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ORIGINAL ARTICLE

C/EBP homologous protein deficiency aggravates acute pancreatitis and associated lung injury

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Abstract

AIM: To investigate the pathophysiological role of C/EBP homologous protein (CHOP) in severe acute pancreatitis and associated lung injury.

METHODS: A severe acute pancreatitis model was induced with 6 injections of cerulein (Cn, 50 μg/kg) at 1-h intervals, then intraperitoneal injection of lipopolysaccharide (LPS, 7.5 mg/kg) in CHOP-deficient (*Chop*$^{-/-}$) mice and wild-type (WT) mice. Animals were sacrificed under anesthesia, 3 h or 18 h after LPS injection. Serum amylase, lipase, and cytokines [interleukin (IL)-6 and tumor necrosis factor (TNF)-α], pathological changes, acute lung injury, and apoptosis in the pancreas were evaluated. Serum amylase and lipase activities were detected using a medical automatic chemical analyzer. Enzyme-linked immunosorbent assay kits were used to evaluate TNF-α and IL-6 levels in mouse serum and lung tissue homogenates. Apoptotic cells in sections of pancreatic tissues were determined by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) analysis. The mouse carotid arteries were cannulated and arterial blood samples were collected for PaO_2 analysis. The oxygenation index was expressed as PaO_2/FiO_2.

RESULTS: Administration of Cn and LPS for 9 and 24 h induced severe acute pancreatitis in *Chop*$^{-/-}$ and WT mice. When comparing *Chop*$^{-/-}$ mice and WT mice, we observed that CHOP-deficient mice had greater increases in serum TNF-α (214.40 ± 19.52 pg/mL *vs* 150.40 ± 16.70 pg/mL; $P = 0.037$), amylase (4236.40 ± 646.32 U/L *vs* 2535.30 ± 81.83 U/L; $P = 0.041$), lipase (1678.20 ± 170.57 U/L *vs* 1046.21 ± 35.37 U/L; $P = 0.008$), and IL-6 (2054.44 ± 293.81 pg/mL *vs* 1316.10 ± 108.74 pg/mL; $P = 0.046$) than WT mice. The histopathological changes in the pancreases and lungs, decreased PaO_2/FiO_2 ratio, and increased TNF-α and IL-6 levels in the lungs were greater in *Chop*$^{-/-}$ mice than in WT mice (pancreas: *Chop*$^{-/-}$ *vs* WT mice, hemorrhage, $P = 0.005$; edema, $P = 0.005$; inflammatory cells infiltration, $P = 0.005$; total scores, $P = 0.006$; lung: hemorrhage, $P = 0.017$; edema, $P = 0.017$; congestion, $P = 0.017$; neutrophil infiltration, $P = 0.005$, total scores, $P = 0.001$; PaO_2/FiO_2 ratio: 393 ± 17.65 *vs* 453.8, $P = 0.041$; TNF-α: $P = 0.043$; IL-6, $P = 0.040$). Results from TUNEL analysis indicated increased acinar cell apoptosis in

mice following the induction of acute pancreatitis. However, *Chop*$^{-/-}$ mice displayed significantly reduced pancreatic apoptosis compared with the WT mice (201.50 ± 31.43 *vs* 367.00 ± 47.88, P = 0.016).

CONCLUSION: These results suggest that CHOP can exert protective effects against acute pancreatitis and limit the spread of inflammatory damage to the lungs.

Key words: C/EBP homologous protein; Acute pancreatitis, Lung injury; Cytokines; Apoptosis

Core tip: We found that mice lacking C/EBP homologous protein (CHOP) had aggravated acute pancreatitis-induced increases in the severity of pancreatic pathology, pancreatitis-associated lung injury, and cytokines interleukin-6 and tumor necrosis factor-α levels compared with wild-type (WT) mice. Pancreatic apoptosis was also lower in *Chop*$^{-/-}$ mice than in WT mice during acute pancreatitis. These results suggest that CHOP exerts protective effects against acute pancreatitis and limits the spread of inflammatory damage to the lungs.

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INTRODUCTION

Acute pancreatitis is an acute inflammatory process of sudden onset, and occurs in the peripheral and internal areas of the pancreas. At an early stage of the disease, acute pancreatitis induces multiple organ system dysfunction syndromes in the lung, kidney, liver, and other organs. These are major contributory factors to the high mortality rate of severe acute pancreatitis[1,2]. Acute pancreatitis can spread systemically through systemic inflammatory responses[3]. Acute lung injury occurs frequently in acute pancreatitis and is a major component of acute pancreatitis-associated multiple organ dysfunction[4]. The overproduction of several cytokines and non-cytokine inflammatory mediators may account for the systemic manifestations of acute pancreatitis[5-7].

Acinar cell damage initiates acute pancreatitis, resulting in local activation of the immune system and local inflammation of the pancreas[5]. The local pro-inflammatory response to acinar cell damage is counteracted by an anti-inflammatory response; however, uncontrolled local inflammation can lead to generalized inflammation and systemic inflammatory response syndrome[8]. Pancreatic acinar cell damage during acute pancreatitis generally occurs through a combination of apoptosis and necrosis[9]. Recent studies have shown that mild acute pancreatitis is associated with extensive apoptotic acinar cell death, whereas severe acute pancreatitis involves acinar cell necrosis with minimal apoptosis[10,11]. Acinar necrosis-induced inflammation can progress systemically, causing multiple organ failure and death. Studies have proposed that acinar apoptosis might protect the pancreas from local and systemic cytokine release[10,11].

C/EBP homologous protein (CHOP), also known as G1 arrest and DNA damage 153 (Gadd153), is the 19.2-kDa protein product of DNA damage-induced transcript 3 (Ddit3) and a key regulator of stress responses. CHOP is known to play an important role in the induction of apoptosis. Overexpression of CHOP promotes apoptosis in several cell lines, whereas CHOP-deficient (*Chop*$^{-/-}$) cells are resistant to endoplasmic reticulum (ER) stress-induced apoptosis[12]. Experiments using *Chop*$^{-/-}$ mice revealed that CHOP-mediated apoptosis contributes to the pathogenesis of a number of ER stress-related diseases[13]. ER stress is the most potent inducer of CHOP expression and CHOP is known as a pro-apoptotic factor[14,15]. It is also involved in several physiological adaptive processes[15,16], including mitochondrial[17] and oxidative stress[18], amino acid starvation[19], and differentiation of keratinocytes[20] and osteoblasts[21]. Studies have reported CHOP upregulation in a murine model of acute pancreatitis[8,22]; however, the mechanism(s) and functional consequences of acute pancreatitis-induced CHOP expression are not well-understood. We hypothesized that CHOP plays an important role in acute pancreatitis and influences acute pancreatitis-induced systemic inflammation and acute lung injury. In this study, we used *Chop*$^{-/-}$ mice to investigate the pathogenesis of acute pancreatitis.

MATERIALS AND METHODS

Animal experiments

All animal care and experimental procedures were approved by the Animal Care Committee of the College of Medicine, National Taiwan University. Mice deficient in CHOP (*Chop*$^{-/-}$) on a C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME, United States). Adult male *Chop*$^{-/-}$ mice and wild-type (WT) C57BL/6 mice weighing 18-25 g were used in this study. Mice were housed at a constant temperature of 20 ℃-22 ℃ with a 12 h:12 h light-dark cycle.

A mouse model of severe acute pancreatitis induced by cerulein (Cn) and lipopolysaccharide (LPS) has been well-established[20,21]. The *Chop*$^{-/-}$ and WT mice were injected with 6 doses of Cn (50 μg/kg; Sigma, St Louis, MI, United States) at 1-h intervals, and then intraperitoneal injection with LPS (7.5 mg/kg; derived from *Escherichia coli* 0111:B4, Sigma), to induce severe acute pancreatitis. Animals were sacrificed under anesthesia (tribromoethanol, 250 mg/kg, dissolved in 2-methyl-2-butanol) by intraperitoneal injection at 3 h or 18 h after LPS injection, and their pancreases and lungs were dis-

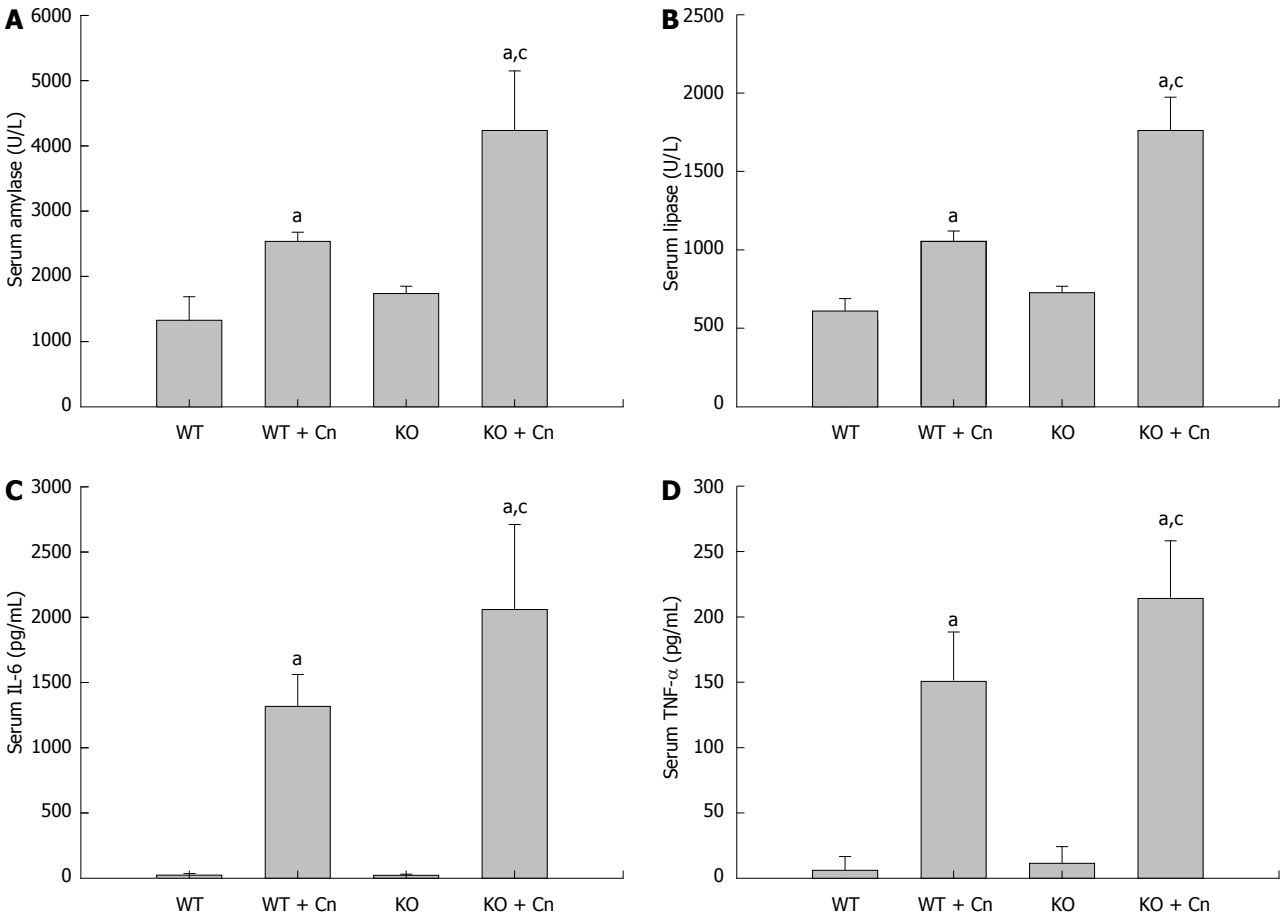

Figure 1 Mice deficient in C/EBP homologous protein displayed increased serum amylase, lipase, interleukin-6, and tumor necrosis factor-α. Acute pancreatitis was induced using cerulein (Cn) and lipopolysaccharide (LPS) in Chop$^{-/-}$ (KO) and wild-type (WT) mice. Serum levels of amylase (A), lipase (B), interleukin (IL)-6 (C), and tumor necrosis factor (TNF)-α (D), were detected 24 h (A-C) and 9 h (D) after induction of acute pancreatitis. Data are presented as mean ± SEM (n = 6). The data are normally distributed. $^{a}P < 0.05$ compared with WT mice without pancreatitis; $^{c}P < 0.05$ *vs* WT mice with pancreatitis.

sected immediately[23,24]. Blood samples were collected for amylase, lipase, and cytokine assays. After rinsing with saline and blotting on paper, segments of the tissues were fixed and embedded in paraffin wax for histological analysis. Other tissue parts were fully homogenized. The lung tissue homogenates were stored in liquid nitrogen before use to evaluate tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels.

Histological examination

To evaluate the morphological severity of acute pancreatitis, the pancreas was fixed in 10% formaldehyde for 24 h, embedded in paraffin, and stained with hematoxylin and eosin. A pathologist who was blinded to the treatment protocol scored the tissues for edema, inflammatory infiltration, and hemorrhage in 10 fields, each on a scale of 0-3. Grades of edema were 0, absent or rare; 1, edema in the interlobular space; 2, edema in the intralobular space; 3, isolated island shape of pancreatic acinus. Inflammation was graded as 0, absent; 1, mild; 2, moderate; 3, severe. Parenchymal hemorrhage was graded as 0, absent; 1, mild; 2, moderate; 3, severe. To evaluate the morphological severity of acute pancreatitis-associated lung injury, lung tissue was rapidly removed and immersed in 10% formalin. Two areas of the lung, one central and one peripheral, were embedded in paraffin. Histological sections were stained with hematoxylin and eosin. Pulmonary alterations were scored by an experienced pathologist in a blind manner. Polymorphonuclear cellularity, pulmonary edema, congestion, necrosis, and hemorrhage were graded, each on a scale of 0-3.

Measurement of PaO_2/FiO_2 ratio

Twenty-four hours after LPS injection, mice were anesthetized with tribromoethanol (250 mg/kg) dissolved in 2-methyl-2-butanol by intraperitoneal injection. The mouse carotid arteries were cannulated and arterial blood samples were collected for PaO_2 analysis. The oxygenation index was expressed as PaO_2/FiO_2.

Analysis of cell apoptosis

Apoptotic cells in sections of pancreatic tissues were determined using a TdT-Frag ELTM DNA fragmentation detection kit (Oncogene Research Products, Boston, MA, United States) according to the manufacturer's instruction. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) analysis was conducted to detect cells containing labeled DNA frag-

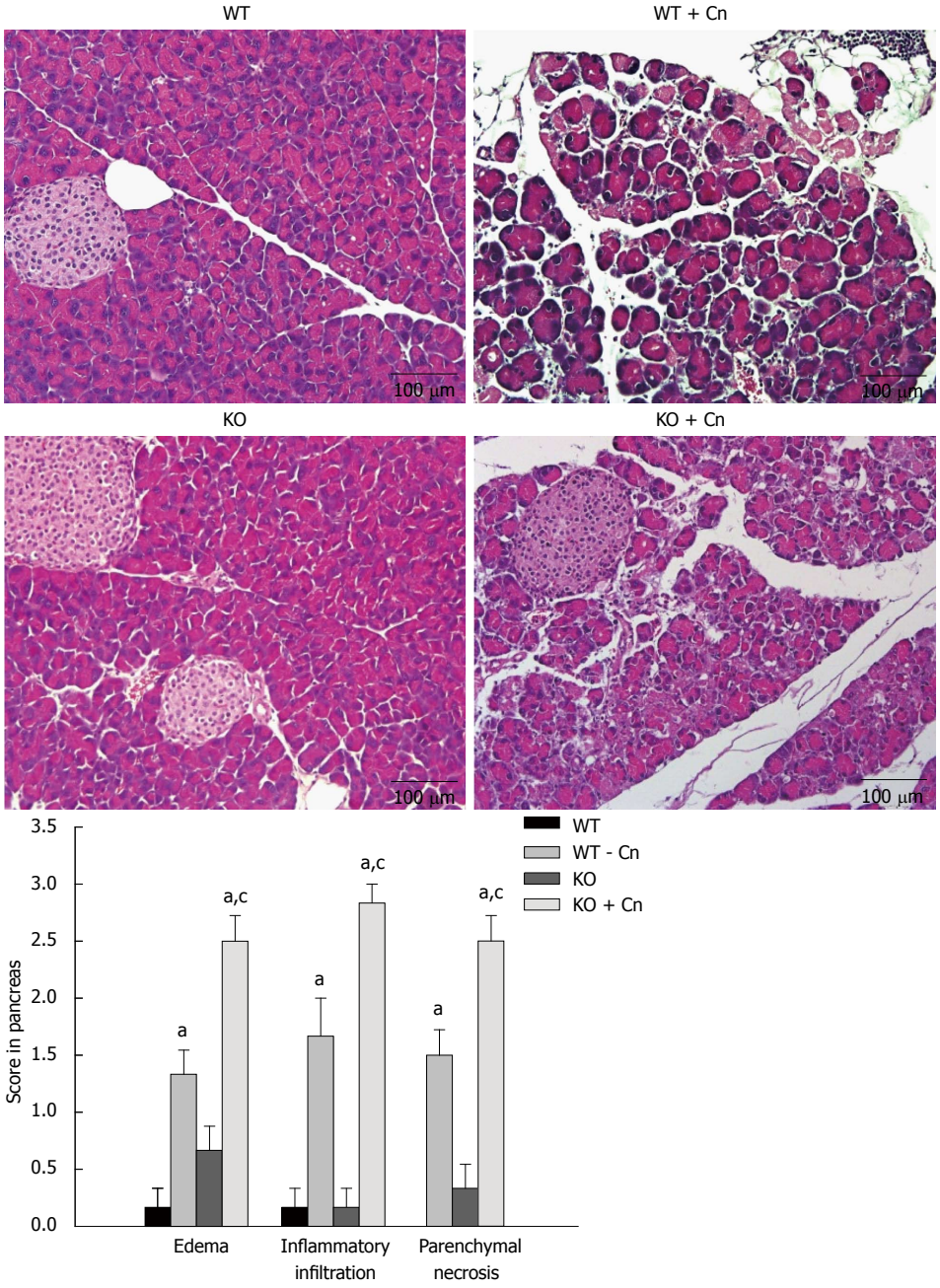

Figure 2 Experimental acute pancreatitis in *Chop*$^{-/-}$ mice. Acute pancreatitis was induced using cerulein (Cn) and lipopolysaccharide (LPS) in *Chop*$^{-/-}$ (KO) and wild-type (WT) mice. Histological examination was performed 24 h after induction of acute pancreatitis. Representative histological changes in pancreatic sections stained with hematoxylin and eosin are shown. Scale bar = 100 μm. Pathological changes in the pancreas were scored. Data are presented as mean ± SEM (n = 4). The data are normally distributed. $^{a}P < 0.05$ *vs* WT mice without pancreatitis; $^{c}P < 0.050$ *vs* WT mice with pancreatitis.

ments. These were revealed as green staining in cell nuclei, indicating the internucleosomal cleavage of DNA.

Measurements of serum amylase, lipase, and cytokines

Serum amylase and lipase activities were detected using a medical automatic chemical analyzer. Enzyme-linked immunosorbent assay kits were used to evaluate the levels of TNF-α (R and D Systems) and IL-6 (Assaypro) in mouse serum and lung tissue homogenates following the induction of acute pancreatitis.

Statistical analysis

Data are expressed as mean ± SEM. Statistical comparisons between experimental groups were performed using one-way analysis of variance test followed by the two-tailed Student t test. A P value < 0.05 was considered significant.

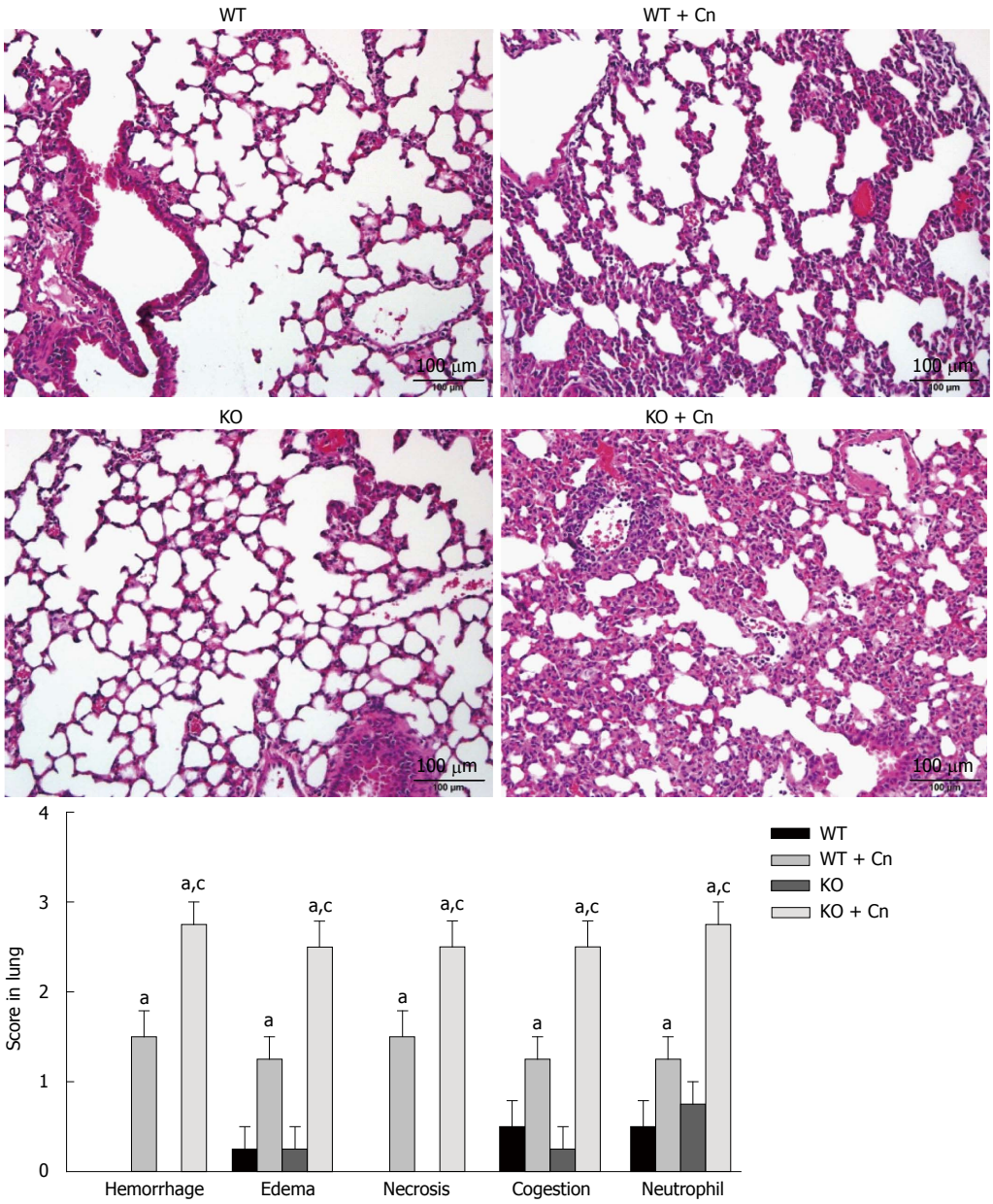

Figure 3 Experimental acute pancreatitis-associated lung injury in *Chop*$^{-/-}$ mice. Acute pancreatitis was induced using cerulein (Cn) and lipopolysaccharide in *Chop*$^{-/-}$ (KO) and wild-type (WT) mice. Histological examination was performed 24 h after induction of acute pancreatitis. Representative histological changes in lung sections stained with hematoxylin and eosin are shown. Scale bar = 100 μm. Pathological changes in lungs were scored. Data are presented as mean ± SEM (n = 4). The data are normally distributed. $^{a}P < 0.050$ *vs* WT mice without pancreatitis; $^{c}P < 0.05$ *vs* WT mice with pancreatitis.

RESULTS

Mice deficient in CHOP displayed acute pancreatitis-induced increases in serum amylase, lipase, IL-6, and TNF-α

Administration of Cn and LPS for 9 and 24 h induced severe acute pancreatitis in *Chop*$^{-/-}$ and WT mice. Following induction of acute pancreatitis, mice displayed increased serum amylase and lipase activities and TNF-α and IL-6 levels (Figure 1). When comparing *Chop*$^{-/-}$ mice and WT mice, we observed that CHOP-deficient mice demonstrated significantly greater increases in serum TNF-α (214.40 ± 19.52 pg/mL *vs* 150.40 ± 16.70 pg/mL; P = 0.037), amylase (4236.40 ± 646.32 Units/L *vs* 2535.30 ± 81.83 Units/L; P = 0.041), lipase (1678.20 ± 170.57 Units/L *vs* 1046.21 ± 35.37 Units/L; P = 0.008), and IL-6 (2054.44 ± 293.81 pg/mL *vs* 1316.10 ± 108.74 pg/mL; P = 0.046) than WT mice (Figure 1).

Mice deficient in CHOP displayed increased acute pancreatitis-induced changes in lung histopathology and TNF-α and IL-6 levels

After administration of Cn and LPS for 24 h, mice showed features of typical acute pancreatitis in the pancreas, including the expansion of interlobular and intralobular spaces by moderate to severe interstitial edema, extensive infiltration of inflammatory cells, and pancreatic hemorrhage (Figure 2). They also displayed

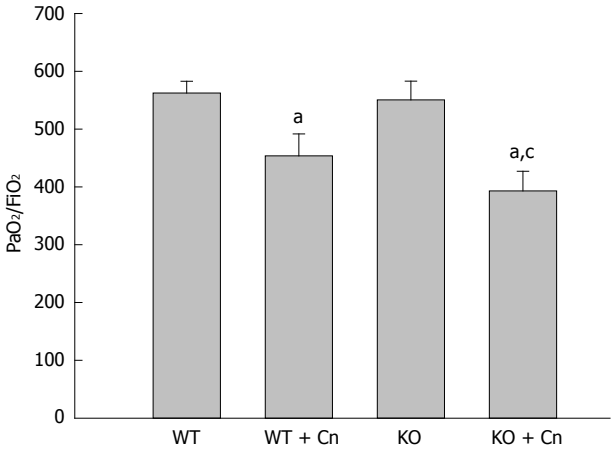

Figure 4 The PaO_2/FiO_2 ratio in *Chop*$^{-/-}$ mice with acute pancreatitis. Acute pancreatitis was induced by using cerulein (Cn) and lipopolysaccharide in *Chop*$^{-/-}$ (KO) mice and wild-type (WT) mice. The PaO_2/FiO_2 ratio was detected 24 h after induction of acute pancreatitis in mice. Data are presented as mean ± SEM (n = 6). The data are normally distributed. $^{a}P < 0.05$ *vs* wild type mice without pancreatitis;. $^{c}P < 0.05$ *vs* wild type mice with pancreatitis.

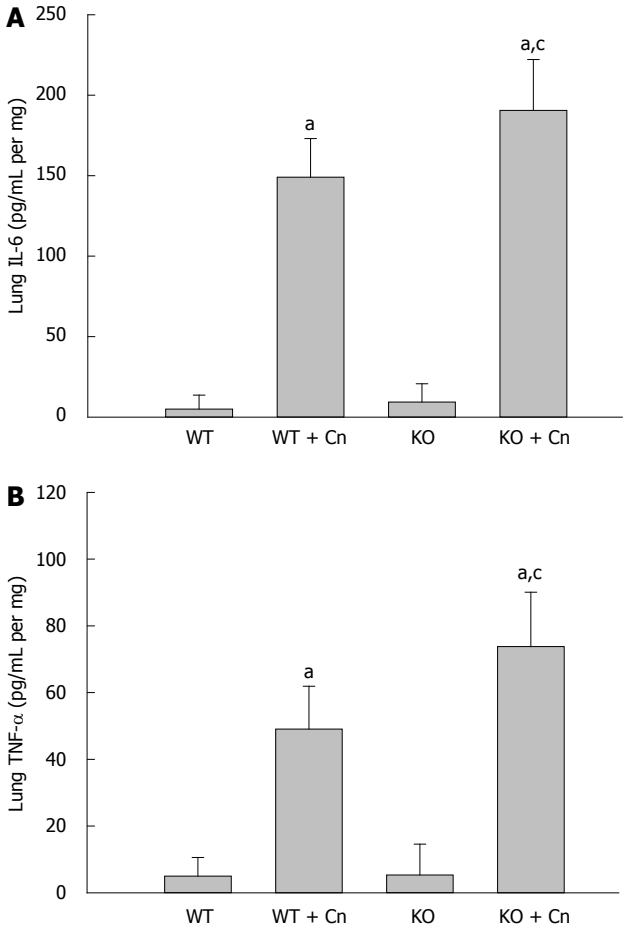

Figure 5 Mice deficient in C/EBP homologous protein displayed increased interleukin-6, and tumor necrosis factor-α in the lungs. Levels of tumor necrosis factor (TNF)-α (A), and interleukin (IL)-6 (B) were detected 9 h (A) and 24 h (B) after induction of acute pancreatitis in *Chop*$^{-/-}$ and wild-type (WT) mice. Data are presented as mean ± SEM (n = 6). The data are normally distributed. $^{a}P < 0.050$ *vs* WT mice without pancreatitis; $^{c}P < 0.05$ *vs* WT mice with pancreatitis.

pulmonary changes such as marked pulmonary edema, inflammatory infiltration, and alveolar collapse (Figure 3), and decreased PaO_2/FiO_2 ratio (an oxygenation index) (Figure 4). The levels of TNF-α and IL-6 in the lungs were markedly increased in mice with acute pancreatitis (Figure 5). Histopathological changes in the pancreases and lungs, decreased PaO_2/FiO_2 ratio, and the increases of levels of TNF-α and IL-6 in the lungs were greater in *Chop*$^{-/-}$ mice than in WT mice (pancreas: *Chop*$^{-/-}$ *vs* WT mice, hemorrhage, $P = 0.005$, edema, $P = 0.005$, inflammatory cells infiltration, $P = 0.005$, total scores, $P = 0.006$; lung: hemorrhage, $P = 0.017$, edema, $P = 0.017$, congestion, $P = 0.017$, neutrophils infiltration, $P = 0.005$, total scores, $P = 0.001$; PaO_2/FiO_2 ratio: 393 ± 17.65 *vs* 453.8, $P = 0.041$; TNF-α: $P = 0.043$; IL-6, $P = 0.040$).

Mice deficient in CHOP showed reduced acute pancreatic-induced apoptosis in the pancreas

Results from TUNEL analysis indicated increased acinar cell apoptosis (Figure 6) in mice following the induction of acute pancreatitis. However, *Chop*$^{-/-}$ mice displayed significantly reduced pancreatic apoptosis *vs* the WT mice (201.50 ± 31.43 *vs* 367.00 ± 47.88, $P = 0.016$; Figure 6).

DISCUSSION

In a murine model, acute pancreatitis and its associated lung injury induced by Cn/LPS injection are compatible with the clinical manifestations of severe acute pancreatitis with lung damage[25,26]. In our previous study, we demonstrated the upregulation of pancreatic CHOP expression following Cn/LPS-induced acute pancreatitis. In the present study, we evaluated the pathophysiological role of CHOP in acute pancreatitis and its associated acute lung injury. Mice deficient in CHOP displayed increases in the severity of pancreatic pathology, increased activities of serum amylase and lipase, increased levels of pancreatitis-induced systemic inflammatory cytokines such as TNF-α and IL-6, and increased acute lung injury compared with WT mice. Moreover, mice with Cn-induced acute pancreatitis displayed low levels of apoptosis and high levels of necrosis[27]. It is known that severe acute pancreatitis is primarily associated with necrosis and, to a lesser extent, with apoptosis in acinar cells. Mild acute pancreatitis, however, is primarily associated with apoptotic acinar cell death[10]. In this study, we observed higher numbers of TUNEL-positive pancreatic cells in WT mice than in *Chop*$^{-/-}$ mice. These results suggest that CHOP mediates the increases in pancreatic cell apoptosis induced by Cn and LPS.

CHOP has been found to be upregulated during ER stress, which is typically associated with cellular death and associated organ dysfunction[28]. Recent evidence has shown that CHOP exerts diverse functional effects in addition to regulation of apoptosis. CHOP contributes to inflammation, the generation of reactive oxygen species, and altered cellular interaction within the extracellular matrix[29]. Lozon *et al*[30] found that *Chop*$^{-/-}$ mice displayed

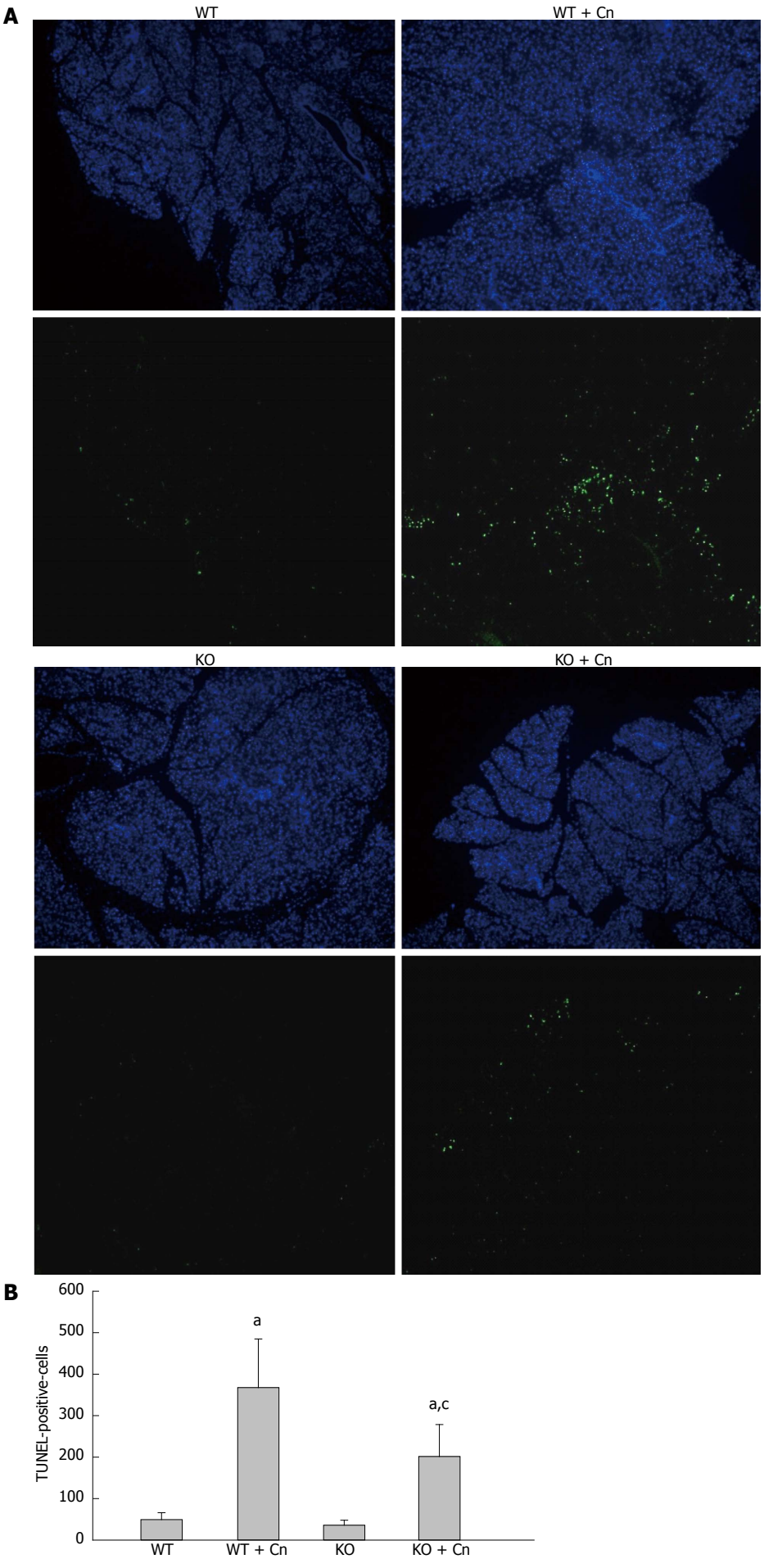

Figure 6 Mice deficient in C/EBP homologous protein displayed decreased apoptosis in the pancreas after induction of acute pancreatitis. Acute pancreatitis was induced using cerulein (Cn) and lipopolysaccharide in *Chop*$^{-/-}$ (KO) and wild-type (WT) mice. We performed transferase-mediated dUTP-biotin nick-end labeling (TUNEL) analysis 9 h after induction of acute pancreatitis. Results indicated the presence of TUNEL-positive cells (A, B) in the pancreas. Data are presented as mean ± SEM (n = 6). The data are normally distributed. $^{a}P < 0.050$ *vs* WT mice without pancreatitis; $^{c}P = 0.016$ *vs* WT mice with pancreatitis.

increased lung edema and permeability, indicating that CHOP plays a protective role after prolonged hyperoxia. Here we observed that CHOP exerts protective effects in a murine model of Cn/LPS-induced acute pancreatitis. This finding is in contrast to those of Suyama *et al*[31], who reported the role of the ER stress-CHOP pathway in the acceleration of pancreatitis. We applied a different protocol from the study by Suyama *et al*[31] to induce severe acute pancreatitis, observing that experimental acute pancreatitis and acute pancreatitis-associated lung injury induced by 6 episodes of Cn injection and one subsequent LPS injection are compatible with the clinical manifestations of severe acute pancreatitis with lung damage[25,32-34]. In the study of Suyama *et al*[31], 12 doses of Cn and 3 doses of LPS were used to induce acute pancreatitis; they did not, however, evaluate the systemic inflammatory cytokines and the severity of lung injury. Acute lung injury is a major component of acute pancreatitis-associated morbidity and mortality[34]. Moreover, Kubisch and Logsdon have examined the effects of secretagogues on the function of acinar cells and the unfolded protein response components including CHOP[35]. They found that the cholecystokinin analog CCK8 significantly increased amylase secretion and mRNA expression of CHOP; however, the pathophysiological role of CHOP was still unclear. In our study, we observed that pancreatic and systemic inflammatory responses and acute lung injury during Cn/LPS-induced acute pancreatitis were greater in CHOP-deficient mice than in their WT counterparts. Taken together, these findings suggest that endogenous CHOP may play a systemic anti-inflammatory role and reduce the severity of acute lung injury during acute pancreatitis.

In conclusion, the present findings demonstrate for the first time that CHOP plays a significant role in the induction of apoptosis and prevention of systemic inflammation and acute lung injury during severe acute pancreatitis.

COMMENTS

Background

Severe acute pancreatitis is a life-threatening disease. Studies have reported C/EBP homologous protein (CHOP) upregulation in a murine model of acute pancreatitis. However, the mechanism(s) and functional consequences of acute pancreatitis-induced CHOP expression are not well-understood.

Research frontiers

Overexpression of CHOP promotes apoptosis in several cell lines. The authors hypothesized that CHOP plays an important role in acute pancreatitis and influences acute pancreatitis-induced systemic inflammation and acute lung injury.

Innovations and breakthroughs

The authors found for the first time that mice lacking CHOP have aggravated acute pancreatitis-induced increases in the severity of pancreatic pathology, pancreatitis-associated lung injury, and cytokine interleukin-6 and tumor necrosis factor-α levels compared with wild-type mice. Pancreatic apoptosis was also lower in *Chop*$^{-/-}$ mice than in wild-type mice during acute pancreatitis.

Applications

CHOP exerts protective effects against acute pancreatitis and limits the spread of inflammatory damage to the lungs.

Terminology

CHOP, C/EBP homologous protein, also known as G1 arrest and DNA damage 153 (Gadd153), is the 19.2-kDa protein product of DNA damage-induced transcript 3 (Ddit3) and a key regulator of stress responses. CHOP is known to play an important role in the induction of apoptosis.

Peer review

The authors aimed to investigate the pathophysiological role of CHOP in severe acute pancreatitis and associated lung injury. This is a unique study and the acquired data support that CHOP has a protective effect against acute pancreatitis.

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