time as the first and twelfth cerulein injections, respectively.

Histological analysis. For histological analysis, pancreatic tissue was fixed overnight in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin & eosin (H&E).

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling assay. For the detection of apoptosis, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed using an *in situ* apoptosis detection kit (Wako, Osaka, Japan).

RT-PCR analysis. Mice were killed at the indicated times after treatment. Pancreases were removed and stored at −80 °C. Total RNA from pancreas was isolated using the acid guanidium thiocyanate phenol–chloroform extraction procedure as described [16]. cDNA was synthesized using a Superscript one-step RT-PCR system (Invitrogen Life Technologies, Carlsbad, CA). The primers used for PCR were as follows: sense primer, 5′-ACTCATTGTGGCTGTGGAGAAG-3′ and antisense primer, 5′-GCCGTCTTTCATTACACAGGAC-3′ for IL-1β (GenBank Accession No. M15131); sense primer, 5′-ACACGTCTTGCCCTCATTAT CTGC-3′ and antisense primer, 5′-CCACTCCTTGTTTCTCTCCACG-3′ for caspase-1 (GenBank Accession No. NM009807); sense primer, 5′-GC GTTGGGTTTTTGTAGATGCC-3′ and antisense primer, 5′-ATGTGC TGTCTGATGTCTGGTG-3′ for caspase-11 (GenBank Accession No. Y13089); sense primer, 5′-GAAAGGATGGTTAATGATGCTGAG-3′ and antisense primer, 5′-GTCTTCAATGTCCGCATCCTG-3′ for BiP (GenBank Accession No. AJ002387); sense primer, 5′-CATACACCACC ACACCTGAAAG-3′ and antisense primer, 5′-CCGTTTCCTAGTT CTTCCTTGC-3′ for CHOP (GenBank Accession Number X67083). The primer sets for IL-1β, caspase-1, caspase-11, BiP, and CHOP were expected to give PCR products of 384, 372, 439, 231, and, 357 bp, respectively. PCR consisted of an initial denaturation cycle at 94 °C for 5 min, followed by the 30 cycles at 94 °C for 15 s, annealing at 55 °C for 30 s, and elongation at 68 °C for 1 min. An additional cycle at 72 °C for 7 min completed the amplification process. The primers used for detection of G3PDH mRNA were as follows: sense primer, 5′-GGAAAGC TGTGGCGTGATG-3′ and antisense primer, 5′- CTGTTGCTGTA GCCGTATTC-3′. The primers used for detection of XBP-1 mRNA were as follows: sense primer, 5′-AAACAGAGTAGCAGCGCAGACTGC-3′ and antisense primer, 5′-GGATCTCTAAAACTAGAGGCTTGGTG-3′. The primer sets for G3PDH and XBP-1 were expected to give PCR products of 380 and 600 bp, respectively. PCR consisted of an initial denaturation cycle at 94 °C for 5 min, followed by 27 cycles consisting of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 30 s. An additional cycle at 72 °C for 7 min completed the amplification process. Amplified PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Serum amylase activity. Substrate for measurement of pancreatic amylase activity was 2-chloro-4-nitrophenyl-4-galactopyranosylmaltoside (Gal-G2-CNP) (CicaLiquid-N p-AMY, Kanto Chemical Co., Inc. Tokyo, Japan).

Western blot analysis. Pancreas samples were homogenized in lysate buffer (Hepes 50 mmol/L, pH 7.4, NaCl 150 mmol/L, Triton X-100 0.1%, glycerol 10%, NaF 1 mmol/L, sodium orthovanadate 2 mmol/L, ethylenediaminetetraacetic acid 1 mmol/L, and a protease inhibitor cocktail [1:100 dilution; Sigma–Aldrich]). Extracts (12–15 μg of protein per lane) were applied to 12% polyacrylamide gel electrophoresis and transferred to an Immobilon polyvinylidene difluoride filter (Millipore, Billerica, MA). Primary antibodies to the following antigens (made in rabbit) were used at the indicated dilutions: Caspase-3 (Cell Signaling Technology, Inc. Danvers, MA), 1:1000; Caspase-9 (Cell Signaling Technology), 1:1000; and

Actin (Sigma–Aldrich Corp.) 1:1000. An anti-rabbit immunoglobulin G antibody conjugated with horseradish peroxidase (Amersham Biosciences Corp., Piscataway, NJ) was used for detection. Unpaired Student's *t* tests were used to calculate *P* values.

Results

Pancreatitis in Chop$^{+/+}$ and Chop$^{-/-}$ Mice

To analyze the relationship between the severity of pancreatitis and the ER-CHOP pathway, we induced experimental pancreatitis by cerulein or by cerulein + LPS. In saline-treated mice, no pathologic changes were observed in the histology of pancreas of *Chop*$^{+/+}$ and *Chop*$^{-/-}$ mice (Fig. 1A and B). Both *Chop*$^{+/+}$ and *Chop*$^{-/-}$ mice treated with cerulein showed evidence of interstitial edema and the accumulation of a huge number of vacuole-like structures distributed throughout the cytoplasm (Fig. 1C and D). The degree of pancreatic inflammation in *Chop*$^{-/-}$ mice was almost the same as in *Chop*$^{+/+}$ mice. Using both cerulein and LPS, Ding et al. reported the successful establishment of a mouse model for severe pancreatitis [17]. We used this method to investigate whether *Chop* is involved in this model. Severe inflammatory changes, such as the destruction of acinar cells and the infiltration of inflammatory cells were diffusely observed in the pancreas of *Chop*$^{+/+}$ mice treated with cerulein + LPS (Fig. 1E). However, such inflammatory changes were obviously attenuated in the pancreas of *Chop*$^{-/-}$ mice (Fig. 1F).

Amylase activity in Chop$^{+/+}$ and Chop$^{-/-}$ mice

To assess the degree of pancreatitis, we analyzed the serum amylase activity of *Chop*$^{+/+}$ and *Chop*$^{-/-}$ mice in which had been induced pancreatitis by cerulein or cerulein + LPS. In *Chop*$^{+/+}$ mice, serum amylase levels were 8085 ± 1761 IU/L ($n = 2$) and $10{,}690 \pm 692$ U/L ($n = 3$) after treatment with cerulein or cerulein + LPS, respectively, while the serum level from saline treatment was 940 ± 35 U/L ($n = 3$) (Fig. 2A). There was a tendency for the induction levels of serum amylase from cerulean + LPS to be higher than that from cerulein. In *Chop*$^{-/-}$ mice, the serum amylase levels were 7940 ± 482 IU/L ($n = 3$) and 7920 ± 1032 U/L ($n = 3$) from treatment with cerulein or cerulein + LPS, respectively; while the serum level from saline treatment was 990 ± 159 U/L ($n = 3$) (Fig. 2B). Thus, there was no significant difference between the induction levels of serum amylase in *Chop*$^{+/+}$ and *Chop*$^{-/-}$ mice after stimulation with cerulein. But the serum amylase levels returned to the same level as those found in *Chop*$^{+/+}$ mice with cerulein treatment in the absence of CHOP. Thus, there was a significant difference ($p < 0.01$) between serum amylase levels in *Chop*$^{-/-}$ and *Chop*$^{+/+}$ mice when treated with cerulein + LPS. These results indicated that

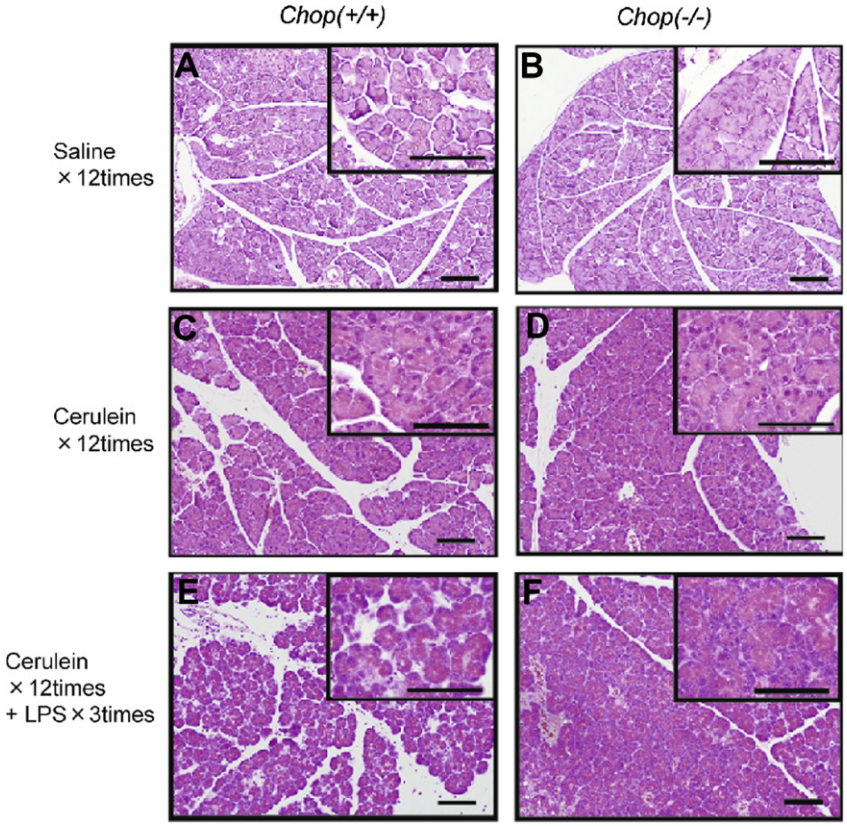

Fig. 1. Histological analysis of experimental pancreatitis in *Chop*$^{+/+}$ and *Chop*$^{-/-}$ mice. Scale bars: 100 μm. (A, B) Saline-treated group. (C, D) Cerulein-treated group. (D, E) Cerulein + LPS treated group.

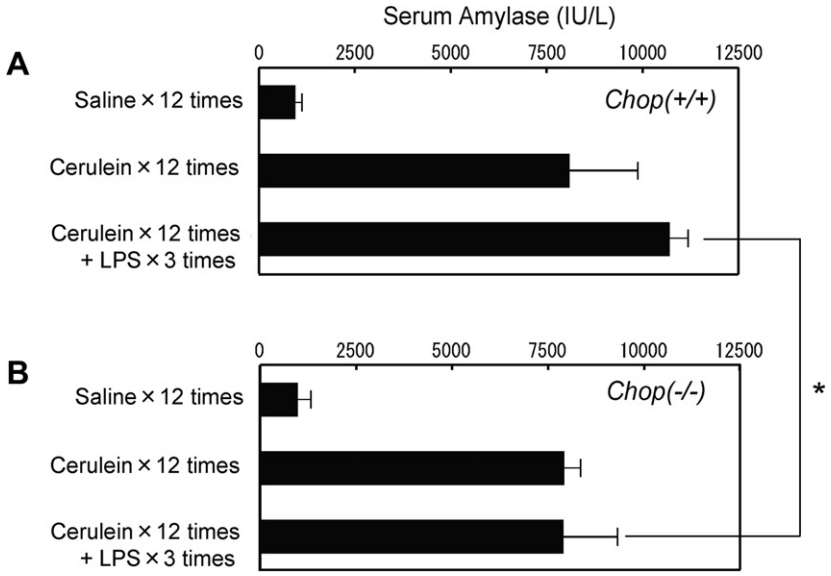

Fig. 2. Analysis of serum amylase activities in $Chop^{+/+}$ and $Chop^{-/-}$ mice of each experimental model of pancreatitis. (A) $Chop^{+/+}$ mice (B) $Chop^{-/-}$ mice In $Chop^{-/-}$ mice treated by cerulein + LPS, the induction of serum amylase is suppressed significantly compared with $Chop^{+/+}$ mice treated by cerulein + LPS ($^{*}P < 0.01$; unpaired Student's t test).

the CHOP pathway is involved in the severe pancreatitis induced by cerulein + LPS.

Induction of ER stress, inflammatory mediators and apoptotic caspases in $Chop^{+/+}$ and $Chop^{-/-}$ mice

ER stress related mechanisms were evaluated after treatment by cerulein or by cerulein + LPS. BiP, XBP-1 and CHOP were activated in $Chop^{+/+}$ mice when treated by cerulein or cerulein + LPS (Fig. 3A). Levels of BiP and XBP-1 were increased also in $Chop^{-/-}$ mice. These results indicated that ER stress is induced by cerulein or cerulein + LPS, regardless of the presence or absence of CHOP.

In $Chop^{+/+}$ mice, caspase-11, caspase-1 and IL-1β were not activated in the cerulein-treated group. However, these inflammatory mediators were increased in $Chop^{+/+}$ mice when treated with cerulein + LPS (Fig. 3A). On the other hand, the expression levels of these inflammatory mediators were much lower in $Chop^{-/-}$ mice. These results indicated that the induction of caspase-11, caspase-1 and IL-1β was mediated by CHOP, and that the milder pancreatitis induced by cerulein + LPS in $Chop^{-/-}$ mice was due to the suppression of IL-1β.

CHOP expression is induced at high levels by ER stress, and CHOP induces apoptosis through activation of caspase-9 and caspase-3 [11]. To investigate whether the CHOP-apoptotic pathway is related to the attenuation of pancreatitis in $Chop^{-/-}$ mice, the expression levels of caspase-9 and caspase-3 were analyzed in each model of experimental pancreatitis. In the cerulein-treated group, there was no induction of caspase-9 and caspase-3 in $Chop^{+/+}$ and $Chop^{-/-}$ mice. In the cerulein + LPS treated group, there was a slight induction of caspase-9 and caspase-3 in $Chop^{+/+}$ and $Chop^{-/-}$ mice (Fig. 3B).

The results of TUNEL assay are shown in Fig. 4. TUNEL positive cells were not observed in the saline-treated $Chop^{-/-}$ and $Chop^{+/+}$ mice. In $Chop^{+/+}$ mice, the number of TUNEL positive cells in the cerulein + LPS treated group was higher than that in the cerulein-treated group. In $Chop^{-/-}$ mice treated with cerulein + LPS, the number of TUNEL positive cells decreased to the same level as that in $Chop^{+/+}$ mice (Fig. 4B). These results suggested that apoptosis was actually induced, although there was no difference in the expression levels of the apoptosis-associated caspases, caspase-9 and caspase-3.

Discussion

Here we showed that the ER stress-CHOP pathway has an important function in the acceleration of acute pancreatitis through the activation of the inflammation-associated caspases pathway, caspase-11, caspase-1 and IL-1β.

We demonstrated the activation of the ER stress pathway as an early event during the development of acute pancreatitis in the cerulein mouse model. Although BiP and XBP-1 were activated by cerulein, there was no difference in the severity of pancreatitis, levels of amylase, or numbers of TUNEL positive cells between $Chop^{+/+}$ and $Chop^{-/-}$ mice. Indeed, CHOP-mediated inflammatory and apoptosis pathways were not activated, as demonstrated by the unchanged levels of caspases and IL-1β. These results suggested that CHOP-mediated pathways are not involved in cerulein pancreatitis.

To analyze the role of CHOP in more detail, we used the more severe pancreatitis model from cerulein and LPS [17]. LPS is a kind of endotoxin that can activate the mononuclear cell system to release cytokines. These cytokines can activate the production of inducible nitric oxide (NO) syn-

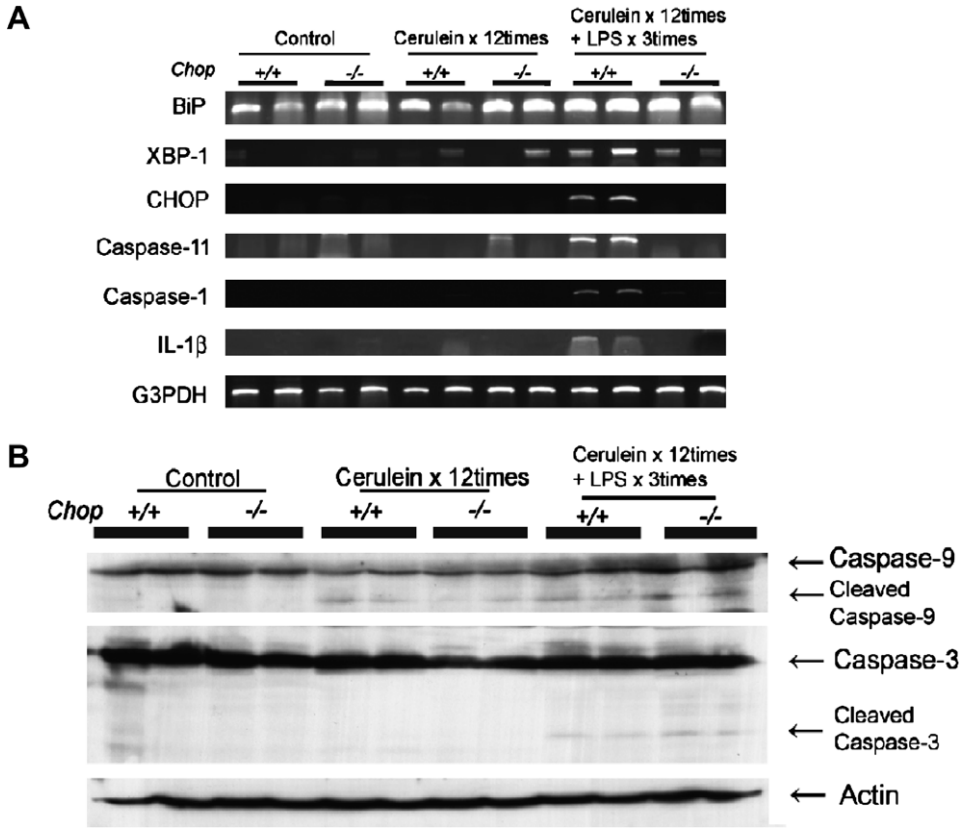

Fig. 3. Analysis of ER stress, inflammatory mediators, and apoptotic caspases in $Chop^{+/+}$ and $Chop^{-/-}$ mice of each experimental model of pancreatitis. (A) RT-PCR analyses of ER stress-associated factors and inflammatory mediators. (B) Western blot analyses of caspase-9 and caspase-3.

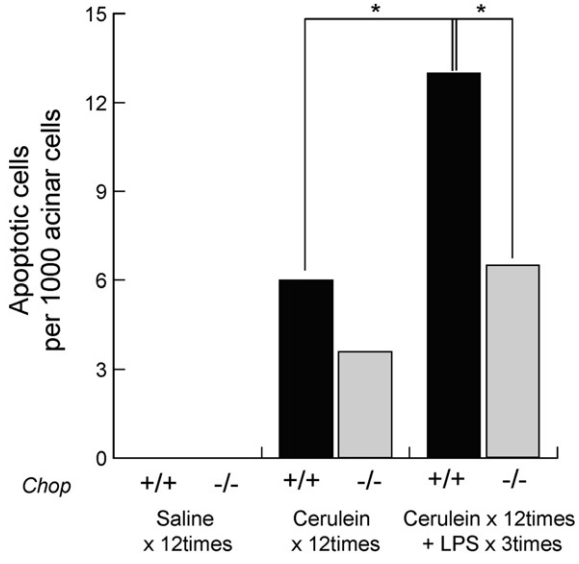

Fig. 4. TUNEL assay in $Chop^{+/+}$ and $Chop^{-/-}$ mice of each experimental model of pancreatitis ($^{*}P < 0.05$; unpaired Student's t test).

thase, resulting in the overproduction of NO. As NO acts as an endothelium-derived relaxing factor and a highly reactive free radical, excessive production of NO causes vasodilatation and increased microvascular permeability, resulting in cellular damage. Thus, treatment with both cerulein and LPS can cause severe pancreatitis in mice. Like in cerulein pancreatitis, BiP and XBP-1 were activated by treatment with cerulean and LPS. The ER stress-CHOP pathway is believed to induce inflammation-associated caspases (caspase-11 and caspase-1). In fact, as shown in Fig. 3, while these caspases and IL-1β were activated in $Chop^{+/+}$ mice, they were considerably down-regulated in $Chop^{-/-}$ mice. The expression of caspase-11 activates caspase-1 and the complex of activated caspase-1 and caspase-11 increases the activation of pro-IL-1β to mature IL-1β. Activated IL-1β is considered to trigger the inflammatory cytokines cascade [13]. In $Chop^{-/-}$ mice, this inflammation cascade through IL-1β was blocked. These results showed that the severity of pancreatitis was attenuated and the level of amylase decreased to the same level as seen in cerulein model in $Chop^{-/-}$ mice. These results also suggest that the CHOP-mediated inflammatory pathway has a major role in the severe pancreatitis induced by cerulein and LPS.

Although treatment with cerulein and LPS-induced apoptosis-associated caspases (caspase-3 and caspase-9), their levels were very low even in the presence of CHOP. Nevertheless, TUNEL positive cells were increased in $Chop^{+/+}$ mice, and decreased in $Chop^{-/-}$ mice. These results suggested that the enhanced apoptosis induced by cerulein and LPS was mediated by CHOP, but not through apoptosis-associated caspases. A similar situation was reported by Endo et al. [12]. In the mouse model of LPS-induced pneu-

monia, a blockade of apoptosis-associated caspases in *Chop*$^{-/-}$ mice was small, but the pneumonia itself was attenuated compared with that in *Chop*$^{+/+}$ mice [12]. Thus, induction of TUNEL positive apoptotic cells may be a reflection of IL-1β induction, another pro-apoptotic molecule. Although the major function of caspase-11 and caspase-1 is believed to regulate cytokine maturation, the function of these caspases is not restricted to the inflammatory cascade [18,19]. Caspase-11 can mediate the activation of both caspase-1 and caspase-3. While caspase-3 mediates induction of apoptosis, caspase-1 is a regulator of apoptosis. Thus, the regulation of the two downstream caspases by caspase-11 may contribute to the development of apoptosis under pathological conditions [19]. IL-1β also promotes the cell death pathway [20].

Concerning the role of apoptosis in experimental pancreatitis, it is generally believed that acinar cells die through both necrosis and apoptosis in experimental models of acute pancreatitis [21,22]. The apoptosis/necrosis ratio varies in different experimental models of pancreatitis. In mouse, the severity of experimental pancreatitis directly correlates with the extent of necrosis and inversely with that of apoptosis [22–24]. Mareninova et al. [25] analyzed the cell death pathway in cerulein pancreatitis and demonstrated that the high necrosis and little apoptosis in the mouse model of cerulein pancreatitis was due to caspases inactivation by XIAP (X-linked inhibitor of apoptosis protein). When a caspase was induced by XIAP inhibitor, embelin, stimulation of apoptosis and decreased necrosis was observed, resulting in normalization of pancreatic histology. In our case, acute pancreatitis became less severe in the absence of CHOP, although apoptosis also decreased. This is apparently contrary to the data described above; that induction of apoptosis can reduce the severity of pancreatitis in mouse. This can be explained as follows. Our data demonstrated that the processing of apoptosis-associated caspases was not observed even in the presence CHOP in the cerulein and LPS model. Similar data that apoptosis-associated caspases were not activated in a cerulein model was reported by Mareninova et al. [25]. On the other hand, we showed that the inflammatory-associated pathway was activated by cerulein + LPS and that this pathway was indeed substantially inhibited in the absence of CHOP. Taken together, these results suggested that the level of apoptosis is too low to influence the severity of pancreatitis induced by cerulein and LPS. Thus, a decrease in apoptosis does not affect the severity of pancreatitis. However, it is possible that enhancement of apoptosis increases the apoptosis/necrosis ratio, resulting in the reduction of the severity of pancreatitis.

To the best of our knowledge, this is the first report showing that the CHOP-caspase-11 pathway has an important role in the acceleration of pancreatitis *in vivo*. Severe acute pancreatitis can be fatal, and the CHOP-inflammation-associated caspases pathway may thus become a new target for therapy against this disease.

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