

**CAAT/Enhancer Binding
Protein-Homologous Protein Deficiency
Attenuates Liver Ischemia/Reperfusion
Injury in Mice**

**(CHOP 欠損はマウスにおける
肝虚血再灌流障害を軽減する)**

和田 征大

平成 30 年 Liver transplantation 掲載予定

主論文

CAAT/Enhancer Binding Protein–Homologous Protein Deficiency Attenuates Liver Ischemia/Reperfusion Injury in Mice

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHOP, CAAT/enhancer binding protein-homologous protein; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; HPRT, hypoxanthine phosphoribosyltransferase; H&E, hematoxylin–eosin; IL-6, interleukin-6; I/R, ischemia/reperfusion; MPO, myeloperoxidase ;SE, standard error; TNF- α , tumor necrosis factor-alpha; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; UPR, unfolded protein response; WT, wild type

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Introduction

Liver ischemia/reperfusion (I/R) injury occurs in liver transplantation, the Pringle maneuver or massive bleeding associated with liver resection, and hemorrhagic and cardiogenic shock (1, 2). This results in liver dysfunction and failure, which can even lead to death via multi-organ system failure. Furthermore, tissue damage of the liver parenchyma induced by I/R can potentially lead to postoperative cancer recurrence (3-7).

The forms of cell death that occurs during liver I/R have been debated. Based on morphological assessment by hematoxylin–eosin (H&E) and TdT-mediated dUTP nick-end labeling (TUNEL) staining, most hepatocyte death occurs via necrosis (8, 9). However, necrosis and apoptosis have common features and mechanisms; in addition, the initiation of the apoptotic process can be converted to necrosis upon ATP depletion at several stages of cell death. Moreover, it is sometimes difficult to distinguish between these two forms (10). On the other hand, Rudiger et al. reported that apoptosis is the central mechanism of liver I/R injury (11). Additionally, many anti-apoptotic strategies against liver I/R injury have been reported (12). Therefore, it is reasonable to approach liver I/R injury from the viewpoint of apoptosis.

Under various abnormal conditions, endoplasmic reticulum (ER) function, which folds newly synthesized proteins is depressed and unfolded or misfolded proteins accumulate in the lumen of endoplasmic reticulum. This situation is referred to as ER stress. The involvement of ER stress in various diseases has been reported, including neuro-degenerative disease, diabetes mellitus (13), cardiovascular disease (14), and chronic kidney disease (15). The unfolded protein response (UPR) is a protective mechanism that mitigates ER stress (16). Accumulated unfolded proteins are eliminated by the UPR in three ways: (i) upregulation of ER chaperones to assist protein folding, (ii) transcriptional attenuation, and (iii) degradation of unfolded proteins. However, when ER stress surpasses this protective mechanism, apoptosis is induced. During ER stress-mediated apoptosis, transcriptional factor CCAAT/enhancer-binding protein homologous protein (CHOP) plays an important role (17).

In CHOP-knockout (CHOP^{-/-}) mice, the improvement of various pathological conditions has been reported. Decreased liver damage in CHOP^{-/-} mice has been shown in an intragastric ethanol feeding model (18), cholestasis-induced liver fibrosis model (19), and LPS-induced acute liver failure model (20). I/R injury in the myocardium (21), kidney (22-25), and neuronal cells (26) was also shown to be attenuated in CHOP^{-/-} mice. However, the relationship between CHOP and liver I/R injury has not been shown. Thus, the aim of this study was to clarify whether CHOP deficiency can attenuate liver I/R injury.

Materials & Methods

Animals

Male C57BL/6 (age, 7–11 weeks; Japan SLC, Tokyo, Japan) and CHOP^{-/-} mice (age, 7–11 weeks; C57BL/6 background) were used. CHOP^{-/-} mice were kindly provided by Professor Tomomi Goto (Kumamoto University, Japan). Food and water were administered *ad libitum*, and mice were maintained under constant environmental conditions with a 12 h light/dark cycle. Protocols for all experiments were approved by the animal research committee of Kyoto University. Animals were cared for in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Liver warm I/R injury model

Mice were anesthetized by isoflurane inhalation and placed on a heating plate to maintain their body temperature during operation. After laparotomy, the vessels supplying 70% of the left side of the liver were disrupted using a micro atraumatic vascular clamp, and subsequently, the abdomen was closed. Before closure, sodium pentobarbital (60 mg/kg) was administered intraperitoneally for sedation during the period of ischemia. After 60 min, the clamp was removed, and reperfusion was initiated. Sham control mice underwent the same procedure without vascular occlusion. Mice were sacrificed at different time points after reperfusion, and liver tissue and blood were collected.

Assessment of hepatocellular damage

Serum aminotransferase (AST) and serum alanine aminotransferase (ALT) levels were measured to assess hepatocellular damage.

Liver histology

Formalin-fixed, paraffin-embedded sections were cut at a thickness of 4 μ m, and the slides were stained with H&E. Histological damage by liver I/R was blindly scored with Suzuki's criteria on a scale from 0 to 4 (27).

TdT-mediated dUTP nick-end labeling (TUNEL) Assay

TUNEL assays were performed on 4 μ m sections using an Apoptosis in situ Detection Kit (Wako, Osaka, Japan) to detect apoptotic and necrotic cells in liver tissues 6 h after reperfusion. The number of TUNEL-positive cells was counted in 10 randomly selected high-power fields (magnification, $\times 400$) per slide (n = 5 per group).

Quantitative real-time RT-PCR

Total RNA was extracted from liver tissue using TRIzol (Invitrogen Japan, Tokyo, Japan) and the RNeasy Mini Kit with on-column DNA digestion (Qiagen, Valencia, CA, USA). In accordance with the manufacturers' protocol, total RNA was reverse transcribed to cDNA using an Omniscript RT Kit (Qiagen, Valencia, CA, USA) with oligo (dT)₈₋₁₂ primers (Invitrogen Japan). Real-time RT-PCR was performed with a KAPA SYBR FAST qPCR Kit (Nippon Genetics, Tokyo, Japan) and StepOnePlus system (Applied Biosystems, Foster City, CA, USA). The primers used in this study are shown in Table 1. Expression levels are provided as relative ratios to the expression level of *HPRT*.

Western blot analysis

Western blot analysis was conducted as previously described(28), using the following primary antibodies and dilutions: anti-GADD153 (CHOP) (no. sc-7351; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:200; anti-cleaved caspase-3 (no. 9664; Cell Signaling Technology, Danvers, MA, USA) at 1:1000; anti-Bax (no. 2772; Cell Signaling Technology) at 1:1000; anti-Bcl-xL (no. 2762; Cell Signaling Technology) at 1:1000; anti-Bcl-2 (no. sc-7382; Santa Cruz Biotechnology) at 1:500; and anti- β -actin antibody (no. sc-; 47778 Santa Cruz Biotechnology) at 1:1000. The intensity of the bands was quantified with Image J.

Immunohistochemistry

The specimens were fixed in 10% formalin and embedded in paraffin. Subsequently, they were deparaffinized, and endogenous peroxidase was quenched with 3% hydrogen peroxide in PBS at room temperature for 10 min. The antigen was retrieved by incubation in citric acid buffer at 120°C for 10 min. After being blocked, the sections were incubated with the primary antibody recognizing anti-CHOP (ab11419; Abcam) diluted at 1:100 at room temperature for 2 h and anti-myeloperoxidase (ab9535; Abcam) diluted at 1:50 at 4°C overnight and then with labeled polymer in an Envision + System HRP (Dako Japan, Tokyo, Japan) at room temperature for 1 h. The sections were examined after incubation with a Liquid DAB Substrate Chromogen System (Dako Japan) for 5 min.

Statistical analysis

All data are expressed as means $\pm$ SE. Statistical analysis was performed using Student's *t*-test to compare mean values between groups. A *P* value less than 0.05 was considered statistically significant. Analyses were performed by JMP pro 11 (SAS Institute, Cary, NC, USA).

Results

CHOP expression increases in wild-type mice following liver I/R injury

Wild-type (WT) mice subjected to liver I/R injury were sacrificed 3 and 6 h after reperfusion to ascertain whether this condition upregulates CHOP expression in the mouse liver. RT-PCR demonstrated that CHOP mRNA expression increased by 16-fold at 3 h after reperfusion compared to that in sham-operated mice (Fig. 1A). Consistent with mRNA levels, CHOP protein expression was elevated at 3 and 6 h after reperfusion by western blotting, whereas the CHOP protein was only minimally expressed in sham-operated mice (Fig. 1B). Then, to clarify which cell type within the liver upregulates CHOP after ischemia reperfusion, we performed immunohistochemical staining for CHOP. In sham-operated livers, CHOP protein was faintly expressed in the cytosol of hepatocytes. Three hours after reperfusion, CHOP was strongly expressed in the cytosol and nucleus of hepatocytes, while nonparenchymal cells were not stained. At 6 h after reperfusion, CHOP expression of hepatocytes decreased compared to that at 3 h after reperfusion. CHOP expression of the cytoplasm diminished, while the nucleus stained strongly, and nonparenchymal cells also expressed CHOP.

I/R injury induces ER stress in both WT and CHOP^{-/-} mice

To confirm whether ER stress is induced by liver I/R injury in both WT and CHOP^{-/-} mice, animals were sacrificed 3 and 6 h after reperfusion. The ER stress marker *GRP78* was assessed by RT-PCR. *GRP78* expression was significantly increased 3 h after reperfusion in both WT and CHOP^{-/-} mice. Three hours after reperfusion, *GRP78* expression slightly increased in CHOP^{-/-} mice, compared to that in WT mice (Fig. 2A). The ATF4-CHOP pathway, which is known to be the main pathway for CHOP induction, was also activated. The mRNA level of *ATF4*, which is upstream of CHOP, also increased 3 h after reperfusion in both WT and CHOP^{-/-} mice (Fig. 2B). Similar to CHOP expression, *GRP78* and *ATF4* mRNA expression peaked at 3 h after reperfusion. These results indicated that liver I/R injury induces ER stress in both WT and CHOP^{-/-} mice.

CHOP deficiency attenuates liver I/R injury

To test whether CHOP deficiency attenuates liver damage induced by I/R, liver sections and blood samples from animals 6 h after I/R were evaluated. Serum AST and ALT levels were significantly increased in WT mice (ALT: 8437 ± 699 IU/L, AST: 3787 ± 312 IU/L) compared to that in CHOP^{-/-} mice (ALT: 5083 ± 830 IU/L, AST: 2543 ± 259 IU/L) at 6 h after reperfusion ($P < 0.05$) (Fig. 3A). On the basis of H&E staining of livers 6 h after reperfusion, extended necrotic areas and vacuolization were attenuated in CHOP-deficient mice compared with WT mice. The Suzuki score significantly decreased in CHOP^{-/-} mice. (Fig. 3B). Then, we

assessed neutrophil infiltration induced by liver I/R injury by immunohistochemical staining for MPO. Numbers of MPO-positive cells were significantly decreased in the livers of CHOP^{-/-} mice at 6 h after reperfusion, as compared to WT mice (Fig. 3C).

Liver I/R injury-induced apoptosis and necrosis are decreased in CHOP^{-/-} mice

We next evaluated hepatocyte death induced by I/R injury at 6 h after reperfusion in both WT and CHOP^{-/-} mice. The number of TUNEL-positive cells was significantly decreased in CHOP^{-/-} mice (47 ± 6 (WT) vs. 24 ± 2 (CHOP^{-/-}) per HPF, $P < 0.05$). Most of the TUNEL-positive cells were hepatocytes, and both the nucleus and cytoplasm were stained, demonstrating a staining pattern indicative of necrotic cells (Fig. 4A). To ascertain whether apoptosis was involved in this process, cleaved caspase-3 was analyzed by western blotting. Cleaved caspase-3 was expressed at a low level in sham-operated mice. Cleaved caspase-3 expression increased after I/R in both groups of animals; however, the expression of this marker was significantly higher in WT mice than in CHOP^{-/-} mice (Fig. 4B).

CHOP deficiency attenuates hepatocyte apoptosis induced by I/R injury through the intrinsic apoptosis pathway

The expression levels of Bcl-2 family proteins—Bcl-2, Bcl-xL, and Bax—were determined by western blotting. Compared to that in sham-operated mice, the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL decreased, and the expression of the pro-apoptotic protein Bax was increased at 6 h after reperfusion. After I/R injury, Bax expression was significantly suppressed in CHOP^{-/-} mice compared to that in WT mice (Fig. 5A). Meanwhile, Bcl-2 was expressed at a slightly higher level in CHOP^{-/-} mice than in WT mice, and the expression of Bcl-xL was virtually identical in both groups (Fig. 5B, C).

Discussion

Several relationships between ER stress and liver I/R injury have been reported. The small molecule chaperone 4-phenyl butyric acid, which ameliorates ER stress, improves liver I/R injury (29, 30). In addition, some drugs attenuate liver I/R injury via the ER stress pathway (31). Improvement of liver I/R injury by inhibiting the ER stress pathway, involving CHOP, has also been reported (32). Furthermore, in an animal model of traumatic hemorrhagic shock, which can be regarded as a mild liver I/R model, ER stress was induced to a greater extent than oxidative stress. This suggests a deep involvement of ER stress and liver I/R injury (33).

The relationship between CHOP and I/R injury of other organs has also been reported. In the kidney and cerebellum, elevation of CHOP expression induced by I/R injury continues until after 24 h (22, 34), while CHOP expression peaks at 0.5 h after reperfusion in myocardial

reperfusion injury (21). In our liver I/R injury model, CHOP expression peaked at 3 h after reperfusion at mRNA and protein levels. However, immunohistochemistry revealed that liver nonparenchymal cells expressed CHOP later than hepatocytes, in which CHOP expression peaked at 3 h after reperfusion. Although improvement of liver injury was mild, we confirm that CHOP deficiency attenuates I/R injury of the liver as shown in other organs.

CHOP regulates the intrinsic apoptosis pathway via Bcl-2 family proteins, and these proteins, including Bcl-2, Bcl-xL, and Bax, are involved in liver I/R injury (35-37). Our results show that liver I/R injury was attenuated in CHOP-deficient mice through the inhibition of Bax expression and the attenuation of apoptosis. The pro-apoptotic molecule Bax, which stimulates cytochrome c release from mitochondria and increases caspase-3 activity, occurs downstream of CHOP and plays an important role in CHOP-mediated apoptosis. In addition, Bax can induce apoptosis independent of other anti-apoptotic molecules (35). Ben-Ari et al. showed that liver I/R injury was attenuated in Bax^{-/-} mice (35). Recently, the relationship between CHOP and inflammation has been reported. In I/R injury, inflammatory cytokines such as TNF- α , IL-6 and IL-1 β were suppressed in myocardia of CHOP^{-/-} mice (21) and IL-1 β was also suppressed in kidney tissue of CHOP^{-/-} mice (22). In a study of steatohepatitis, the relationship between CHOP and the NF κ B pathway was shown in human primary hepatocyte (38). We assessed the inflammatory response including these cytokines and pathways, but could not find a significant difference between the groups. However, the significant difference in neutrophil infiltration in both groups suggested the involvement of CHOP in some inflammatory response. One of the reasons for this result is probably the mildness of improvement of liver damage in this study.

However, it should be carefully considered whether inhibiting the UPR itself leads to alleviation of liver I/R injury in clinical practice. Zhou et al. showed that the role of the UPR in the mouse liver I/R model differs between a 30 min ischemia group and a 90 min ischemia group. Specifically, this response was pathogenic in the 90 min ischemia model, but was protective in the 30 min group (39). This report reminds us that the UPR is originally a protective mechanism. In the clinical setting, severe liver damage, as induced in the animal model of warm I/R injury, is rare because liver resection is carefully performed to mitigate postoperative liver failure, and hemorrhagic and cardiogenic shock are not a complete interruption of the blood flow. During liver resection, moderate I/R injury in small remnants of the liver causes liver failure, not severe I/R injury itself. Portal clamping (6) or intraoperative blood loss (40, 41) might affect tumor recurrence even if they are not fatal during the acute phase. For these reasons, liver warm I/R injury needs to be improved, even if it does not readily occur with severity. Meanwhile, since the degree of liver damage is different from that of the animal model, we must carefully address the relationship between the UPR and liver I/R injury in clinical situations. Elucidation of the function of CHOP in liver I/R injury should facilitate

the investigation of this relationship.

After renal I/R injury in CHOP^{-/-} mice, several mechanisms for ameliorating injury have been reported (22-25). Noh et al. showed that the Bax/Bcl-2 ratio is significantly decreased in the I/R kidney in CHOP^{-/-} mice (22). In contrast, Chen et al. did not find any significant difference in the expression of Bax and Bcl-2 between CHOP^{-/-} and WT mice (25). Noh et al. suggested that this difference was due to the degree of kidney injury. In the future, further elucidation is also expected regarding the mechanism through which liver I/R injury is alleviated in CHOP^{-/-} mice (22).

In conclusion, we have for the first time shown that CHOP deficiency attenuates liver I/R injury. These results may contribute to further investigation of a therapy against liver I/R injury associated with the endoplasmic reticulum stress pathway.

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Table 1. Sequences of primers used for real-time RT-PCR

target	Forward	Reverse
CHOP	5'-TATCTCATCCCCAGGAAACG	5'-GGGCACTGACCACTCTGTTT
GRP78	5'-GAAAGGATGGTTAATGATGCTGAG	5'-GTCTTCAATGTCCGCATCCTG
ATF4	5'-ATGGCCGGCTATGGATGAT	5' -CGAAGTCAAACCTCTTTCAGATCCATT
HPRT	5'-TCAACGGGGGACATAAAAAGT	5'-TGCATTGTTTTACCAGTGTCAA

Figure 1

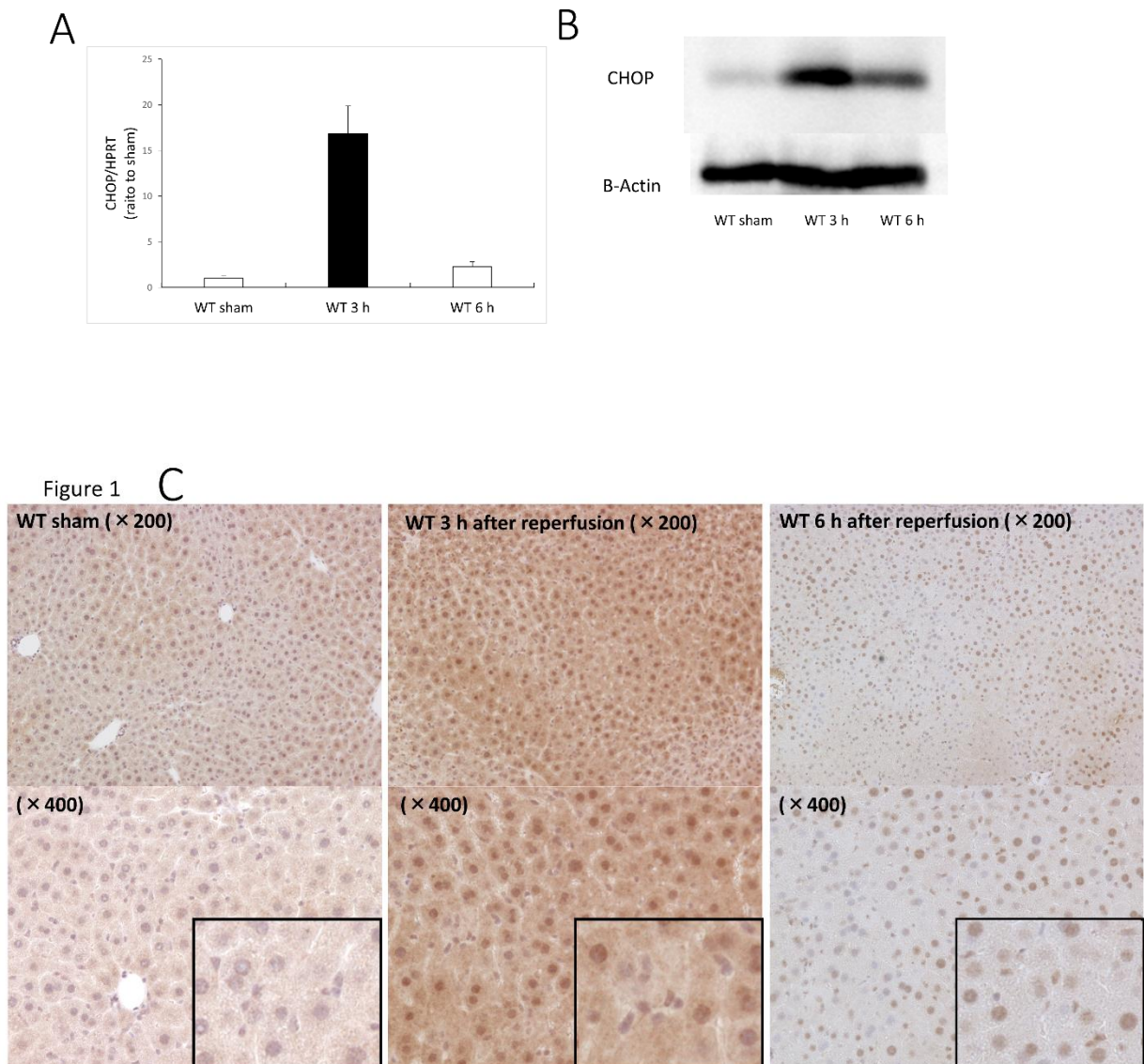


Fig. 1. Liver warm ischemia reperfusion (I/R) injury induces CCAAT/enhancer-binding protein homologous protein (CHOP) expression in wild type (WT) mice. WT mice were subjected to 70% liver I/R for 60 min and were sacrificed at 3 and 6 h after reperfusion. (A) Quantitative real-time PCR and (B) western bolt analysis of *CHOP* expression. (C) Immunohistochemistry of *CHOP* expression. Data are shown as the mean $\pm$ SE (n = 3). *P < 0.05. Sham: sham-operated mice.

Figure 2

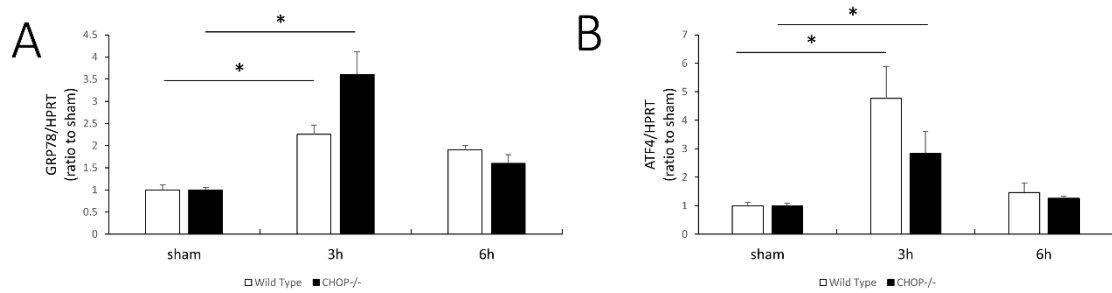


Fig. 2. Expression of the endoplasmic reticulum (ER) stress marker glucose-regulated protein (GRP) 78 and ATF4 in both WT and CHOP^{-/-} mice subjected to liver I/R injury. Livers were harvested at 3 and 6 h after reperfusion. Expression levels of GRP78 (A) and ATF4 (B) were determined by quantitative real-time PCR. Data are shown as the mean $\pm$ SE (n = 4). *P < 0.05.

Figure 3

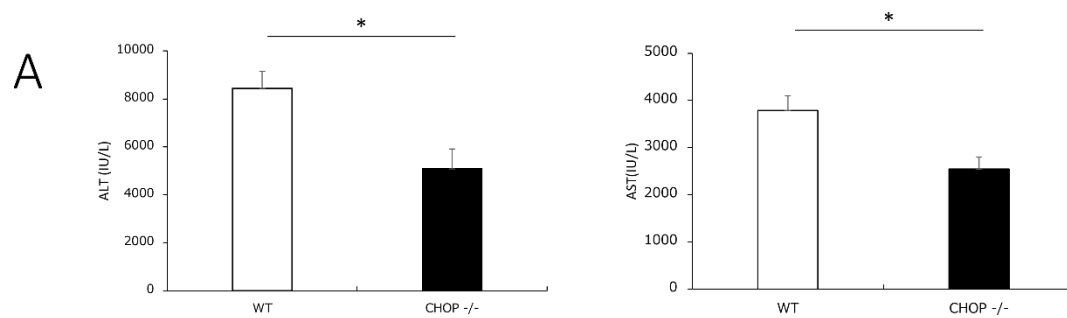


Figure 3

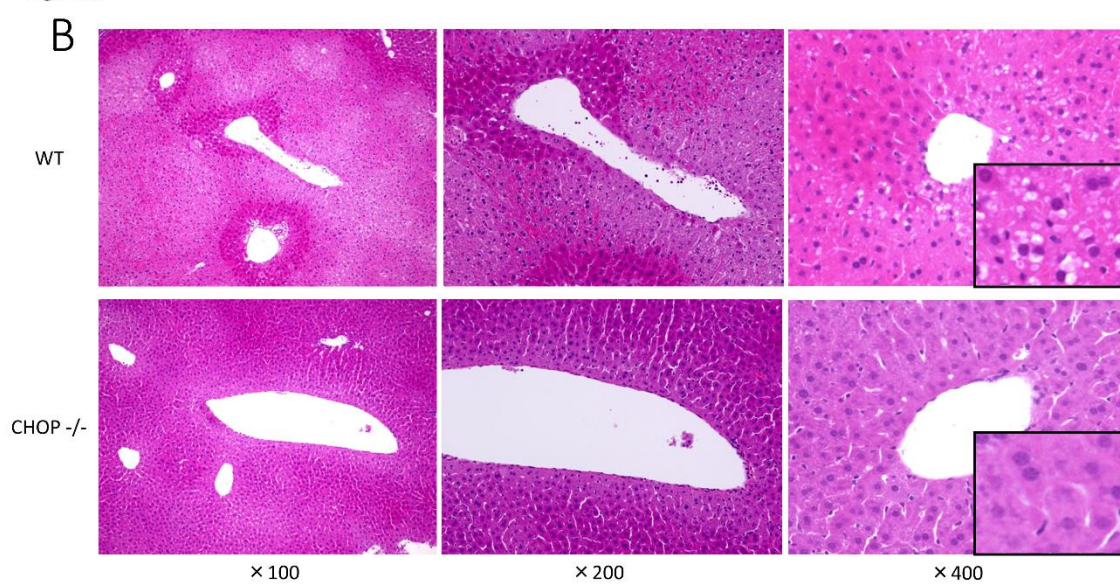


Figure 3

B

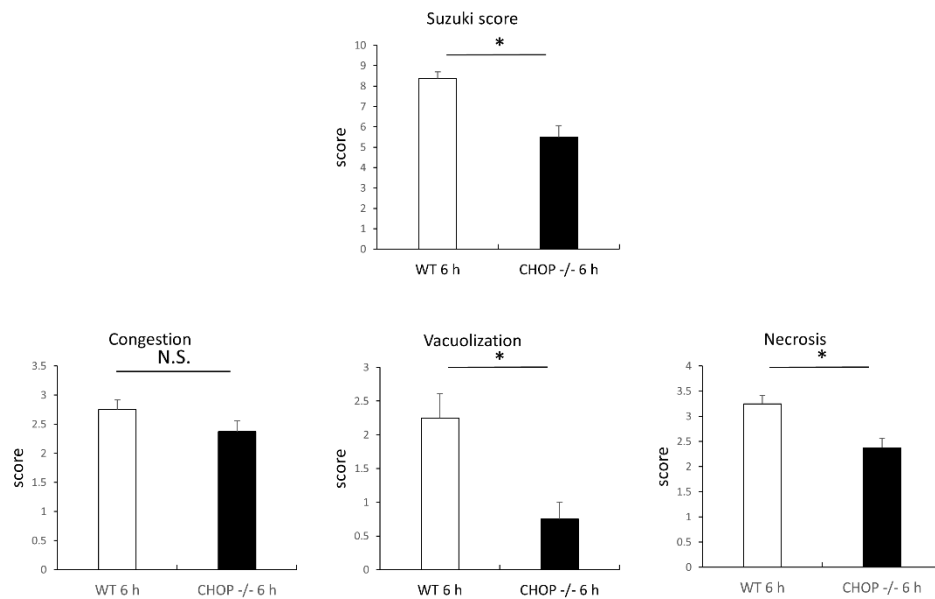


Figure 3

C

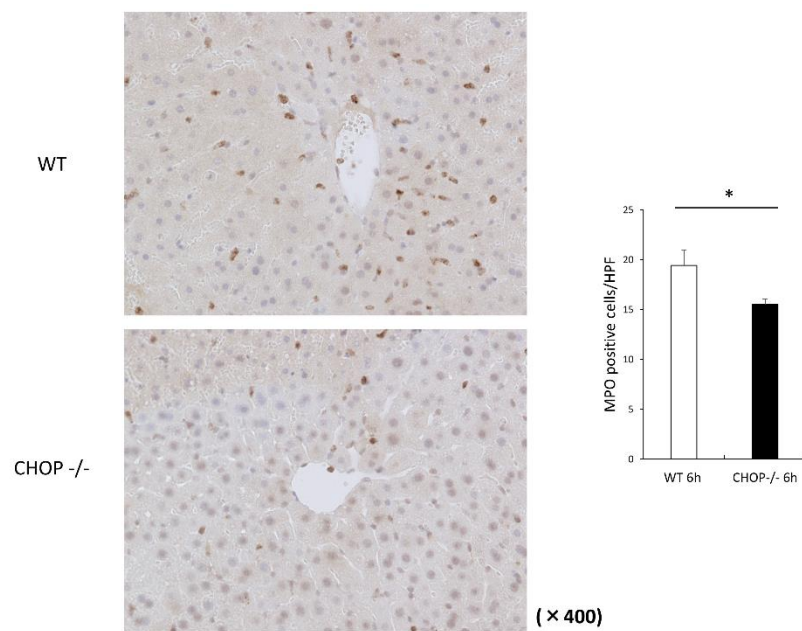


Fig. 3. CHOP deficiency attenuates liver warm liver I/R injury. (A) Serum aminotransferase (AST) and serum alanine aminotransferase (ALT) levels was measured after 60 min of ischemia and 6 h after reperfusion using WT and CHOP^{-/-} mice. (B) Representative H&E-stained liver sections 6 h after reperfusion in WT and CHOP^{-/-} mice (original magnification, ×100, ×200, and ×400) and quantification of Suzuki's criteria of the liver damage at 6 h after reperfusion. (C) Immunohistochemical staining for MPO-positive cells in livers with I/R injury and quantitation of hepatic MPO accumulation. Data are shown as the mean ± SE (n = 8). *P < 0.05.

Figure 4 A

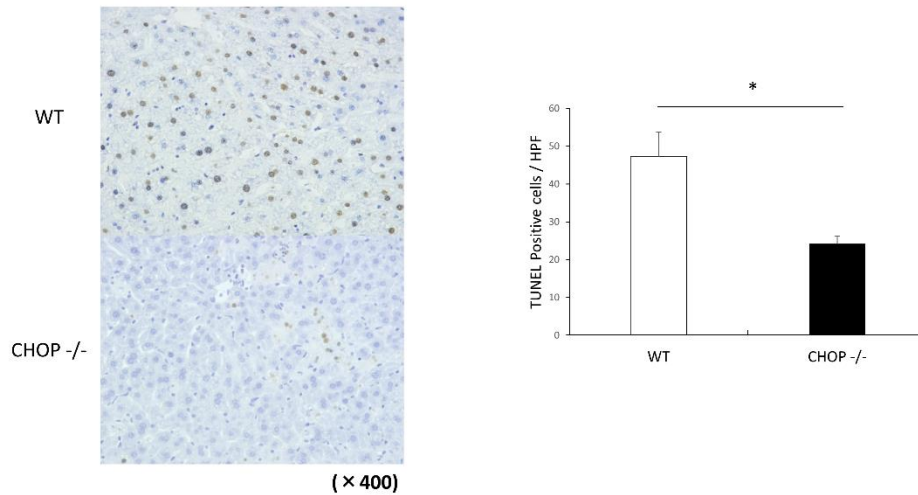


Figure 4 B

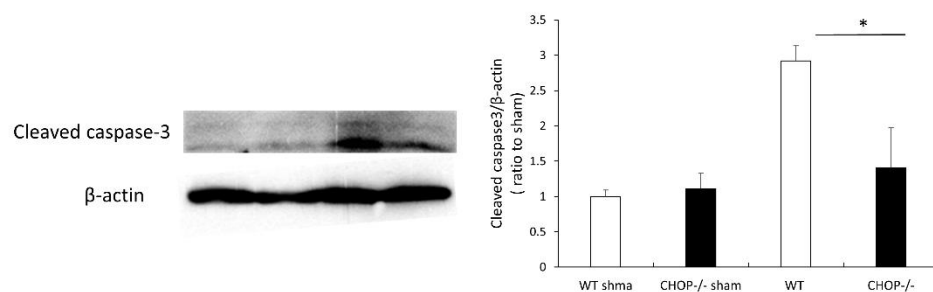


Fig. 4. (A) TUNEL staining of liver sections 6 h after reperfusion in a model of liver I/R injury, comparing WT and CHOP-/- animals. Hepatocyte necrosis and apoptosis were analyzed (original magnification, ×400). The number of TUNEL-positive cells in each high-power field was evaluated. Data are shown as the mean ± SE (n = 5). *P < 0.05. (B) Cleaved caspase-3 expression at 6 h after reperfusion was analyzed by western blotting, and densitometry analysis was performed for quantification. Data are shown as the mean ± SE (n = 5). *P < 0.05. HPF = high-power field.

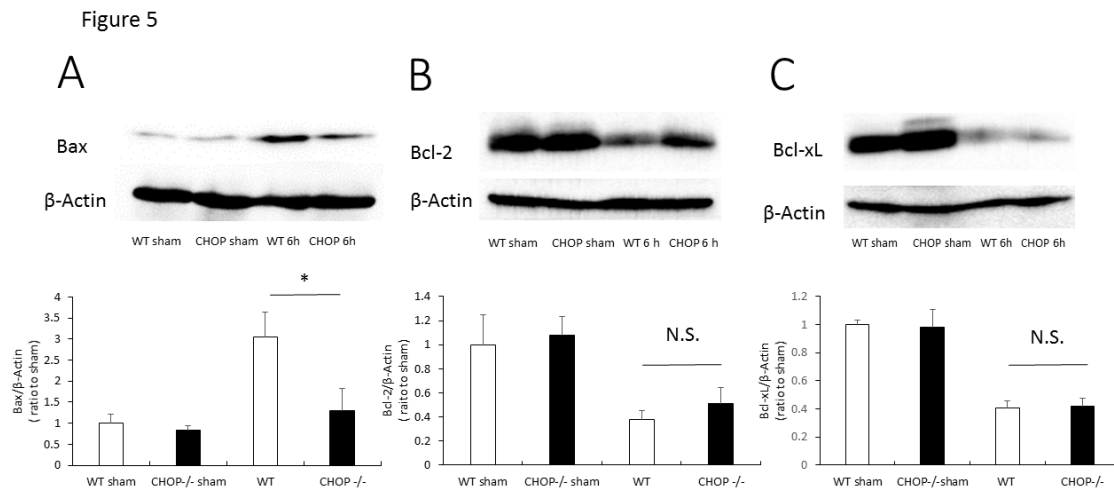


Fig. 5. Expression of apoptotic markers in a mouse model of liver warm I/R injury. Bax expression increased with I/R injury, and this was attenuated in CHOP^{-/-} mice (A). The expression of Bcl-2 and Bcl-xL was not significantly different between WT mice and CHOP^{-/-} mice (B, C). The expression of these proteins was evaluated by western blotting, and densitometry analysis was performed to quantify the expression of these proteins. Data are shown as the mean $\pm$ SE (n = 5). *P < 0.05, N.S. = not significant. Sham: sham-operated mice.