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Inhibition of the dephosphorylation of eukaryotic initiation factor 2α ameliorates murine experimental pancreatitis

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ABSTRACT

Background: Endoplasmic reticulum (ER) stress in the pancreas is closely associated with the development of acute pancreatitis. However, the role of the protein kinase RNA-like ER kinase (PERK) in this disease is not fully understood. We investigated whether an inhibitor of the dephosphorylation of eukaryotic initiation factor 2α, salubrinal, could improve murine experimental pancreatitis through the amelioration of ER stress.

Methods: Acute pancreatitis was induced by the intraperitoneal administration of cerulein (50 μg/kg) six times at 1-h intervals followed by lipopolysaccharide (10 mg/kg). Salubrinal was administered intraperitoneally immediately after lipopolysaccharide injection and 3 h later. Mice were sacrificed 24 h after the first injection of cerulein, and serum amylase and proinflammatory cytokines were measured. The severity of pancreatitis was evaluated histologically using a scoring system. The expression levels of ER stress-related proteins were evaluated by Western blotting.

Results: The administration of salubrinal significantly attenuated the increase in serum amylase levels and improved histologically assessed pancreatitis. The serum levels of proinflammatory cytokines were significantly suppressed in salubrinal-treated mice, as was the expression of glucose-regulated protein 78, CCAAT/enhancer-binding protein homologous protein, and cleaved caspase-3.

Conclusions: The amelioration of ER stress through augmentation of the PERK-signaling pathway may be a therapeutic target for the treatment of acute pancreatitis.

Introduction

Acute pancreatitis (AP) is a disorder of the pancreas that is characterized by inflammation, edema, and cell necrosis of the exocrine glands, which produces a range of disease severity. Although AP usually has a favorable prognosis, severe forms can have a mortality rate of up to 30% [1]. The pathogenesis of AP has been investigated and pathological intra-acinar trypsinogen activation has been proposed as the key event in the pathogenesis of the disease [2]. However, some studies using trypsinogen-7 knockout mice have demonstrated the possible existence of other trypsinogen-independent pathogenic mechanisms [3,4].

Pancreatic acinar cells contain abundant endoplasmic reticulum (ER) in order to fulfill their physiological function of producing large amounts of digestive enzymes. A variety of newly synthesized digestive enzymes is transported to the ER and bind to chaperone proteins, which facilitate the correct folding and posttranslational modifications of the synthesized proteins. These are then assembled into zymogen granules in the Golgi and are secreted into the extracellular environment [5]. These ER processes are sensitive to various environmental alterations and cellular disturbances, including inflammatory stimuli, oxidative stress, and increased protein secretion. Disruption of the protein-folding homeostasis in the ER leads to the accumulation of unfolded and misfolded proteins in its lumen, a condition known as ER stress. To cope with this cellular stress and restore ER homeostasis, eukaryotic cells have developed the unfolded protein response (UPR) [6,7].

The UPR is a set of coordinated signaling pathways that monitor and maintain ER homeostasis, and mediate three types of responses: reduction of the protein load entering the ER, enhancement of the capacity of the ER to handle unfolded proteins, and

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activation of protein-degradation programs to rid the ER of accumulated unfolded/misfolded proteins. The UPR is initiated by three stress sensors that are expressed constitutively in the ER membrane: protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) [7–9]. During homeostasis, these molecules remain inactive via their association with the chaperone glucose-regulated protein 78 (GRP78). In the presence of excessive unfolded/misfolded proteins, GRP78 disengages from these regulatory molecules and induces their activation. Activated PERK phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α) to reduce protein synthesis. Concomitantly, phosphorylated eIF2α induces activating transcription factor 4 (ATF4), which subsequently transactivates genes that encode other UPR-associated transcription factors, together with the ER protein-folding and protein-degradation systems, and promote cellular homeostasis. Activated ATF6 moves to the Golgi apparatus, where it becomes a transcriptionally active form that increases the transcription of ER chaperones, including GRP78, and protein foldases. The activation of IRE1 causes the splicing and activation of the X-box binding protein 1 (XBP1) to its active transcription factor form (spliced XBP1), which also regulates the expression of various chaperones, foldases, and other protective molecules. Together, the UPR mechanisms clear unfolded/misfolded proteins from the ER and alleviate ER stress. When severe and/or prolonged ER stress occurs, CCAAT/enhancer-binding protein homologous protein (CHOP), a transcription factor that controls genes involved in apoptosis and inflammation and is involved in the PERK branch of the UPR, is activated and contributes to programmed cell death [10].

Pancreatic cells are more sensitive to external stimuli when the ER is disordered [11]. Studies have shown that ER stress is closely associated with the development and progression of AP [12–14]. The structure of the ER can change significantly during the pathogenesis of pancreatitis. Early morphological changes include swelling and the vacuolization of the ER and the loss of ribosomes [15]. The activation of PERK and the phosphorylation of eIF2α have been observed at an early stage of cerulein-induced pancreatitis [16,17]. Although deletion of PERK aggravated the functional impairment of pancreatic acinar cells [18], the therapeutic potential of its modulation has not been studied fully.

In this study, we assessed the therapeutic effect of augmentation of the PERK-signaling pathway using an inhibitor of eIF2α dephosphorylation, salubrinal, in an experimental murine model of pancreatitis, and investigated the molecular mechanisms underlying the effect of this drug on the healing of pancreatic injury.

Materials and methods

Mice

Male C57BL/6 wild-type mice (6–8 weeks of age) were purchased from Japan SLC (Shizuoka, Japan). All mice were housed in specific pathogen-free conditions in the animal facility of Kansai Medical University. All animal experiments were conducted with the approval of the Ethics Committee for the Use of Experimental Animals of Kansai Medical University.

Induction of AP

To induce AP, mice were injected six times intraperitoneally with cerulein (Sigma-Aldrich, St Louis, MO, USA; 50 μg/kg body weight) at 1-h intervals (n = 10 in each group) or with normal saline as control. Lipopolysaccharide (LPS; Sigma-Aldrich) was injected at a dose of 10 mg/kg immediately after the induction of pancreatitis with six cerulein injections, to produce a septic challenge as previously described [19]. Immediately after and 3 h after completion of cerulein and LPS administration, mice were injected intraperitoneally with an inhibitor of eIF2α dephosphorylation, salubrinal (Tocris Bioscience, Bristol, UK), which was dissolved in phosphate-buffered saline (PBS) containing 1% dimethyl sulfoxide (DMSO), at doses of 0.5, 1, 2.5, and 5 mg/kg.

Histological examination

The mice were killed 24 h after the first cerulein or saline injection. Then blood samples were collected and the sera were stored at −30 °C until analysis. Pancreatic tissues were excised for histopathological examination. The tissues were fixed in 10% phosphate-buffered formaldehyde (pH 7.2), embedded in paraffin, before 3.0-μm sections were prepared, stained with hematoxylin and eosin, and examined under light microscopy. The severity of pancreatitis was graded using a scoring system described previously [20]. Briefly, grading for inflammatory cell infiltration was scored as: 0, absent; 1, in ducts (around ductal margins); 2, in the parenchyma (in <50% of the lobules); and 3, in the parenchyma (in >50% of the lobules). Edema was scored as: 0, absent; 1, focally increased between lobules; 2, diffusely increased between lobules; and 3, acini disrupted and separated. Acinar necrosis was scored as follows: 0, absent; 1, periductal necrosis (≤5%); 2, focal necrosis (5–20%); and 3, diffuse parenchymal necrosis (>20–50%). The total score was calculated as the sum of all these scores.

Western blot analysis

Pancreatic tissue samples (n = 5 in each group) were lysed in Tris–EDTA–Na buffer (Sigma-Aldrich). Insoluble materials were removed by centrifugation at 12,000 *g* for 10 min at 4 °C. After determination of the protein concentration using a bicinchoninic acid protein assay kit (Pierce Chemical, Dallas, TX, USA), supernatants were boiled with Laemmli sample buffer (Bio-Rad Laboratories, Osaka, Japan) for 5 min. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Antibodies against eIF2α (1:750), phosphorylated eIF2α (1:750), GRP78 (1:1000 dilution), ATF4 (1:1000), cleaved caspase-3 (1:800), β-actin (1:1000; all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), and CHOP (1:1000; Cell Signaling Technology, Danvers, MA, USA) were used as primary antibodies, and the membranes were incubated overnight at 4 °C. The membranes were washed three times for 5 min with Tris-buffered saline containing Tween 20 (TBS-T) and incubated with secondary antibodies diluted in TBS-T containing 3% bovine serum albumin. Immunoreactive bands were visualized using an enhanced chemiluminescence peroxidase developing solution (Bio-Rad Laboratories) and were recorded on autoradiographic film. Relative band intensity was quantified by densitometric analysis and the respective protein expression was normalized to that of β-actin.

Measurement of serum amylase and cytokine levels

Sera were obtained from the mice that were killed 24 h after the first cerulein or saline injection. Serum amylase levels were measured by a commercial laboratory (SRL Inc., Tokyo, Japan). Serum levels of interleukin (IL)-1β, IL-6, IL-10, tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ) were measured using Bio-Plex Pro cytokine bead assay kits (Bio-Rad Laboratories).

Apoptosis of pancreatic acinar cells

Apoptotic acinar cells in the pancreatic tissue were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using an *in situ* cell death detection kit (Takara Bio, Shiga, Japan), according to the manufacturer's instructions.

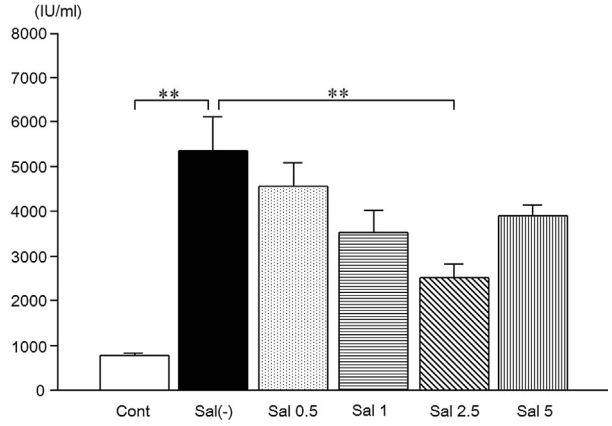

Fig. 1. Serum levels of amylase in mice treated with salubrinal. The amylase levels were significantly decreased in the mice treated with 2.5 mg/kg salubrinal compared with those in vehicle-treated mice (**P < 0.01). The results are expressed as mean ± SE of 10 mice.

Statistical analysis

Student's *t*-test was used to determine the significance of differences observed between two groups. Differences between more than two groups were evaluated by the Tukey–Kramer test. Data are expressed as mean ± standard error (SE). A two-tailed P value < 0.05 was considered significant.

Results

Serum levels of amylase in mice treated with salubrinal

Serum amylase levels were significantly elevated in the mice treated with cerulein and LPS compared with those in the mice treated with vehicle (**P < 0.01; Fig. 1). The administration of 2.5 mg/kg salubrinal significantly decreased serum amylase levels compared with those in vehicle-treated mice with cerulein- and LPS-induced pancreatitis (**P < 0.01).

Severity of pancreatitis of mice treated with cerulein and LPS

The administration of cerulein and LPS induced intense infiltration of inflammatory cells and interstitial edema of the pancreas, accompanied by acinar cell necrosis compared with vehicle-treated mice (Fig. 2A and B). Treatment with salubrinal dose-dependently suppressed pancreatic inflammation (Fig. 2C: 0.5 mg, 2D: 1 mg, 2E: 2.5 mg, 2F: 5 mg). When the severity of AP was graded using a semiquantitative scale, scores for inflammation (Fig. 3A), edema (Fig. 3B), necrosis (Fig. 3C), and total score (Fig. 3D) were all lower in the mice treated with salubrinal than in control mice, although these differences were not significant. However, when the mice treated with each dose of salubrinal were compared separately

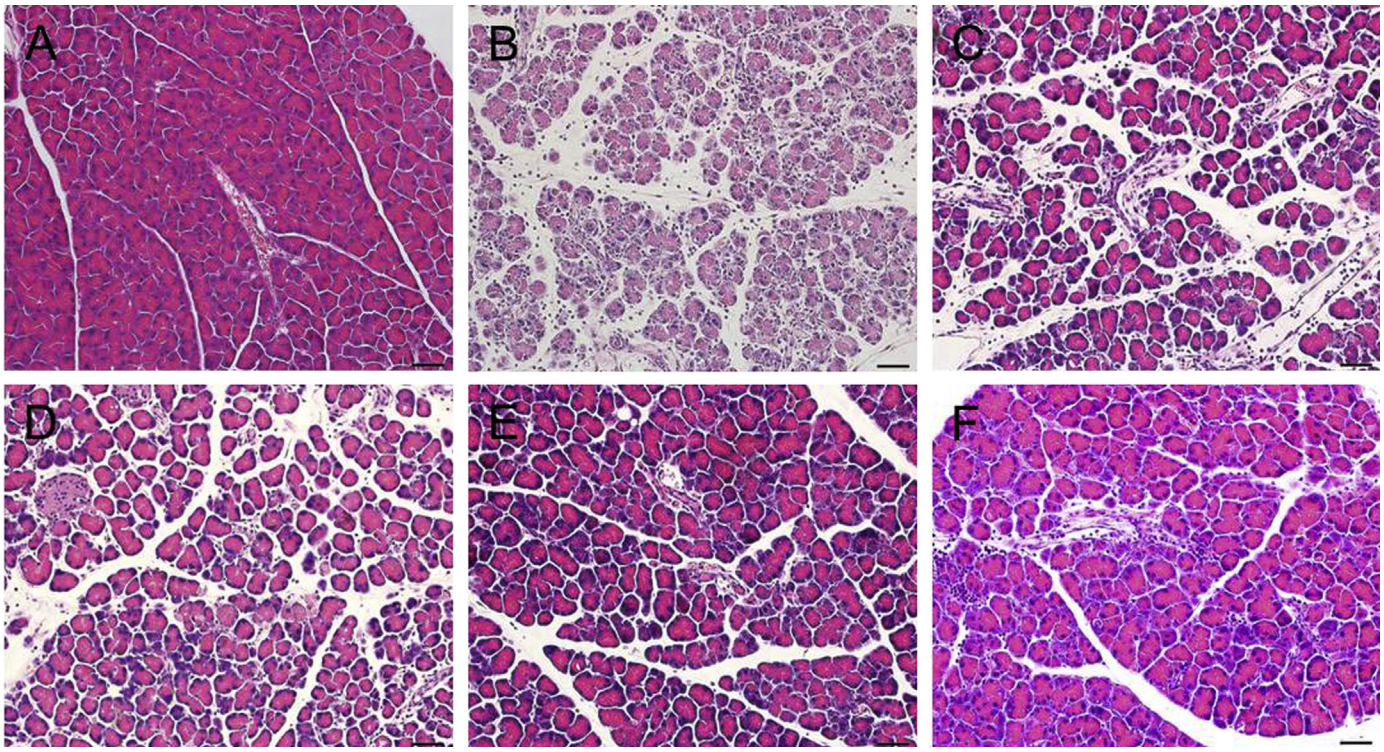

Fig. 2. Histopathological analysis of the pancreas of mice treated with salubrinal. Representative pancreatic sections stained with hematoxylin and eosin are shown after treatment with phosphate-buffered saline (A), cerulein and lipopolysaccharide (B), or treatment with cerulein and lipopolysaccharide plus salubrinal at 0.5 mg/kg (C), 1 mg/kg (D), 2.5 mg/kg (E), and 5 mg/kg (F). The administration of cerulein and LPS induced intense infiltration of inflammatory cells associated with the destruction of the acini, pseudotubular complex formation, and irregular fibrosis in the pancreas. Treatment with salubrinal dose-dependently suppressed pancreatic inflammation. Bars indicate 100 μm.

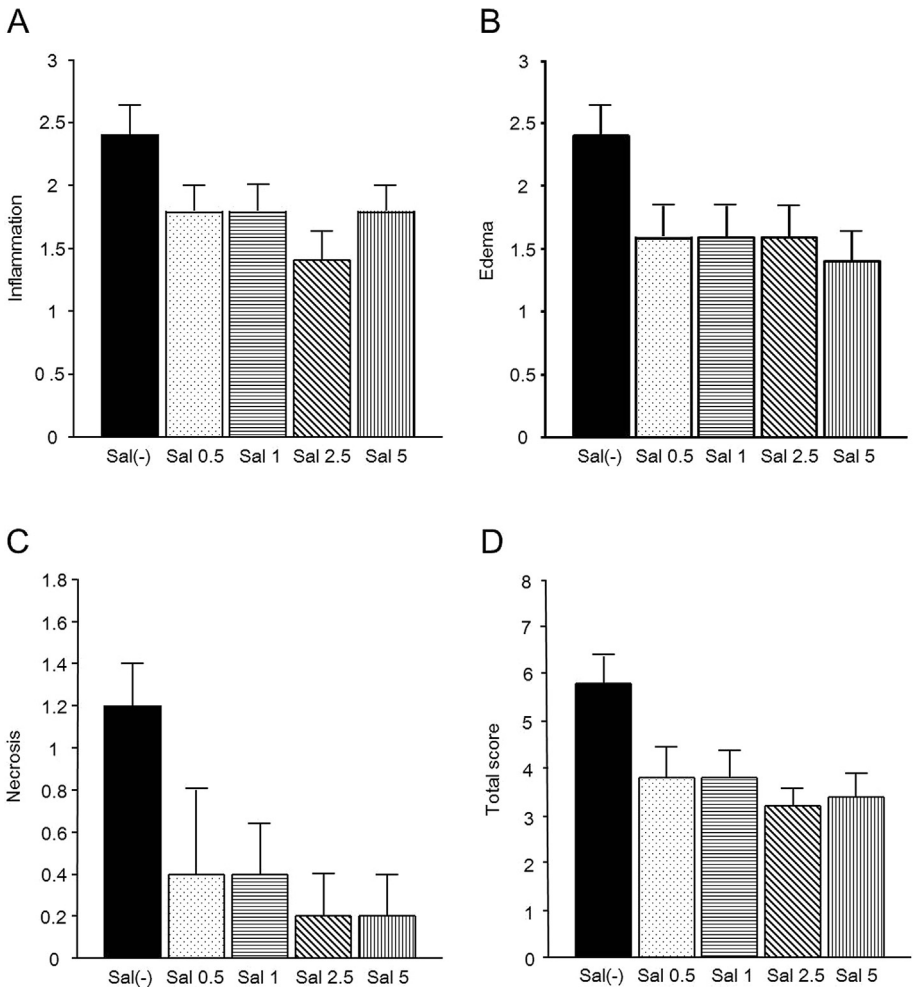

Fig. 3. Histological scoring of the pancreas of the mice treated with salubrinal. The severity of chronic pancreatitis was graded using a semiquantitative system: inflammation (A), edema (B), necrosis (C), and total score (D). All histological scores were lower in the mice treated with salubrinal, although these differences were not significant. The results are expressed as mean ± SE of 10 mice.

with the control mice, there was a significant difference in inflammation ($P < 0.05$), necrosis ($P < 0.05$), and total scores ($P < 0.01$) between the mice treated with 2.5 mg salubrinal and the control mice. Therefore, we proceeded with further experiments using salubrinal at a dose of 2.5 mg/kg.

Serum cytokine levels in the sera of mice with pancreatitis

Serum levels of IL-1β, IL-6, IL-10, TNF-α, and IFN-γ were significantly elevated in the mice treated with cerulein and LPS (**P < 0.01, *P < 0.05; Fig. 4). The administration of 2.5 mg/kg salubrinal significantly decreased the serum levels of IL-1β, IL-6, IL-10, and TNF-α in the treated mice (**P < 0.01, *P < 0.05). There was no significant difference in serum IFN-γ levels between the salubrinal- and vehicle-treated mice.

Expression of ER stress-related proteins in the pancreas of mice with pancreatitis

There was no significant difference in the expression of the eIF2α protein between salubrinal- (2.5 mg/kg) and vehicle-treated mice with cerulein- and LPS-induced pancreatitis. However, the expression of phosphorylated eIF2α protein was elevated in salubrinal-treated mice with pancreatitis compared with that in vehicle-treated mice with pancreatitis (*P < 0.05; Fig. 5A–C). The ratio of phosphorylated eIF2α to total eIF2α was elevated in mice treated with salubrinal (*P < 0.05; Fig. 5D). The chaperone protein GRP78 was downregulated in salubrinal-treated mice with cerulein- and LPS-induced pancreatitis compared with vehicle-treated mice with pancreatitis (*P < 0.05; Fig. 6A). ATF4 protein expression was also lower in mice treated with salubrinal (Fig. 6B), although the decrease was not significant. The expression of CHOP and cleaved caspase-3 protein was significantly lower in salubrinal-treated mice with pancreatitis than in vehicle-treated mice with pancreatitis (*P < 0.05; Fig. 6C and D).

Immunohistochemical analysis of the pancreas of mice with pancreatitis

A considerable number of pancreatic acinar cells positive for TUNEL staining were observed in the mice treated with cerulein and LPS (Fig. 7C). The administration of 2.5 mg/kg salubrinal decreased the number of apoptotic cells (Fig. 7D).

Discussion

In this study, we demonstrated for the first time that augmentation of the PERK signaling pathway via inhibition of the

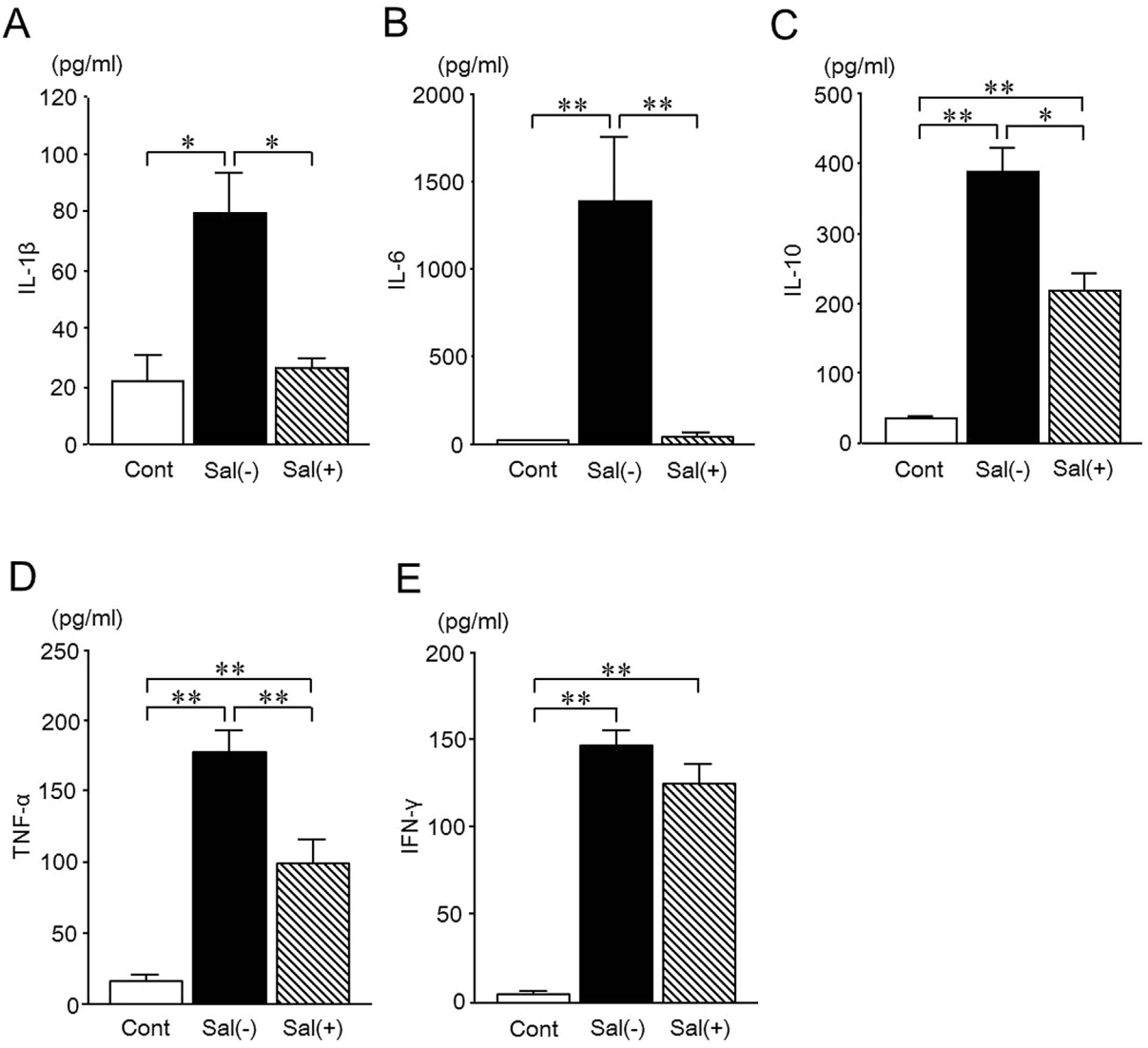

Fig. 4. Serum cytokine levels in the mice treated with salubrinal: IL-1β (A), IL-6 (B), IL-10 (C), TNF-α (D), and IFN-γ (E). Serum levels of IL-1γ, IL-6, IL-10, and TNF-α were significantly decreased in the mice treated with salubrinal (**P < 0.01, *P < 0.05). There was no significant difference in serum IFN-γ levels between the salubrinal- and vehicle-treated mice. The results are expressed as mean ± SE of five mice.

dephosphorylation of eIF2α ameliorated AP induced in mice by administration of cerulein and LPS. The administration of salubrinal, an inhibitor of eIF2α dephosphorylation, led to upregulation of the level of phosphorylated eIF2α protein, which was accompanied by downregulation of GRP78, CHOP, and cleaved caspase-3 in the pancreas compared with that in vehicle-treated mice with pancreatitis.

Polypeptides are folded, with the help of chaperone proteins, in the ER lumen into specific tertiary structures that are essential for their correct function. Because pancreatic acinar cells produce and secrete large quantities of digestive enzymes, they are highly susceptible to ER stress induced by a broad range of factors, including bacterial toxins and proinflammatory cytokines. Enhanced ER stress has been demonstrated in the pancreas of patients with AP and chronic pancreatitis and in animal models of these diseases [12–14,21,22]. When the ER machinery is disrupted by the accumulation of misfolded/unfolded proteins in the ER, the UPR, including the activation of PERK, is initiated. The activation of PERK leads to the phosphorylation of eIF2α and the subsequent suppression of protein translation, thereby reducing the burden of protein entering the ER. PERK-regulated translational control is most important for cells with abundant ER, such as pancreatic β cells, which are crucially involved in insulin secretion. In $PERK^{-/-}$ mice, β cells are preferentially susceptible to ER stress, which results in diabetes [18].

Salubrinal is a selective inhibitor of eIF2α dephosphorylation, which was developed as a protective agent against ER stress-mediated apoptosis [23]. Salubrinal attenuates synthesis of unfolded or misfolded proteins by inhibiting eIF2α dephosphorylation. In neurons, salubrinal can reduce the load of unfolded or misfolded proteins that are retained in the ER under conditions associated with neurodegeneration [24,25]. In rat kidneys, salubrinal has been shown to reduce cyclosporine-induced nephrotoxicity, which can induce ER stress [26]. In the present study, the administration of salubrinal increased the level of phosphorylated eIF2α in the pancreas of mice treated with cerulein and LPS compared with that in mice treated with vehicle. The ratio of phosphorylated eIF2α to total eIF2α was significantly higher in salubrinal-treated mice than it was in vehicle-treated mice, confirming the inhibitory effect of salubrinal on eIF2α

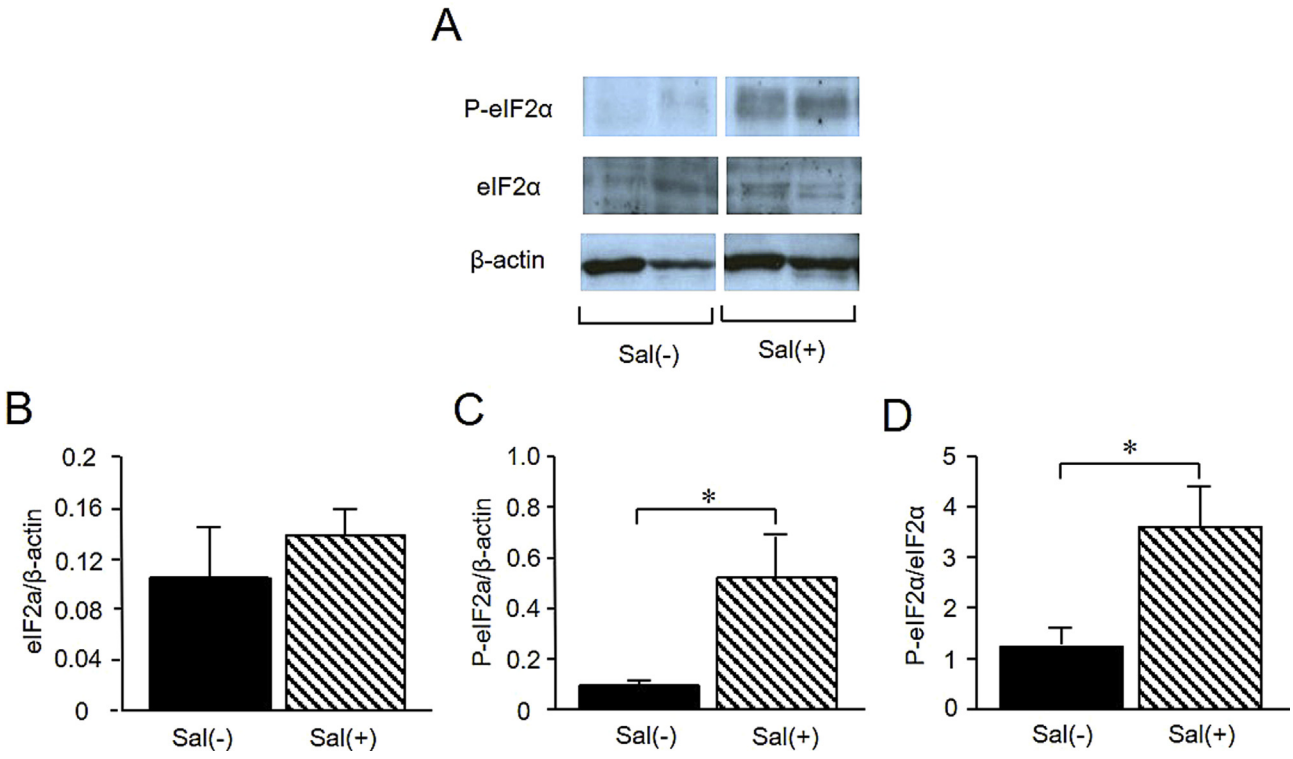

Fig. 5. Western blot analysis of eIF2α protein in the pancreas of the mice treated with salubrinal. Representative Western blot and semiquantitative analysis of eIF2α and phosphorylated eIF2α (P-eIF2α) proteins in the mice with cerulein- and lipopolysaccharide-induced pancreatitis. There was no significant difference in the expression of the eIF2α protein between salubrinal- and vehicle-treated mice with cerulein- and LPS-induced pancreatitis. The levels of P-eIF2α were significantly elevated in salubrinal-treated mice compared with vehicle-treated mice (*P < 0.05). The ratio of P-eIF2α to total eIF2α was also significantly higher in mice treated with salubrinal than in mice treated with vehicle (*P < 0.05). The results are expressed as mean ± SE of five mice.

dephosphorylation. Cerulein- and LPS-induced pancreatitis was attenuated in mice treated with salubrinal, an effect that was presumably mediated via sustained eIF2α phosphorylation reducing the protein load entering the ER, thereby protecting pancreatic acinar cells from ER stress. Although the main target of salubrinal is thought to be pancreatic acinar cells, effect of salubrinal on immune cells can not completely excluded because we analyzed the inflamed pancreas of mice, which contained inflammatory cells.

In mice treated with salubrinal, acute pancreatitis was ameliorated in association with the decrease in proinflammatory cytokines such as IL6 and TNF-α. However, there was no significant difference in serum IFN-γ levels between the salubrinal- and vehicle-treated mice. IFN-γ is produced by NK cell and T cells, but not by pancreatic acinar cells. On the other hand, IL-1β, IL6, IL-10, and TNF-α are produced not only by immune cells, but also by pancreatic acinar cell [27,28]. As salubrinal decreased ER stress in pancreatic acinar cells, we suppose that decreased ER stress may lead to suppression of proinflammatory cytokine release by pancreatic acinar cells.

In general, chaperone proteins disengage from proteins once they achieve the correct conformation. Therefore, they accumulate in association with misfolded proteins, and chaperone production is upregulated in response to increased misfolding. The detection of increased levels of chaperones, particularly GRP78, is commonly used as a marker of ER stress [29]. In this study, the expression of GRP78 protein was upregulated in the pancreas of mice with cerulein- and LPS-induced pancreatitis, as reported previously [30]. Interestingly, although salubrinal alone did not increase or decrease expression of GRP78 in *in vitro* experiments [23], the level of GRP78 expression was significantly lower in salubrinal-treated mice than it was in vehicle-treated mice. This reduced expression of GRP78 seems to reflect the attenuation of ER stress induced by inhibition of dephosphorylation of eIF2α in the pancreas. Indeed, the proportion of phosphorylated eIF2α protein was upregulated in salubrinal-treated mice compared with that in vehicle-treated mice.

Phosphorylated eIF2α initiates the translational control of preexisting mRNAs, and is the method by which most mRNAs are translationally repressed. However, phosphorylated eIF2α paradoxically augments the translation of ATF4, which is a transcriptional activator that regulates a wide range of genes and plays a crucial role in cell adaptation to stress conditions [31]. During long-term ER stress, however, ATF4 may also stimulate the expression of CHOP, which is responsible for initiation of the apoptotic cascade [10]. Thus, ATF4 mediates production of the transcriptional factor CHOP.

CHOP is a cellular stress sensor that can be induced in response to a series of physiological or stress conditions such as ER stress, nutrient deprivation, DNA damage, cellular growth arrest, and hypoxia. In the present study, we used a model of AP induced by administration of cerulein and LPS, in which the CHOP-mediated pathway plays a major role in induction of pancreatic inflammation [32]. The administration of salubrinal suppressed the expression of CHOP and led to the attenuation of pancreatitis despite the upregulation of phosphorylated eIF2α. This may be the result of lower expression of ATF4, although there was no significant difference in the expression of the ATF4 protein between salubrinal- and vehicle-treated mice. However, because the three signaling branches of UPR all lead to CHOP transcription, the suppression of CHOP expression may have been caused by the suppression of other UPR mediators such as IRE1 and ATF6.

The shutdown of translation induced by transient pancreatic

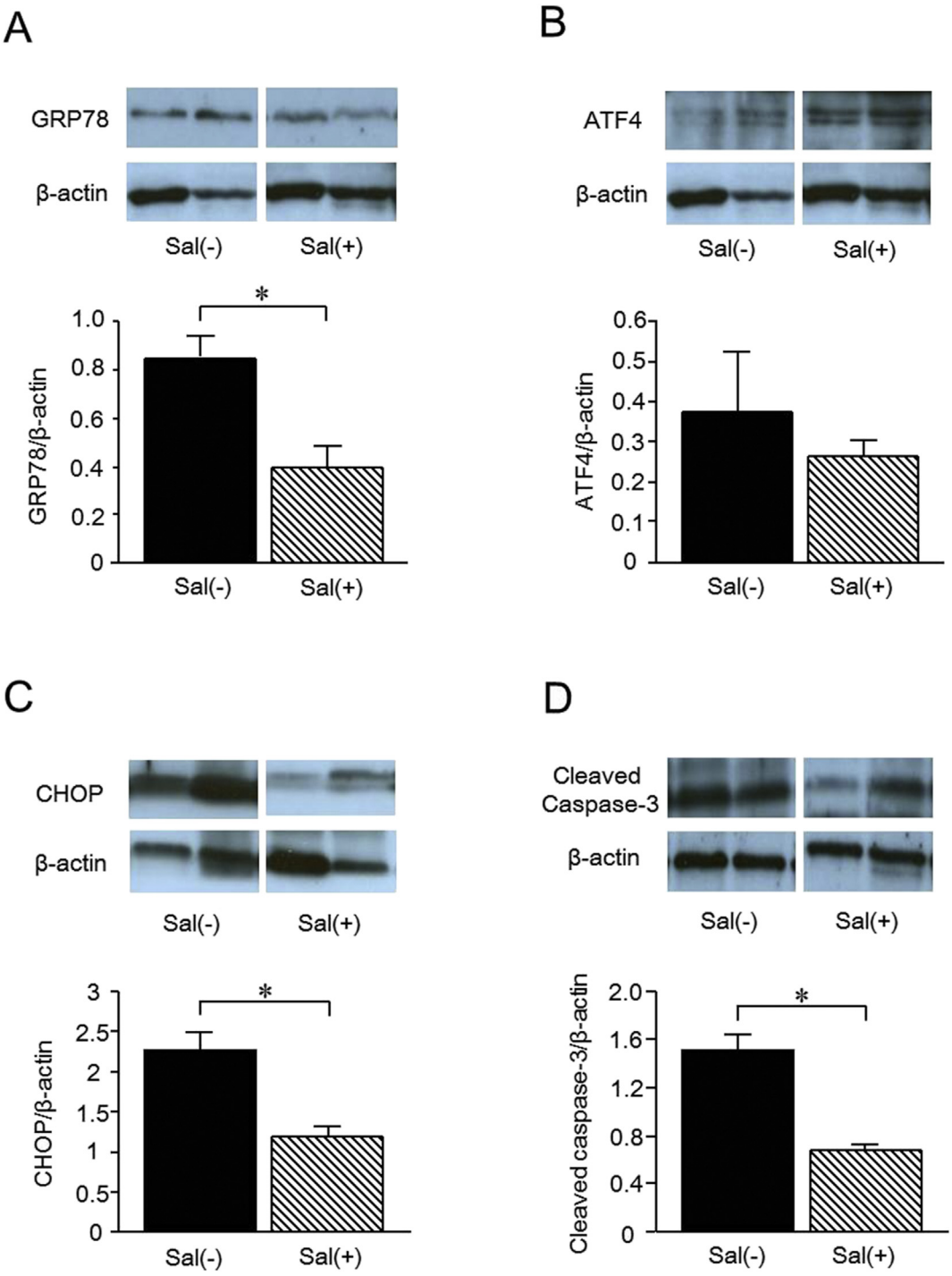

Fig. 6. Western blot analysis of ER stress-related proteins GRP78 (A), ATF4 (B) CHOP (C), and cleaved caspase-3 (D) in pancreas of mice treated with salubrinal. The protein levels of GRP78, CHOP, and cleaved caspase-3 were significantly lower in mice treated with salubrinal than in mice treated with vehicle (*$P < 0.05$). The protein levels of ATF4 tended to be lower in mice treated with salubrinal than in mice treated with vehicle. The results are expressed as mean ± SE of five mice.

acinar cell damage may play different roles in the pathological processes of pancreatitis. During the acute phase of pancreatitis, the suppression of protein synthesis can be viewed as a cytoprotective response triggered by cerulein and LPS administration. However, pancreatic acinar cells in which ER functioning is impaired can only cope with the most severe form of stress when their ability for protein synthesis is sufficiently restored to permit new protein synthesis. The beneficial level and duration of activation of eIF2α phosphorylation are unknown, and the long-term effects of sustained inhibition of eIF2α dephosphorylation must be evaluated.

In conclusion, we demonstrated that modulation of the PERK-signaling pathway by suppressing the dephosphorylation of eIF2α attenuated cerulein- and LPS-induced pancreatitis. Our results suggest that the augmentation of the PERK-signaling pathway is a potential therapeutic target for the treatment of AP.

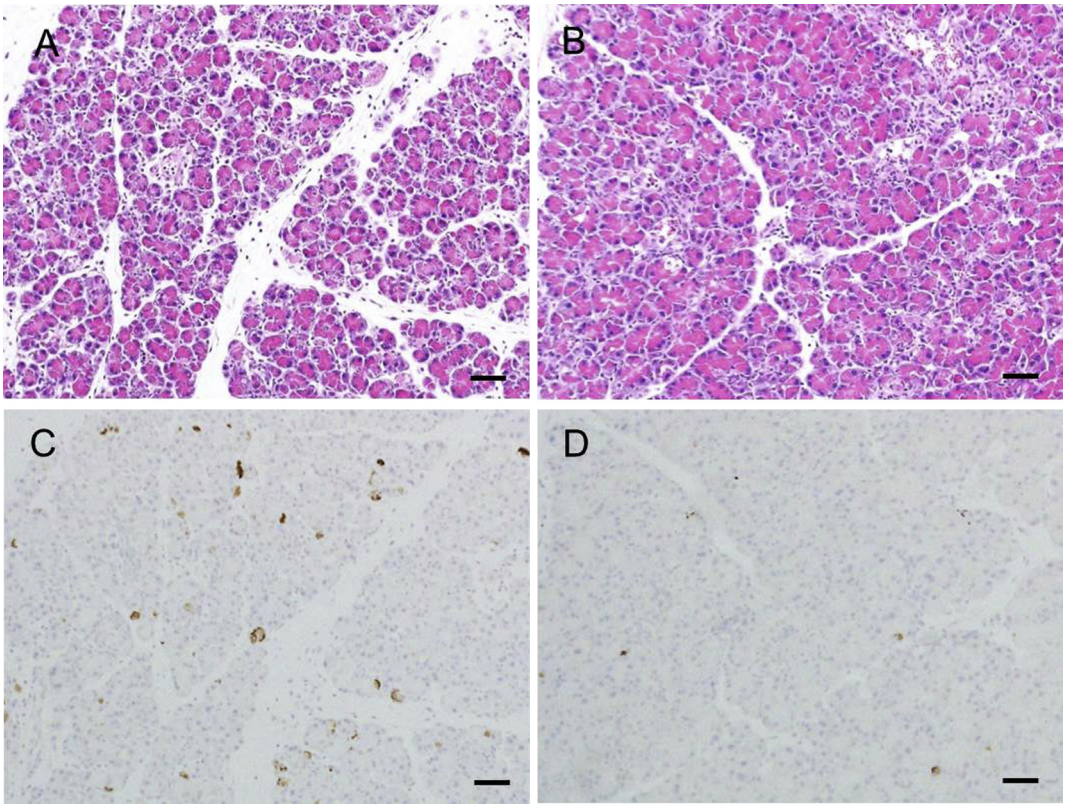

Fig. 7. Immunohistochemical staining of the pancreas of mice treated with salubrinal. Representative pancreatic sections stained with hematoxylin and eosin (A, B) and by the TUNEL method (C, D) are shown. A considerable number of pancreatic acinar cells positive for TUNEL staining were observed in the mice treated with cerulein and lipopolysaccharide (C). The administration of 2.5 mg/kg salubrinal decreased the number of apoptotic cells (D). Bars indicate 100 μm.

Acknowledgements

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