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C/EBP homologous protein (CHOP) gene deficiency attenuates renal ischemia/reperfusion injury in mice

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ABSTRACT

C/EBP homologous protein (CHOP), a transcription factor for the expression of apoptosis-related genes, plays an important role in endoplasmic reticulum (ER) stress-related organ diseases, including diseases of the kidney. Here, we investigated the role of CHOP in ischemia/reperfusion (I/R)-induced acute kidney injury using CHOP-knockout ($CHOP^{-/-}$) and wild type ($CHOP^{+/+}$) mice. Fifteen or thirty minutes of bilateral renal ischemia (I/R) insult resulted in necrotic and apoptotic tubular epithelial cell death, together with increases in plasma creatinine (PCr) and blood urea nitrogen (BUN) concentrations. After I/R, BiP/GRP78 and CHOP expressions in the kidney gradually increased over time. CHOP expression was greater in the outer medulla than that in the cortex and localized intensely in the nucleus. I/R caused apoptosis of tubular epithelial cells in both $CHOP^{-/-}$ and $CHOP^{+/+}$ mice. The number of apoptotic cells after I/R was lower in $CHOP^{-/-}$ mice than that in $CHOP^{+/+}$ mice. Consistent with the degree of apoptosis, I/R-induced kidney morphological and functional damages were milder in $CHOP^{-/-}$ than that in $CHOP^{+/+}$ mice. The cleavage of procaspase-3 and the induction of Bax protein after I/R were lower in $CHOP^{-/-}$ than that in $CHOP^{+/+}$ mice. In contrast, the expression levels of Bcl-2, Bcl-xL, cIAP2, Mcl-1, and XIAP were higher in $CHOP^{-/-}$ than that in $CHOP^{+/+}$ mice. These results indicate that I/R induces ER stress, leading to the activation of CHOP-associated apoptosis signals, resulting in renal functional and histological damages.

1. Introduction

The endoplasmic reticulum (ER) is a site of protein synthesis and folding. A variety of stresses, including ischemia/reperfusion (I/R) injury, perturb the ER function of cells [1]. To cope with these perturbations, cells activate various self-defense systems, attenuate protein translation to reduce new protein synthesis and the accumulation of unfolded proteins, upregulate ER chaperones, and degrade unfolded proteins [1,2]. If ER stress is beyond its self-defense capacity, cells activate apoptotic signals to quickly remove damaged organelles [3]. Transcriptional activation of C/EBP homologous protein (CHOP) is a key event in ER stress-mediated apoptosis. CHOP represses the expression of Bcl-2 proteins as well as activates calcium signaling pathways, causing mitochondrial damage, cytochrome c release, and caspase cascade activation [4].

Acute kidney injury (AKI) is a significant medical problem [5]. Mortality by severe AKI conditions exceeds 50% in some studies. I/R is a main cause of AKI in many clinical settings, including kidney transplantation and cardiac bypass surgeries, and is commonly used for developing animal experimental models [5,6]. I/R in the kidney induces necrosis and apoptosis of tubular epithelial cells, leading to acute renal failure [7]. Many reports have demonstrated that this I/R-induced cell death is associated with apoptosis [8,9]. Therefore, we hypothesized that CHOP would respond to I/R injury. In the present study, we investigated the role of CHOP in I/R-induced AKI and its underlying mechanisms by using CHOP-knockout ($CHOP^{-/-}$) and wild type ($CHOP^{+/+}$) mice. Here, we report that CHOP deficiency attenuates I/R-induced AKI through the inhibition of the ER-associated apoptotic signaling pathway.

2. Materials and methods

2.1. Animal preparation

All experiments were conducted in 8–10 week old male *CHOP* deficient ($CHOP^{-/-}$) and wild-type ($CHOP^{+/+}$) mice (Jackson Lab; Bar Harbor, MA, USA). The study was approved by the Institutional Animal Care and Use Committee of Kyungpook National University. Mice were allowed free access to water and standard chow. Mice were anesthetized with pentobarbital sodium (60 mg/kg body weight; Sigma-Aldrich) before surgeries. To induce 15 or 30 min of bilateral renal ischemia, both kidneys were exposed through flank incision sites,

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and then renal vascular pedicles were clamped with non-traumatic microaneurysm clamps (Roboz Surgical Instruments). After 30 min, clamps were removed and blood reflow was confirmed visually. Sham operation was performed using the same surgical procedures as for ischemia except for the clamping of renal pedicles. Body temperature was maintained at 36.5–37.0 °C throughout procedures.

Kidneys were either snap-frozen in liquid nitrogen for western blot analysis or perfusion-fixed in PLP (4% paraformaldehyde, 75 mM L-lysine, 10 mM sodium periodate; Sigma; St. Louis, MO, USA) for histological studies.

2.2. Measurements of plasma creatinine (PCr) and blood urea nitrogen (BUN) concentrations

The concentrations of PCr and BUN were measured using a Vitros250 analyzer (Johnson & Johnson).

2.3. Western blot analysis

Western blot analyses were performed in kidneys snap-frozen in liquid nitrogen, as previously described [10]. The following primary antibodies were used: anti-BiP/GRP78 (Cell Signaling), anti-CHOP (Cell Signaling), anti-cIAP2 (Santa Cruz Biotech), anti-Mcl-1 (Santa Cruz Biotech), anti-XIAP (AnaSpec), anti-caspase-3 (Cell Signaling), anti-Bcl-xL (BD Transduction Labs), anti-Bcl-2 (Cell Signaling), anti-Bax (Cell Signaling), anti-Ly6G (eBioscience), anti-IL-1β (Santa Cruz Biotech) and anti-actin (Santa Cruz Biotech) antibodies. Densities of blots were measured using the ImageJ program (NIH).

2.4. Histology

PLP-fixed kidneys were embedded in paraffin and cut into 4-μm sections. Sections were stained with periodic acid Schiff stain (PAS). Images of the cortex and outer medulla in the kidney were captured using i-Solution software (iMT technology). Tubule damage was scored by the following criteria: 0, no damage; 1, mild damage with the rounded epithelial cells and dilated tubular lumen; 2, moderate damage with flattened epithelial cells, dilated lumen, and congestion of lumen; and 3, severe damage with flat epithelial cells lacking nuclear staining and congestion of the lumen. More than 50 tubules per kidney ($n = 3–4$) were analyzed.

2.5. Immunohistochemistry

Immunohistochemistry staining was performed as described previously [10] using anti-CHOP (Cell Signaling) antibodies. Images were captured using a Leica DM2500 microscope (Leica).

2.6. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

TUNEL assay was performed using an in situ cell death detection kit (Fluorescein, Roche) according to the manufacturer's instruction. In brief, 4-μm kidney sections were deparaffinized and re-hydrated. Afterward, the sections were incubated with TUNEL reagent mixture for 30 min at room temperature and then washed with PBS three times for 5 min each. Nuclei were stained with 4,6 diamidino-2-phenylindole (DAPI) for 1 min. Images were observed under a microscope (Leica DM2500). DAPI- and TUNEL-positive images were collected, respectively, and merged with i-Solution software. TUNEL-positive cells were counted in 10 fields (0.1 mm^2 per field) per kidney ($n = 3–4$).

2.7. Statistical analysis

Results are expressed as the mean ± SEM. Statistical differences among groups were calculated using the Student's *t*-test and one-way analysis of variance for comparison between two groups. Differences between groups were considered statistically significant at a p-value < 0.05.

3. Results

3.1. I/R induces ER stress in the kidney

To test whether I/R in kidney induces ER stress, BiP/GRP78 and CHOP expressions were determined in the kidney by western blot at 0 (without reperfusion), 1.5, 4, and 24 h and 9 days after 30 min of bilateral renal ischemia or 24 h after sham-operation. CHOP was expressed at very low levels in the sham-operated kidneys (Fig. 1A). CHOP expression increased after ischemia and then slightly decreased 1.5 h after reperfusion. It then rebounded to peak 24 h after reperfusion and then remained at high levels until 9 days after reperfusion decreased (Fig. 1A). The upregulation of BiP/GRP78 has been considered as a marker for ER stress. The expression of BiP/GRP78, a molecular chaperone against ER stress, was similar to CHOP expression, but BiP/GRP78 expression was highest at 4 h after ischemia (Fig. 1A). Since segments of kidney tubules are differentially susceptible to I/R injury, we determined the kidney sites that express BiP/GRP78 and CHOP by immunohistochemical staining. BiP/GRP78 protein was faintly expressed in sham-operated kidneys (Fig. 1B). Twenty-four hours after ischemia the intensity of BiP/GRP78 protein expression became stronger than that of sham, and then 9 days after ischemia, the intensity of BiP/GRP78 decreased (Fig. 1B). BiP/GRP78 protein was localized on the cytosol of tubular epithelial cell (Fig. 1B). Faint CHOP protein expression was detected in the sham-operated kidneys (Fig. 1C). After ischemia, CHOP protein was detected in the cytosol and nucleus of kidney tubule cells, and nuclear CHOP expression became stronger over time after ischemia (Fig. 1C). I/R-induced CHOP expression was greater in the tubules of the outer medulla than in those of the cortex (Fig. 1A and C).

3.2. CHOP gene deletion protects kidney structure and function against I/R

To test whether CHOP gene deletion alters kidney susceptibility to I/R injury, CHOP-deficient mice were subjected to 15 or 30 min of bilateral renal ischemia. I/R injury resulted in severe tubular cell damages, leading to the disruption, congestion, and dilation of tubules (Fig. 2A and B). Twenty-four hours after 15 or 30 min of ischemia, tubular cell damages in the outer medulla were much more severe than that in the cortex (Fig. 2A and B). The damage scores in the kidney subjected to 15 min of ischemia were much lower than that subjected to 30 min of ischemia (Fig. 2A and B). After 15 min of ischemia, damage scores in the cortex and OM of $CHOP^{-/-}$ mice were significantly lower than those in $CHOP^{+/+}$ mice (Fig. 2B). Unlike the results of 15 min of ischemia, damage scores after 30 min of ischemia in the outer medulla (OM), but not in the cortex, of $CHOP^{-/-}$ mice were not significantly different when compared with those in $CHOP^{+/+}$ mice (Fig. 2A). It may be due to the different tubular cell susceptibilities to I/R injury between the cortex and the OM; tubular epithelial cells in the OM are much more susceptible to I/R injury than those in the cortex [7]. Therefore, there were no differences in injury scores in the OM between $CHOP^{+/+}$ mice subjected to 30 min of ischemia and $CHOP^{-/-}$ mice. Consistent with the structural damages, ischemia significantly impaired renal functions and increased PCr and BUN concentrations in both $CHOP^{+/+}$ and $CHOP^{-/-}$ mice 4 and 24 h after 30 min of ischemia (Fig. 3A and B). These increases in PCr and BUN concentrations were significantly milder in $CHOP^{-/-}$ than that in $CHOP^{+/+}$ mice 24 h, but not 4 h, after 30 min of ischemia (Fig. 3A and B). After 15 min of ischemia, PCr and BUN concentrations gradually increased in $CHOP^{+/+}$ mice overtime, but those $CHOP^{-/-}$ mice peaked at 4 h after the ischemia and then returned to normal levels 24 h (Fig. 3C and D). After sham-operation, PCr and BUN concentrations in $CHOP^{-/-}$ mice were not significantly different compared to those in

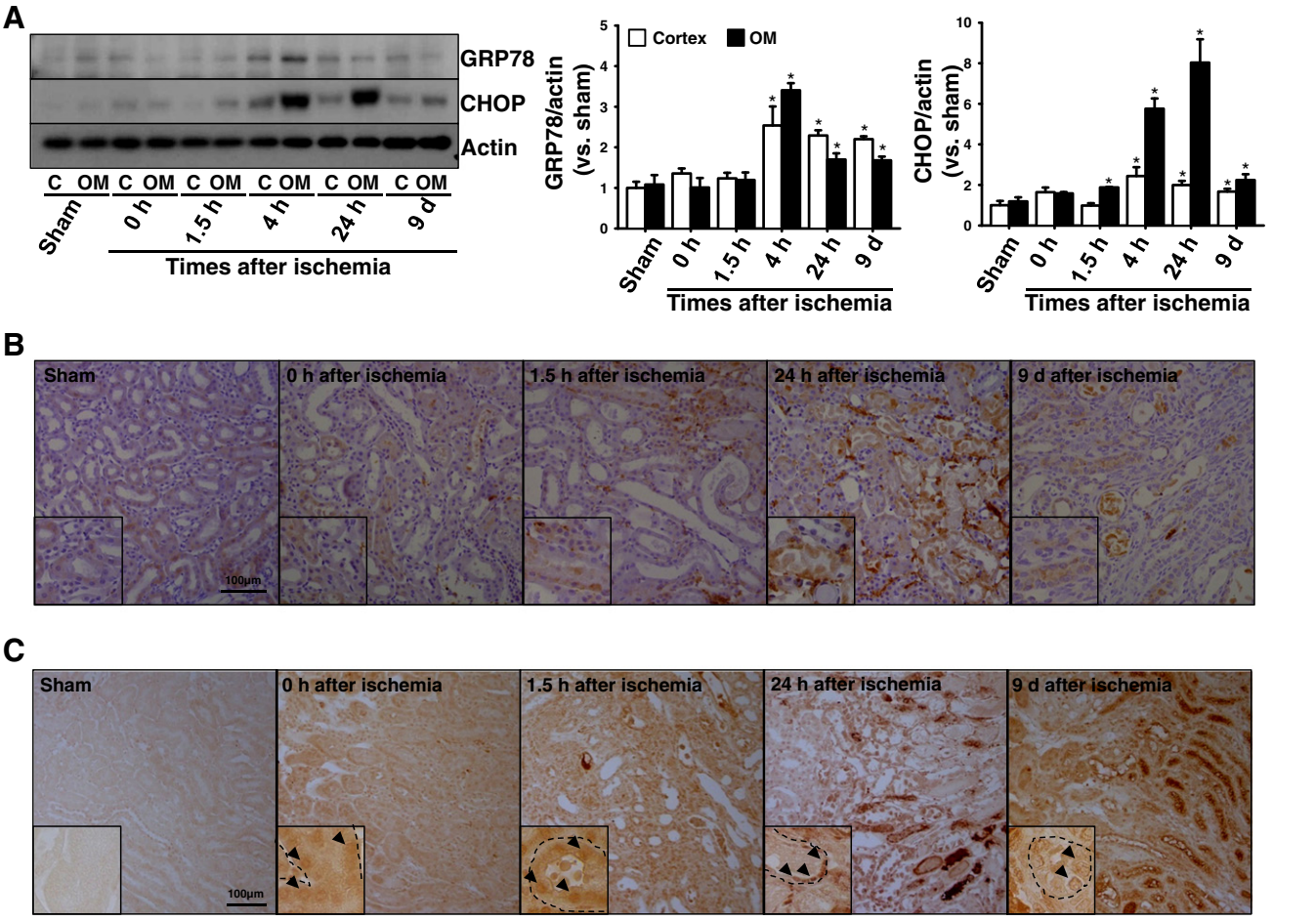

Fig. 1. BiP/GRP78 and CHOP expression in kidney tissues of I/R-induced mice. C57BL/6 male mice were subjected to either 30 min of ischemia or sham operation. Kidneys were harvested 0, 1.5, 4, and 24 h and 9 days after ischemia. (A) Expressions of BiP/GRP78 and CHOP were determined by western blot in the cortex (C) and outer medulla (OM). Blot densities of BiP/GRP78 and CHOP were normalized with actin. Localizations of BiP/GRP78 (B) and CHOP (C) proteins were evaluated by immunohistochemical staining. Sections subjected to BiP/GRP78 were counter stained with hematoxylin to visualize nuclei. Data are presented as means ± SE (n = 6). *, $p < 0.01$ vs. respective sham.

CHOP$^{+/+}$ mice (Fig. 3A to D). These results indicate that CHOP deficiency protects renal tubular cells against I/R injury.

3.3. CHOP gene deletion reduces tubule cell apoptosis after I/R

Thirty minutes of ischemia resulted in significantly increased TUNEL-positive apoptotic cells in kidney tubular cells in the cortex and OM (Fig. 4). Twenty-four hours after 30 min of ischemia, TUNEL-positive cells were less observed in the cortex of *CHOP*$^{-/-}$ mice compared to *CHOP*$^{+/+}$ mice. However, in the OM, there was no difference between the number of TUNEL-positive cells in *CHOP*$^{+/+}$ and *CHOP*$^{-/-}$ mice (Fig. 4A).

Since CHOP regulates the intrinsic apoptosis pathway through the inhibition of Bcl-2 [11], we determined the expression levels of the Bcl-2 family of proteins. I/R significantly reduced the expression of the anti-apoptotic proteins Bcl-2, Bcl-xL, Mcl-1, XIAP, and cIAP2, whereas it enhanced the expression of the pro-apoptotic protein Bax (Fig. 5). The I/R-induced reduction of anti-apoptotic protein levels was significantly milder in *CHOP*$^{-/-}$ mice than that in *CHOP*$^{+/+}$ mice (Fig. 5). In addition, the I/R-induced enhancement of the pro-apoptotic protein Bax was significantly milder in *CHOP*$^{-/-}$ mice (Fig. 5). Ischemia activated caspase-3 cleavage, which is a consequence of an enhanced pro-apoptotic to anti-apoptotic protein ratio (Fig. 5). This I/R-induced caspase-3 cleavage was significantly milder in *CHOP*$^{-/-}$ mice than that in *CHOP*$^{+/+}$ mice (Fig. 5). These results suggest that CHOP deficiency protects against the apoptosis of kidney epithelial cells following I/R injury.

3.4. CHOP gene deletion reduces inflammatory response after I/R

Finally we determined whether CHOP gene deletion reduces I/R-induced inflammatory responses. Thirty minutes of ischemia increased IL-1β and Ly6G expression in the kidneys of both CHOP$^{-/-}$ and CHOP$^{+/+}$ mice (Fig. 6). The I/R-induced increases of IL-1β and Ly6G expression were significantly less in *CHOP*$^{-/-}$ mice than that in *CHOP*$^{+/+}$ mice (Fig. 6).

4. Discussion

I/R-induced apoptosis is linked to ER stress in various organs [12–14]. When cells undergoing severe ER stress fail to repair ER dysfunction, cell homeostasis is restored via the activation of the apoptotic pathways; CHOP, caspase-4/caspase-12, and c-Jun NH2-terminal kinase (JNK) signaling pathways [15,16]. Under normal conditions, CHOP is expressed at very low levels, whereas stresses such as I/R injury, oxidative stress, and hypoxia, result in dramatically increased cellular CHOP expression [9,17,18]. In the present study, BiP/GRP78 expression was highest at 4 h after ischemia, while CHOP expression was highest at 24 h after ischemia. It suggests that cells activate first the BiP/GRP78 chaperone system to cope with mild stress which occurs early after I/R insult, and then activate secondarily the CHOP system when injury level is too high to be defended by the ER chaperones. Therefore, BiP/GRP78 expresses earlier periods after ischemia than CHOP. BiP/GRP78 is quickly synthesized under ER stress that leads to the accumulation of unfolded/misfolded proteins in the ER [1]. We found that I/R induces

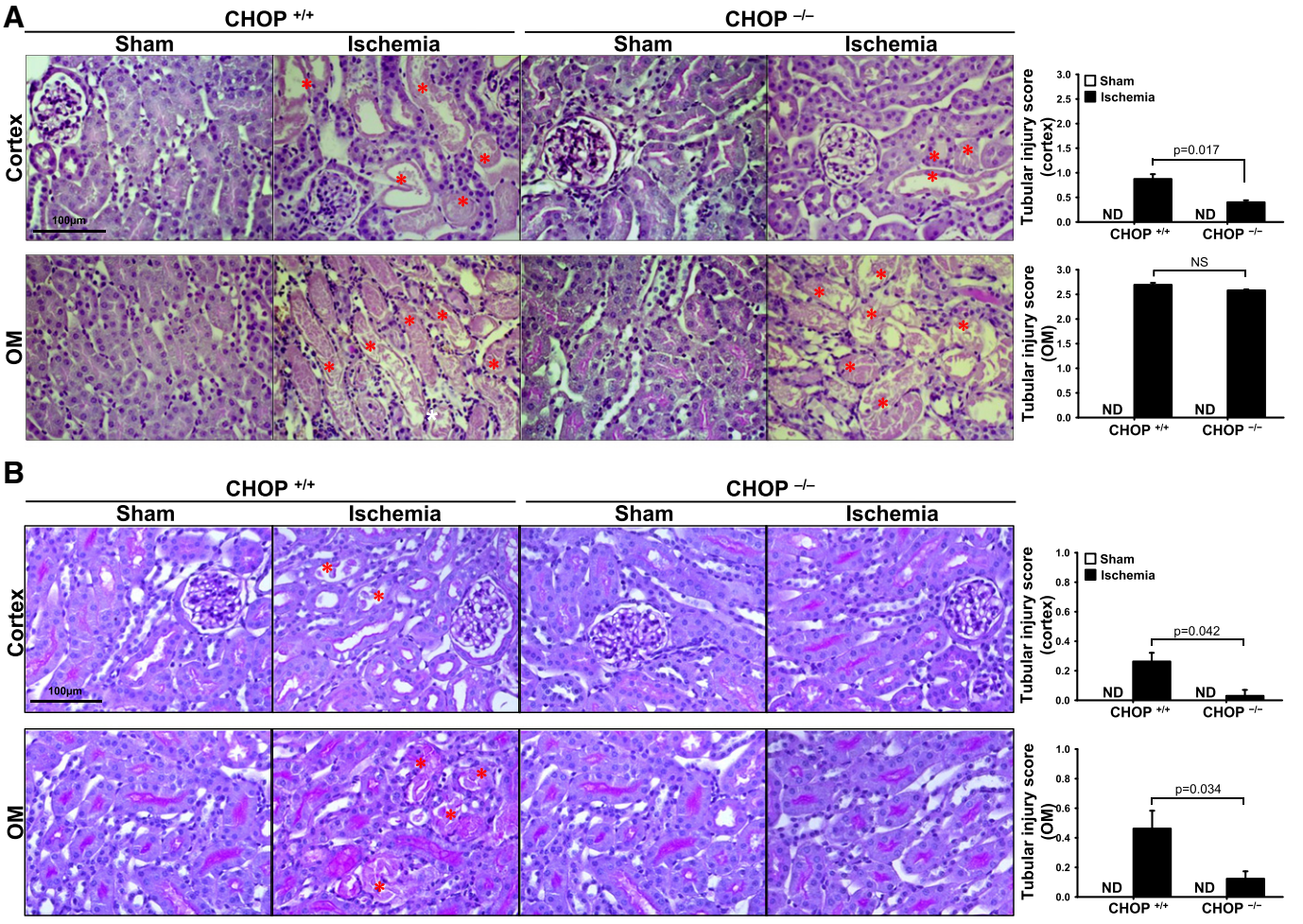

Fig. 2. Histological changes in CHOP$^{+/+}$ and CHOP$^{-/-}$ mouse kidneys 24 h after ischemia. CHOP$^{+/+}$ and CHOP$^{-/-}$ mice were subjected to either sham operation or 30 min (A) and 15 min (B) of bilateral renal ischemia. Twenty-four hours after ischemia, kidneys were harvested and PAS-stained. Pictures were taken of the cortex and the outer medulla (OM). Tubular damage scores in the cortex and the outer medulla were determined as described in the Materials and methods section. Asterisks indicate injured tubules. Data are presented as means ± SE (n = 6). ND, non-detectible.

apoptosis of the tubular epithelial cells and elevates CHOP expression, and I/R-induced apoptosis was reduced by CHOP-gene deletion. In addition, CHOP was localized to the nucleus after ischemia. These results indicate that I/R insult induced ER stress in the kidney tubular epithelial cells, leading to CHOP-mediated apoptosis in the kidney.

Apoptosis is triggered through two signaling pathways, an extrinsic and intrinsic pathway. The extrinsic pathway begins outside the cell via the activation of pro-apoptotic receptors on the cell surface [19]. The intrinsic pathway is initiated from within the cell. CHOP regulates the intrinsic apoptosis pathway by inhibiting Bcl-2 functions [11]. The intrinsic pathway is dependent on the Bcl-2 family of proteins [19,20]. The Bcl-2 protein family consists of three major protein subgroups: the BH3 (Bcl-2 homology)-only proteins (e.g., Bid, Bad), Bax-like proteins (e.g., Bax, Bak), and the Bcl-2-like factors (e.g., Mcl-1, Bcl-xL) [21]. BH3-only and Bax-like proteins have pro-apoptotic roles, while the Bcl-2-like proteins have an anti-apoptotic role. The pro-apoptotic proteins Bax and Bak induce the efflux of cytochrome c from the mitochondria into the cytosol by forming pores in the outer mitochondrial membrane [22]. Cytochrome c then activates caspase-9 with Apaf-1 and pro-caspase-9, leading to the subsequent activation of caspase-3 and caspase-7 [23]. It has been suggested that ER stress recruits Bak and Bax into the ER and initiates apoptosis [24], whereas the overexpression of Bcl-2 in the ER protects renal tubular epithelial cells against ER stress-induced apoptosis [25]. Gaudette et al. reported that Bcl-xL protects against CHOP-dependent apoptosis in neuronal cells [26]. In the kidney and liver, Bi-1, a Bax inhibitor-1, reduces ER stress and I/R injury [8]. In the present study, I/R reduced Bcl-2 and Bcl-xL expression, whereas it increased Bax expression, resulting in an increase in the Bax to Bcl-2 ratio. The post-I/R increase in the Bax to Bcl-2 ratio was less in the *CHOP*$^{-/-}$ mouse kidney compared to that in the *CHOP*$^{+/+}$ mouse kidney. These data indicate that reduced kidney apoptosis in *CHOP*$^{-/-}$ mice may be associated with reduced intrinsic apoptosis. In contrast to our data, Chen et al. did not find any significant difference in the expression of Bax and Bcl-2 between the *CHOP*$^{-/-}$ and *CHOP*$^{+/+}$ mice exposed to 15 min of bilateral renal ischemia [11]. The discrepancies between the results of Chen et al. and those presented here may be owing to the differences in strength and/or duration of the ischemia produced; they induced 15 min of ischemia, while we induced 30 min of ischemia, which can cause more severe kidney tubular epithelial cell death. Renal ischemia/reperfusion induces various types of tubular cell death including necrosis and apoptosis. Although in this study we focused on apoptotic cell death, necrosis also could majorly contribute to tubule cell death after severe I/R. In the present study, the necrotic cell death was also more severe in *CHOP*$^{+/+}$ mice than in *CHOP*$^{-/-}$ mice. It suggests that the apoptosis increased CHOP signal activation could activate other cell death signals secondarily.

XIAP and c-IAP2 bind directly to pro-caspase-9, inhibit the activation of caspases, especially caspase-3, and finally reduce apoptotic cell death [27,28]. Zhang et al. reported that heat shock protein 72, which is a well-known kidney I/R injury protective protein, suppresses I/R-induced

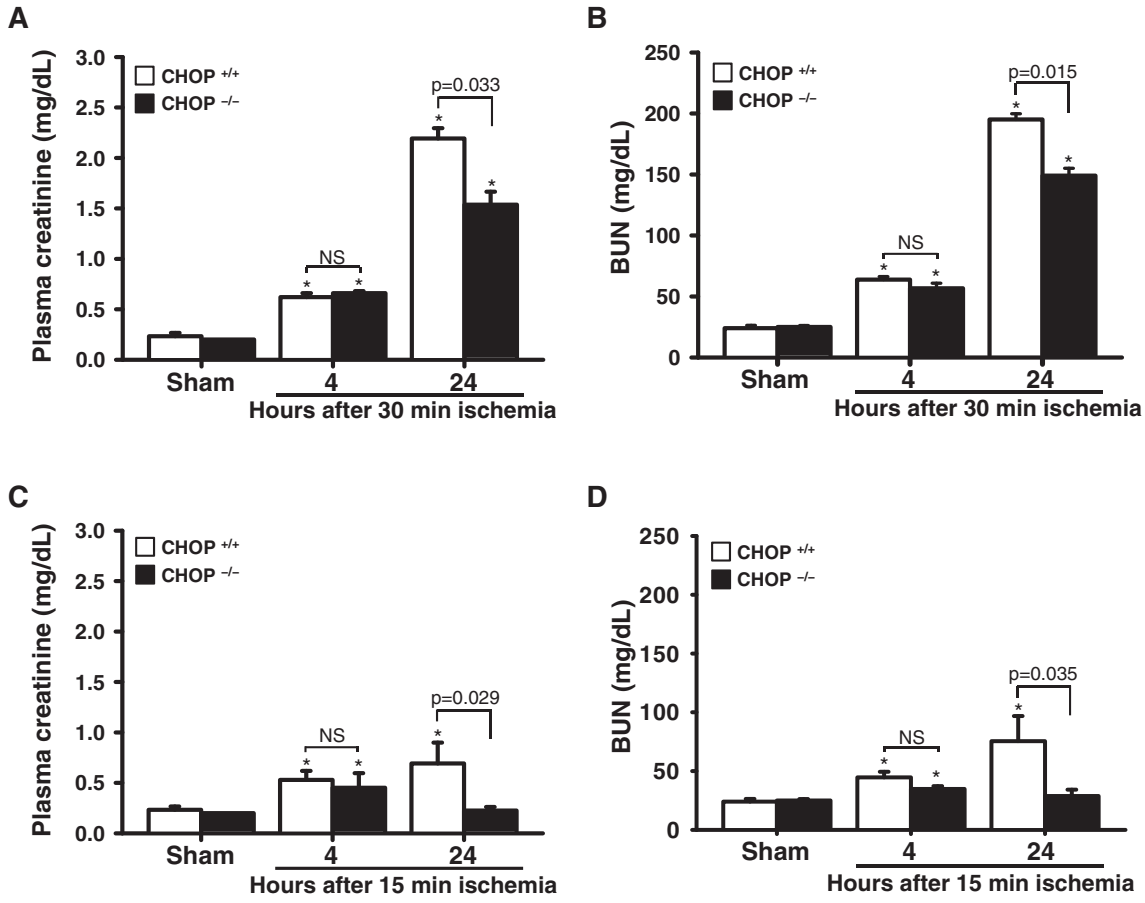

Fig. 3. Concentrations of plasma creatinine (PCr) and blood urea nitrogen (BUN) 4 and 24 h after ischemia in CHOP$^{+/+}$ and CHOP$^{-/-}$ mice. CHOP$^{+/+}$ and CHOP$^{-/-}$ mice were subjected to sham operation or 30 (A and B) and 15 (C and D) min of bilateral renal ischemia. Concentrations of plasma creatinine (PCr, A and C) and blood urea nitrogen (BUN, B and D) were determined 4 and 24 h after ischemia. Data are presented as means ± SE (n = 5–10). *, $p < 0.05$ vs. respective sham.

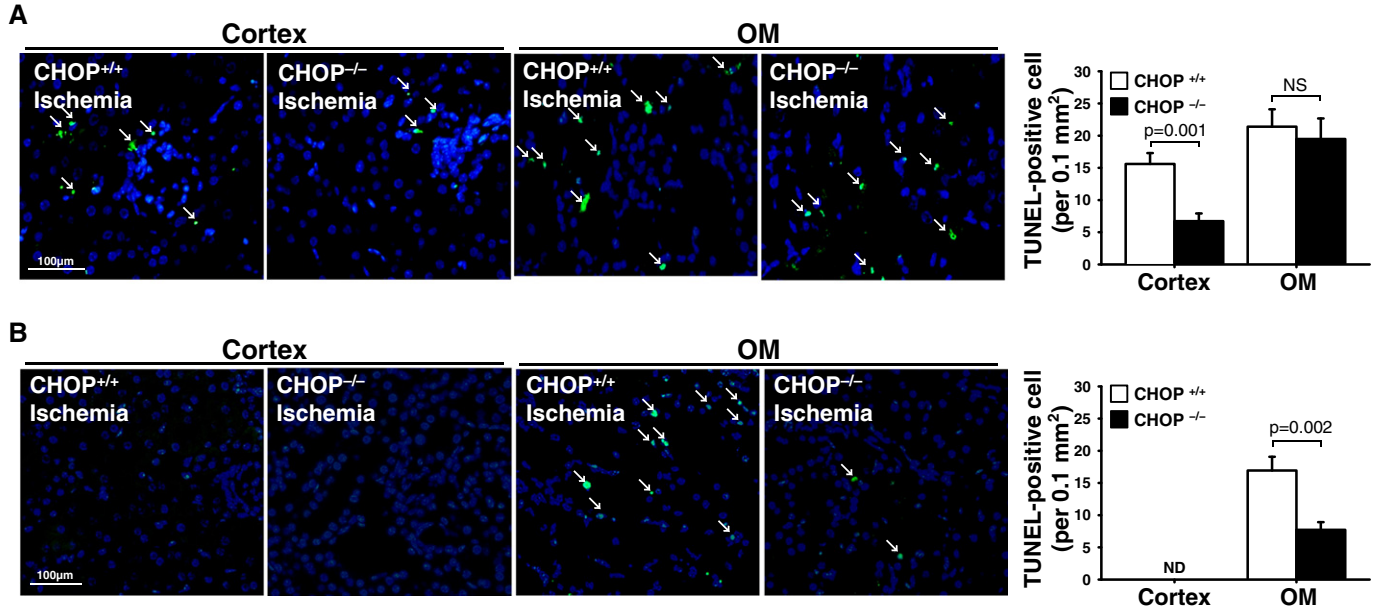

Fig. 4. Apoptosis in CHOP$^{+/+}$ and CHOP$^{-/-}$ mouse kidneys 24 h after ischemia. CHOP$^{+/+}$ and CHOP$^{-/-}$ mice were subjected to either sham operation or 30 (A) and 15 (B) min of bilateral renal ischemia. Twenty-four hours after ischemia, kidneys were harvested. Apoptosis was evaluated by a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Arrows indicate TUNEL-positive cells. Pictures were taken of the cortex and the outer medulla (OM). TUNEL-positive cells were quantified as the number of positive nuclei in a 0.1-mm^2 field of the kidney section (10 fields/kidney). The nuclei were stained blue with 4′,6-diamidino-2-phenylindole (DAPI). Data are presented as means ± SE (n = 6). ND, non-detectible.

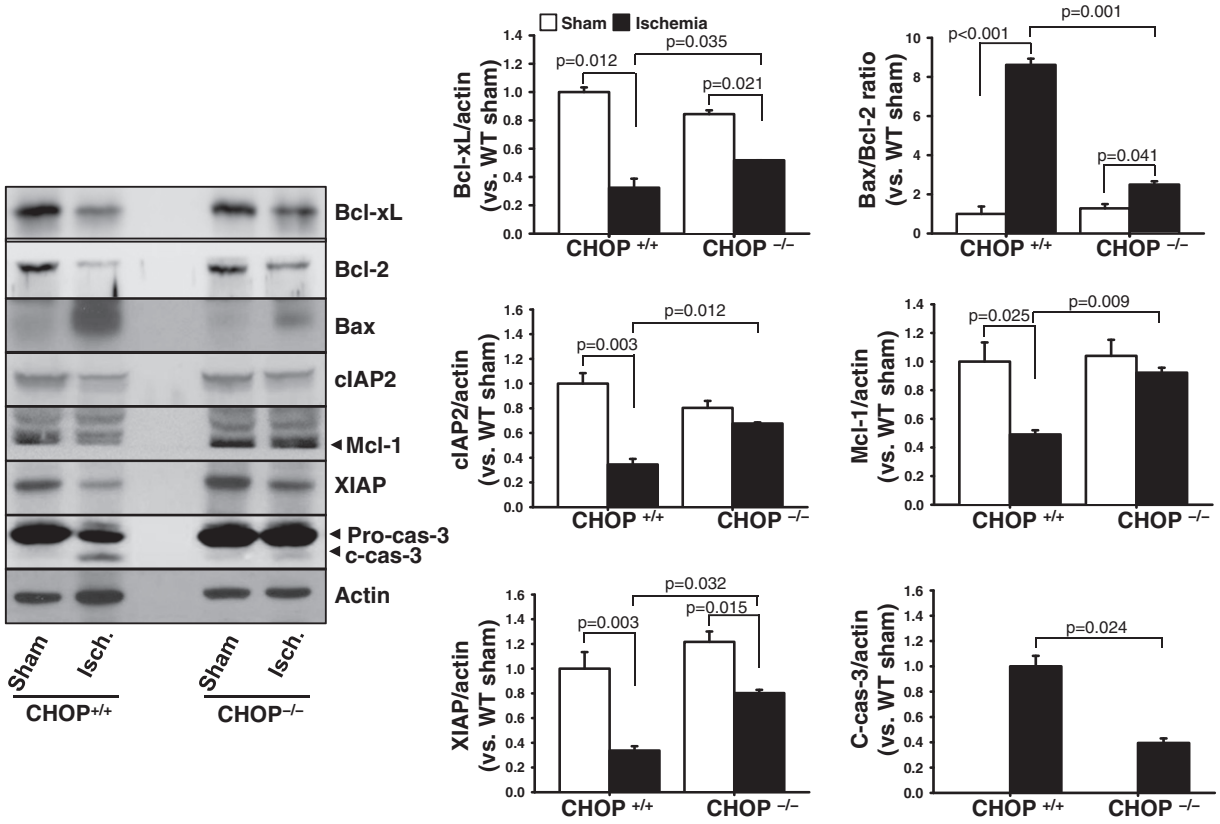

Fig. 5. Expressions of apoptosis-related proteins in CHOP$^{+/+}$ and CHOP$^{-/-}$ mouse kidneys 24 h after ischemia. CHOP$^{+/+}$ and CHOP$^{-/-}$ mice were subjected to either sham operation (Sham) or 30 min of bilateral renal ischemia (Isch.). Twenty-four hours after ischemia, kidneys were harvested. Expressions of Bcl-xL, Bcl-2, Bax cIAP2, Mcl-1, XIAP, and caspase-3 were determined in the kidney by western blot. Sham and I/R-induced protein levels of Bcl-xL, Bcl-2, Bax cIAP2, Mcl-1, XIAP, and caspase-3 were normalized with actin. Data are presented as means ± SE (n = 6).

apoptosis by increasing XIAP stability [29]. Overexpression of XIAP leads to increased cell death resistance, whereas the loss of XIAP increases ER stress-induced cell death, even in the absence of CHOP [30]. In addition, Bcl-2-like anti-apoptotic factors, such as Mcl-1 and Bcl-xL, form heterodimers with Bak and suppress Bak homo-oligomerization and pore formation to protect cells from apoptosis [31,32]. In the cisplatin-induced AKI model, cisplatin treatment induced apoptotic cell death with the depletion of Mcl-1 protein, and overexpression of Mcl-1 prevented apoptosis via the inhibition of Bax activation [33]. In the present study, we found that I/R insult induced caspase-3 activation via reductions in cIAP2, Mcl-1, and XIAP expression, and this post-I/R caspase-3 activation was reduced by CHOP deletion with concomitant reductions in cIAP2, Mcl-1, and XIAP. These results suggest that the inhibition of caspase-3 by CHOP deficiency lowers the kidney susceptibility of CHOP-deficient mice to I/R injury. It has been reported that caspase-3 is activated in the kidney after I/R and that the activation is mediated by the induction of CHOP [34]. Overexpression of CHOP induces apoptosis with a decrease in Bcl-2 expression, while deficiency of CHOP protects cells from apoptosis [2]. Overexpression of Bcl-2 also blocks CHOP-induced apoptosis [2] and has cytoprotective effects in renal injury models [25]. CHOP also contributes to cell cycle inhibition and the generation of reactive oxygen species, which is highly associated with I/R-induced cell death [9,13]. *CHOP*$^{-/-}$ mice also exhibit reduced apoptosis in response to ER stress [35,36].

Inflammation is an important mediator of I/R injury. Recent studies have demonstrated that CHOP is associated with the inflammatory response [37,38]; activation of caspase 11–caspase 1 by CHOP stimulates IL-1 and IL-1β productions in the lung exposed to LPS. In this present study we found that that I/R induced inflammatory response, and the increase of inflammatory response was attenuated by CHOP deficiency,

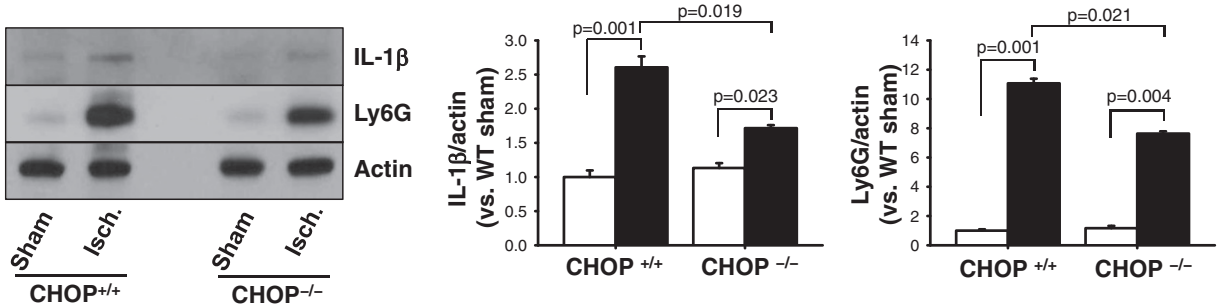

Fig. 6. Expression of inflammatory cytokine in CHOP$^{+/+}$ and CHOP$^{-/-}$ mouse kidneys 24 h after ischemia. CHOP$^{+/+}$ and CHOP$^{-/-}$ mice were subjected to either sham operation (Sham) or 30 min of bilateral renal ischemia (Isch.). Twenty-four hours after ischemia, kidneys were harvested. Expressions of Ly6G and IL-1β were determined in the kidney by western blot. Ly6G and IL-1β bands were normalized with actin. Data are presented as means ± SE (n = 6).

suggesting that reduced susceptibility in CHOP gene-deleted mice is associated with reduced inflammatory response.

Taken together, our findings demonstrate that I/R induces ER stress, leading to the activation of CHOP signaling pathways, and CHOP gene deletion protects the kidney against I/R injury by inhibiting apoptosis of tubular epithelial cells, suggesting that ER stress-mediated CHOP induction may be a promising molecular target for AKI.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Author disclosure statement

No competing financial interests exist.

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