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Transduced PEP-1-Heme Oxygenase-1 Fusion Protein Reduces Remote Organ Injury Induced by Intestinal Ischemia/Reperfusion

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: A fusion protein composed of heme oxygenase-1 (HO-1) and cell-penetrating peptide PEP-1 has been shown to reduce local intestinal injury after intestinal ischemia/reperfusion (I/R). In this study, we investigated the effects of PEP-1-HO-1 fusion protein on remote organ injury induced by intestinal I/R in rats.

Material/methods: We randomly assigned 24 male Sprague-Dawley rats to 3 groups: Sham, I/R, and I/R plus PEP-1-HO-1 treatment (HO). The model of intestinal I/R was established by occluding the superior mesenteric artery for 45 min followed by 120-min reperfusion. In HO group, PEP-1-HO-1 was administered intravenously 30 min before ischemia, while animals in the Sham and I/R groups received the equal volume of physiological saline. At the end of the experiment, lung, liver, and blood samples were collected and analyzed.

Results: Malondialdehyde levels and histological injury scores were increased, and superoxide dismutase activities were decreased in the lung and liver tissues in the I/R group compared with the Sham group ($P<0.05$). Serum levels of alanine aminotransferase, aspartate aminotransferase, tumor necrosis factor-α, interleukin-6, and lung tissue wet weight to dry weight ratio were increased in the I/R group compared with the Sham group ($P<0.05$). NF-κB expression in intestinal tissues was significantly higher in the I/R group than in the Sham group. These changes were significantly reversed by treatment with PEP-1-HO-1.

Conclusions: This study demonstrates that administration of PEP-1-HO-1 has a protective role against lung and liver injury after intestinal I/R, attributable to the reduction of released proinflammatory cytokines regulated by NF-κB.

MeSH Keywords: Cell-Penetrating Peptides • Heme Oxygenase-1 • Reperfusion Injury

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Background

Intestinal ischemia/reperfusion (I/R) injury may develop as a common consequence of acute mesenteric ischemia, hemorrhagic, septic or traumatic shock, thoracoabdominal aneurysm repair, severe burns, or some surgical procedures including small bowel transplantation and abdominal aortic surgery [1]. Intestinal mucosa is highly sensitive to I/R, and it is unavoidably leads to intestinal tissue injury. Furthermore, intestinal I/R not only results in local intestinal insults, but also causes remote organ damage and even multiple organ dysfunction or failure [2], including the liver [3–7], kidneys [6,7], and lungs [6–9].

Although the exact mechanisms regulating remote organ injury after intestinal I/R are not fully elucidated, clinical and experimental studies indicate that oxidant species, activated complements, generated chemokines, and activated neutrophils are responsible for remote organ injury after intestinal I/R [10]. In addition, many studies have demonstrated that circulating cytokines (such as tumor necrosis factor-α (TNF-α)) play a pivotal role in the process [11,12]. Administration of a neutralizing anti-TNF-α antibody before induction of intestinal ischemia alleviates the remote organ injury after intestinal I/R [11,13]. TNF-α is one of the inflammatory genes regulated by a nuclear factor kappa B (NF-κB) family of transcription factors. NF-κB is increasingly implicated in the development of I/R injury, including the brain and heart [14]. In our previous study, we generated a fusion protein composed of HO-1 and PEP-1 proteins, also known as PEP-1-HO-1 fusion protein, and found that PEP-1-HO-1 fusion protein decreased myocardial reperfusion injury via attenuating the production of proinflammatory cytokines regulated by NF-κB [15].

Heme oxygenase-1 (HO-1) is a rate-limiting enzyme in the heme degradation to free iron, carbon monoxide, and biliverdin/bilirubin [16]. Accumulating evidence shows that over-expression of HO-1 by gene therapy or chemical induction can used to protect against I/R-induced injury in many cells or organs [17–19]. Our previous research indicates that transduction of PEP-1-HO-1 fusion protein protects against intestinal I/R injury [20]. However, it is unknown whether PEP-1-HO-1 fusion protein protects against remote organ injury after intestinal I/R.

This study aimed to investigate: (1) whether PEP-1-HO-1 fusion protein protects against remote organ injury after intestinal I/R, and (2) whether PEP-1-HO-1 fusion protein decreases remote organ injury after intestinal I/R via suppressing the production of proinflammatory cytokines regulated by NF-κB.

Material and Methods

Production of PEP-1-HO-1 fusion protein

PEP-1-HO-1 fusion protein was prepared according to our previously described method [15]. The concentration of PEP-1-HO-1 fusion protein was assessed with the Bradford method and then stored at –80°C until use.

Animals and experimental groups

Twenty-four male Sprague-Dawley rats weighing 210–260 g were purchased from the Department of Laboratory Animal Center of Wuhan University, and were raised in individual cages in a temperature-controlled room with alternating 12 h light/dark cycles, and acclimated for a week before the study. All animals were fasted overnight before the experiments but allowed free access to tap water throughout the experimental procedure. The investigation was conducted in conformance to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Research Committee on Ethics of the Zhongnan Hospital of Wuhan University. Animals were randomly assigned to 3 groups (n=8 for each): Sham, I/R, and I/R plus PEP-1-HO-1 fusion protein treatment (HO).

Surgical procedure

All rats were first anesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneally). Additional anesthesia was administered to ensure stable anesthesia. A polyethylene catheter (PE-10) was inserted into the right carotid artery for monitoring mean arterial pressure and removal of arterial blood samples. A cannula in the left iliac vein provided access for the administration of PEP-1-HO-1 fusion protein or physiological saline. The model of intestinal I/R was established according to our previously described method [20]. The small intestine was exteriorized and the superior mesenteric artery (SMA) was dissected carefully and occluded with an atraumatic microvascular clamp (ischemia). After 45 min of ischemia, the clamp was removed for 120 min of reperfusion. Rats in the Sham group underwent the same surgical procedures without clamping. Half of 1 milligram of PEP-1-HO-1 fusion protein was administered via the left iliac vein 30 min before the start of ischemia in the HO group. The same volume of physiological saline instead of PEP-1-HO-1 was given with the same method as in the other groups.

Specimen collection

At the end of the experiment, the animals were killed with a lethal dose of anesthesia, and blood samples were collected via the right carotid artery for subsequent measurement of

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cytokines and enzyme activities. Tissues harvested from the left upper lung lobe and liver were preserved in 10% formalin for determination of histological analysis. The left lower lung lobe was used for the assessment of pulmonary edema. Tissues harvested from the right lung and the liver were immediately stored at -80°C for biochemical analyses.

Assessment of pulmonary edema

The severity of pulmonary edema was estimated by lung tissue wet weight to dry weight (W/D) ratio. The left lower lung lobe was removed and rinsed with saline. The wet weight of the lung tissue was determined and subsequently placed in a drying oven at 80°C for 24 h. After desiccation, the tissue was weighed again to obtain the tissue dry weight. Then, the W/D weight ratio in lung tissue was calculated.

Histological measurement

The isolated lung and liver tissues were fixed in 10% formalin. After embedded in paraffin, sections (5-μm thickness) were cut and stained with hematoxylin and eosin for histological evaluations under a light microscope. Each slide was evaluated by 2 pathologists in a blinded manner. Histologic injury was graded using scoring systems described by Sizlan et al. [6]. Histopathologic changes observed in lung sections were graded into 4 categories. Grade 0, no diagnostic change; grade 1, mild neutrophil leukocyte infiltrations and mild to moderate interstitial congestion; grade 2, moderate neutrophil leukocyte infiltrations, perivascular edema formation, and partial destruction of pulmonary architecture; and grade 3, dense neutrophil leukocyte infiltration and complete destruction of the pulmonary architecture.

Hepatic injury was evaluated by a point-counting method for the severity of hepatic injury with the use of the ordinal scale. Grade 0, minimal or no evidence of injury; Grade 1, mild injury consisting in cytoplasmic vacuolation and focal nuclear pyknosis; Grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, loss of intercellular borders; and Grade 3, severe injury with disintegration of hepatic cords, hemorrhage and neutrophil infiltration.

Assessments of malondialdehyde levels and superoxide dismutase activities in lung and liver tissues

Collected lung and liver tissues were immediately homogenized on ice using a homogenizer. The homogenates were centrifuged for obtaining the supernatants at 3000 rpm for 10 min at 4°C. Malondialdehyde (MDA) levels in the supernatants were evaluated using MDA assay kit (Jiancheng Biologic Project Company, Nanjing, China) according to the manufacturer's instructions. The amount of MDA was expressed as nmol/mg protein. Superoxide dismutase (SOD) activities in the supernatants were determined in accordance with the manufacturer's instructions (Jiancheng Biologic Project Company, Nanjing, China). SOD activity is expressed as U/mg protein.

Assessments of serum alanine aminotransferase, aspartate aminotransferase, and cytokine levels

Blood samples of the right carotid artery were collected in all animal groups. Serum was obtained by centrifuging blood for 10 min at 2500 rpm at 4°C and kept at –20°C until assays were performed. The serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were examined with an OLYMPUS AU1000 automatic analyzer (AusBio Laboratories Co., Ltd. Beijing, China). Values are expressed as U/L. The serum levels of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 were measured by use of enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Values are expressed as pg/ml.

Western blot analysis

NF-κB expression in intestinal tissues was determined by Western blot analysis as previously described by us [15]. Nuclear extracts were obtained from frozen intestinal tissues with protein extraction kit (Pierce, Meridian Road, Rockford, IL, USA) according to the manufacturer's instructions. The protein concentrations in the supernatant were determined by a BCA protein assay kit (Beyotime Biotechnology). Thirty micrograms extracted protein from each sample was loaded into each lane of 10% SDS polyacrylamide gel electrophoresis, then transferred to PVDF membranes (Millipore, Bedford, USA). Membranes were incubated with primary mouse anti-NF-κB p65 (1:500, SC-8008) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The membranes were washed in PBS-0.05% Tween-20 and incubated for 1 h in horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch). After washing in PBS-0.05% Tween 20, immune complexes were visualized using an enhanced chemiluminescence kit (Amersham, Piscataway, NJ) and the density of each band was quantified by densitometry using the Quantity One software (BioRad, Hercules, CA).

Statistical analysis

Statistical analysis was performed using SPSS statistical software (SPSS for Windows, Version 11.5, SPSS Inc, Chicago, IL). Data are expressed as means ±SD. Differences among the groups were analyzed by 1-way ANOVA and Student-Newman-Keul (SNK) test. *P* values of less than 0.05 were considered to be significant.

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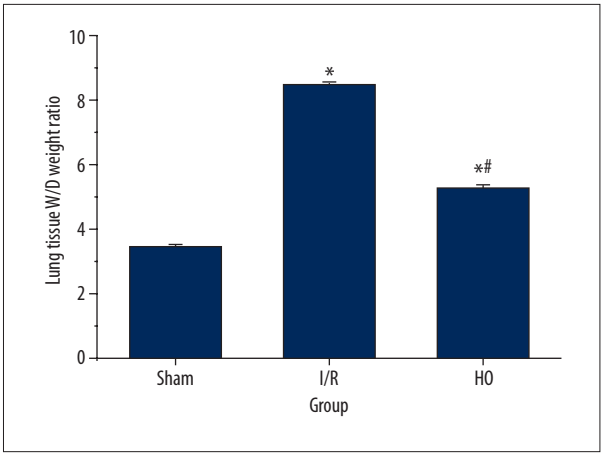

Figure 1. Lung tissue wet weight to dry weight (W/D) ratio in the Sham, ischemia/reperfusion (I/R), and I/R plus PEP-1-HO-1 fusion protein treatment (HO) groups. Data are expressed as means ±SD. * *P*<0.05 *vs.* Sham group. # *P*<0.05 *vs.* I/R group. n=8 rats/group.

Results

Effect of PEP-1-HO-1 on lung tissue W/D weight ratio

In this study, we examined the lung tissue W/D weight ratio, an indicator of lung injury. W/D weight ratio in lung tissues is shown in Figure 1. Lung tissue W/D weight ratio was significantly higher in the I/R and HO groups in comparison to the Sham group (*P*<0.05). In contrast, lung tissue W/D weight ratio was significantly lower in the HO group when compared to the I/R group (*P*<0.05).

Histopathology of the lungs and livers

Representative lung and liver hematoxylin and eosin-stained sections of all groups are shown in Figures 2 and 3. There was no evidence of injury in the Sham animals (Figures 2A, 3A). Intestinal I/R induced apparent lung and liver injury, manifested as histological changes in the lung and liver with edema, hemorrhage and neutrophil infiltration, as well as varying degrees of

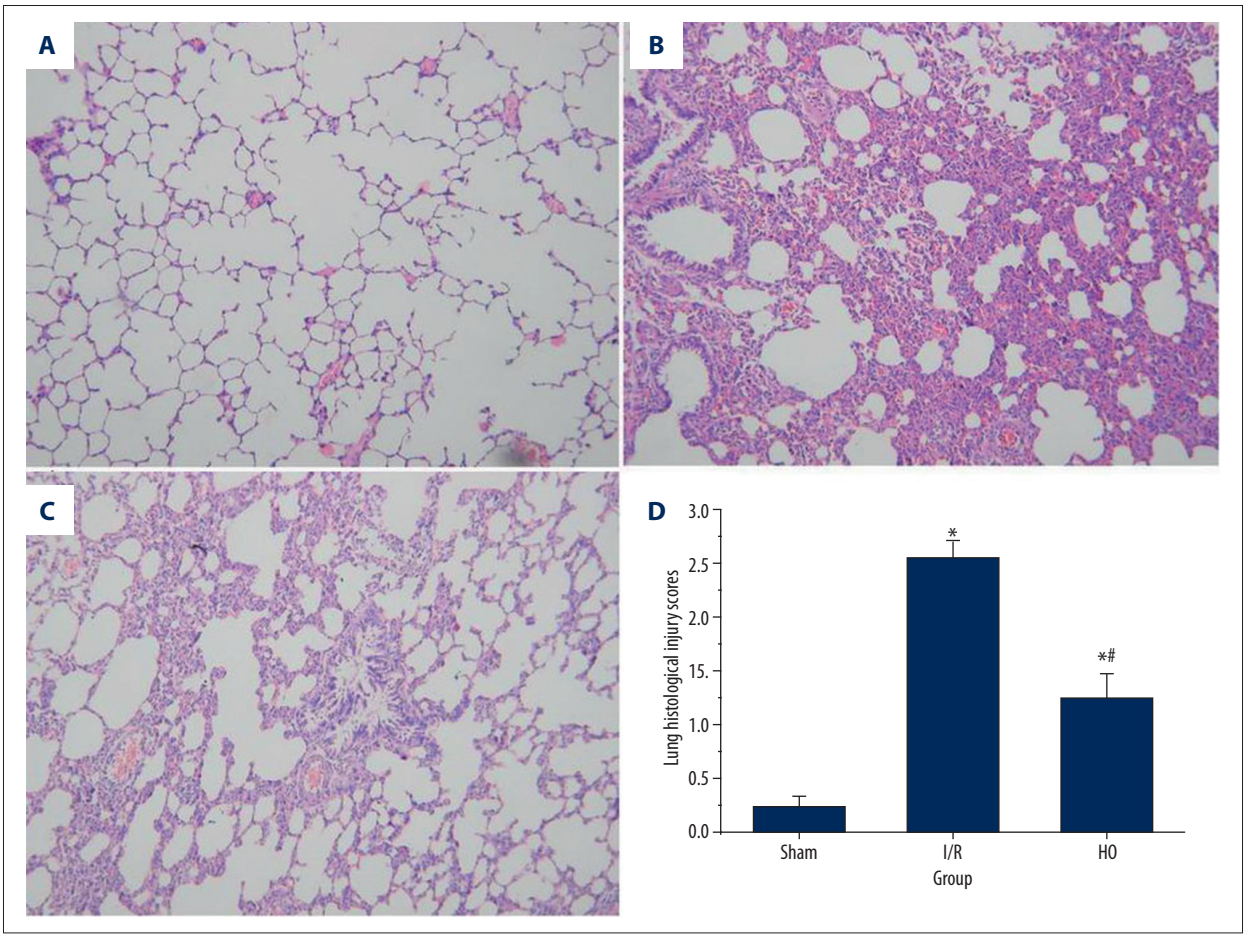

Figure 2. Representative hematoxylin and eosin-stained sections and histological injury scores of lung tissues in all groups. (**A**) Sham group. (**B**) Ischemia/reperfusion (I/R) group. (**C**) I/R plus PEP-1-HO-1 fusion protein treatment (HO) group (Original magnification, ×100). (**D**) Lung histological injury scores in all groups. Data are expressed as means ±SD. * *P*<0.05 *vs.* Sham group. # *P*<0.05 *vs.* I/R group. n=8 rats/group.

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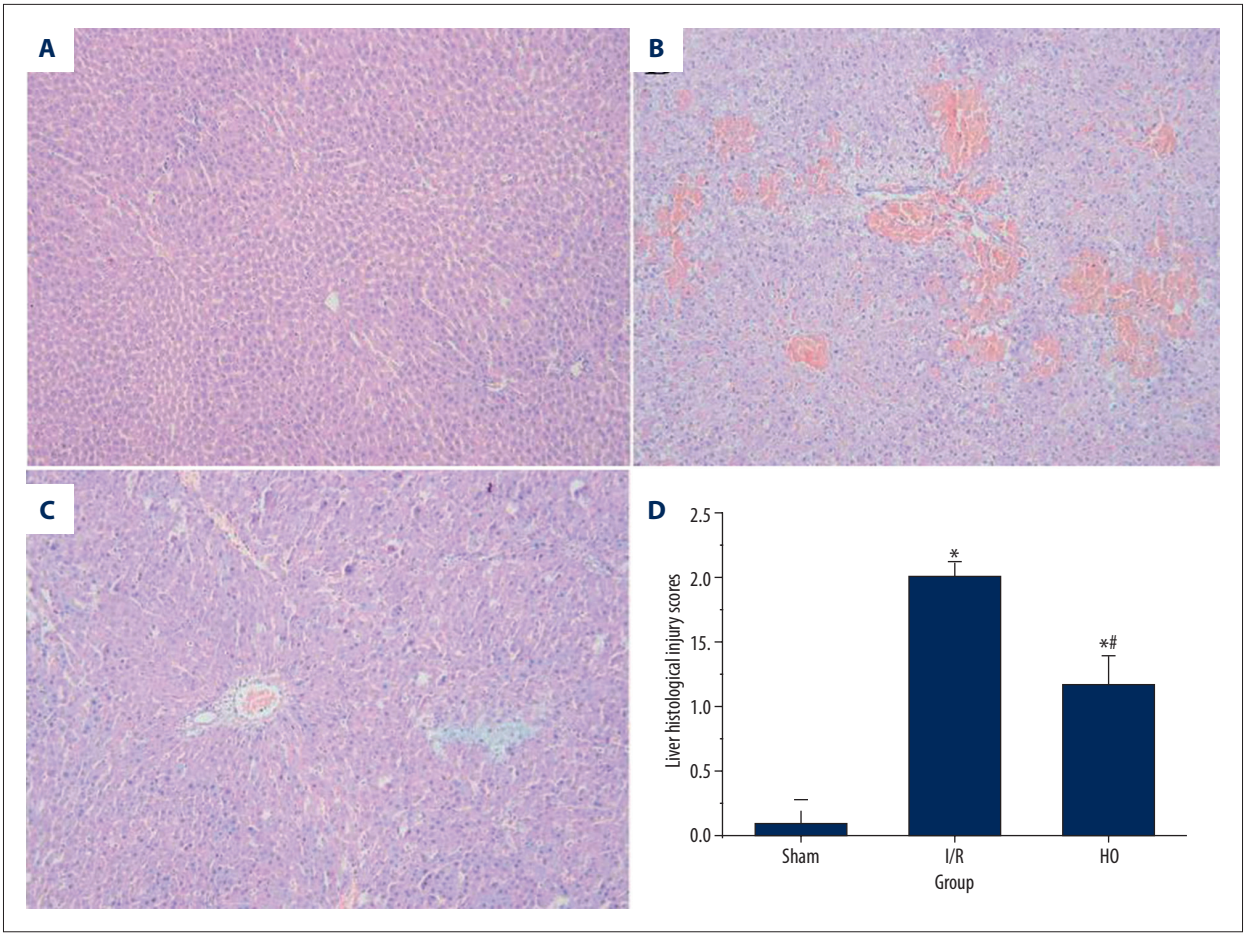

Figure 3. Representative hematoxylin and eosin-stained sections and histological injury scores of liver tissues in all groups. (**A**) Sham group. (**B**) Ischemia/reperfusion (I/R) group. (**C**) I/R plus PEP-1-HO-1 fusion protein treatment (HO) group (Original magnification, ×100). (**D**) Liver histological injury scores in all groups. Data are expressed as means ±SD. * *P*<0.05 *vs.* Sham group. # *P*<0.05 *vs.* I/R group. n=8 rats/group.

structural changes (Figures 2B, 3B), whereas treatment with PEP-1-HO-1 fusion protein obviously suppressed these morphological changes (Figures 2C, 3C). The lung and liver histological injury scores from all groups are shown in Figues 2D and 3D. Lung and liver histological injury scores in the Sham group were low. I/R resulted in increased lung and liver histological injury scores compared to the Sham group (*P*<0.05). Treatment with PEP-1-HO-1 fusion protein attenuated the increase in lung and liver histological injury scores compared to the I/R group (*P*<0.05).

Effects of PEP-1-HO-1 on MDA levels and SOD activities in the lung and liver tissues

MDA levels and SOD activities in the lungs and liver are shown in Figure 4. As shown in Figure 4A and 4B, the lung and liver tissue MDA levels were significantly increased in the I/R and HO groups in comparison to the Sham group (*P*<0.05). Treatment with PEP-1-HO-1 fusion protein reduced the MDA levels when compared to the I/R group (*P*<0.05). As shown in Figure 4C and 4D, the lung and liver tissue SOD activities in the I/R and HO groups were significantly lower than those in the Sham group (*P*<0.05). Treatment with PEP-1-HO-1 fusion protein markedly restored the SOD activities (*P*<0.05).

Effects of PEP-1-HO-1 on levels of serum ALT, AST, TNF-α, and IL-6

Serum levels of ALT, AST, TNF-α, and IL-6 are shown in Figure 5. I/R resulted in an increase of serum levels of ALT, AST, TNF-α, and IL-6 compared to the Sham group (*P*<0.05). Treatment with PEP-1-HO-1 fusion protein attenuated the increase in serum levels of ALT, AST, TNF-α, and IL-6 compared to the I/R group (*P*<0.05).

Effect of PEP-1-HO-1 on NF-κB expression in intestinal tissues

NF-κBp65 expression in intestinal tissues is shown in Figure 6. Western blot analysis showed weak NF-κB p65 positive signals

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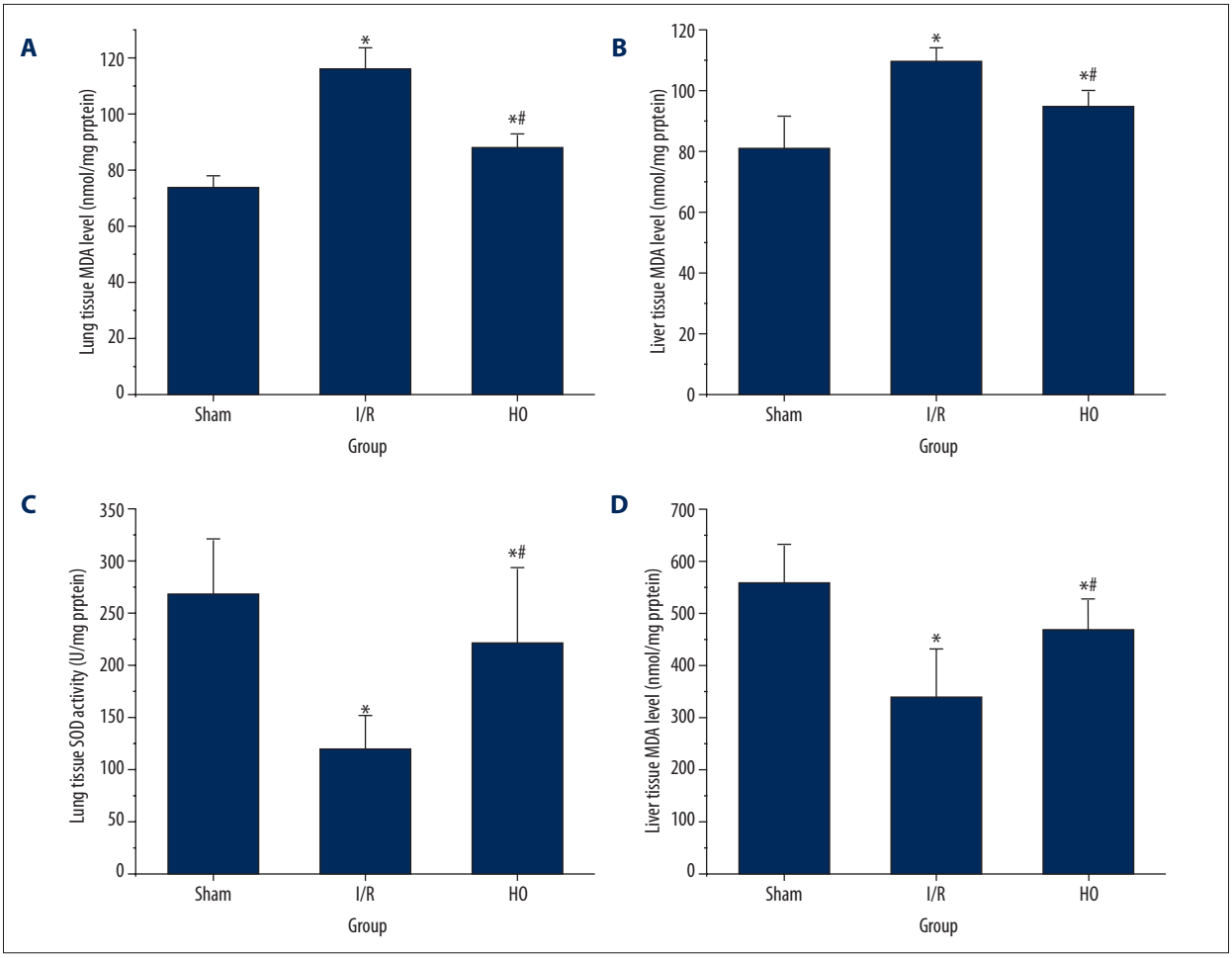

Figure 4. Effects of PEP-1-HO-1 on malondialdehyde (MDA) levels and superoxide dismutase (SOD) activities in lung and liver tissues. (**A**) The lung tissue MDA level of all groups. (**B**) The liver tissue MDA level of all groups. (**C**) The lung tissue SOD activity of all groups. (**D**) The liver tissue SOD activity of all groups. I/R: ischemia/reperfusion. HO: I/R plus PEP-1-HO-1 fusion protein treatment. Data are expressed as means ±SD. * $P<0.05$ *vs.* Sham group. # $P<0.05$ *vs.* I/R group. n=8 rats/group.

in the Sham group. In contrast, significant increase of NF-κB p65 expression was found in the I/R group. Compared with the I/R group, the signals were weakened in the HO group ($P<0.05$).

Discussion

HO-1 over-expression has been shown to attenuate I/R injury in various tissues. To the best of our knowledge, this is the first study using protein transduction technology to transduce the protective protein HO-1 into intestinal tissues for protection against remote organ injury induced by intestinal I/R. In this study, we found that PEP-1-HO-1 fusion protein had a protective role against remote organ damage induced by intestinal I/R, partly due to the decreased production of cytokines regulated by NF-κB.

Intestinal I/R is an important event, which can not only cause local intestinal insults, but also causes remote organ damage and life-threatening multiple organ failure. In various damaged organs, the lung and liver are organs susceptible to intestinal I/R [5,21,22]. In the present study, we found that 45 min of intestinal ischemia followed by 120 min of reperfusion induced the lung and liver damage, manifested as the histological evidence of lung and liver tissue changes and increase of lung and liver histological injury scores. Moreover, the intestinal I/R also initiated an increase in serum ALT and AST levels and the lung tissue W/D weight ratio, a marker of pulmonary edema. Previous studies have shown that oxidative stress has pivotal roles in the etiology of many types of organ damage caused by I/R [23,24]. In this study, we tested the levels of MDA and activities of SOD in the lung and liver tissues. Our results showed that MDA levels were higher in the I/R group in comparison to the Sham group. In contrast, SOD activities were greatly reduced in the lung and liver tissues after I/R injury. MDA is an established marker of oxidative stress. Thus, this indicates that oxidative stress caused by intestinal I/R

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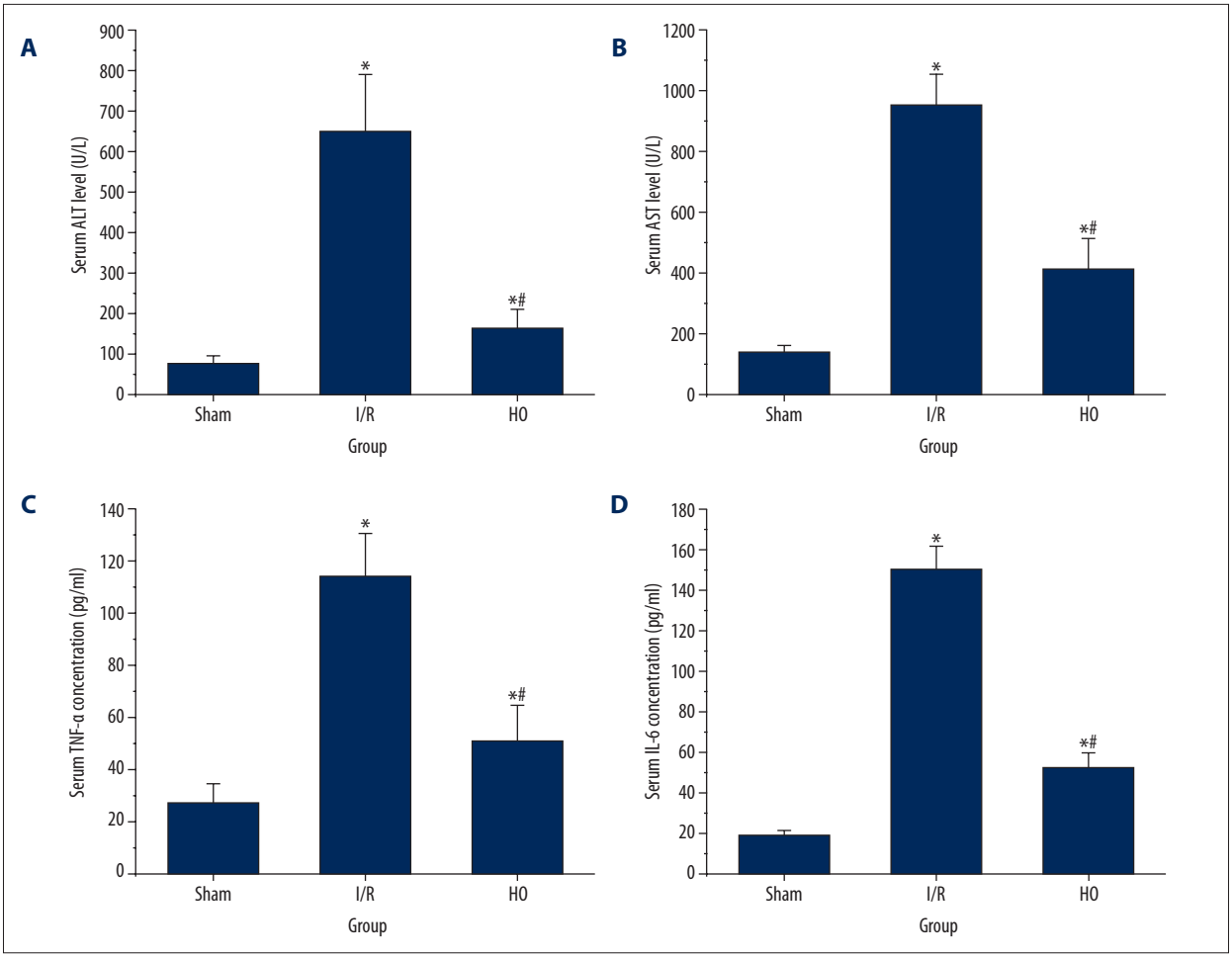

Figure 5. Effects of PEP-1-HO-1 on levels of serum ALT, AST, TNF-α, and IL-6. (**A**) The serum ALT level of all groups. (**B**) The serum AST level of all groups. (**C**) The serum TNF-α concentration of all groups. (**D**) The serum IL-6 concentration of all groups. I/R: ischemia/reperfusion. HO: I/R plus PEP-1-HO-1 fusion protein treatment. Data are expressed as means ±SD. * $P<0.05$ *vs.* Sham group. # $P<0.05$ *vs.* I/R group. n=8 rats/group.

may be an important cause of remote organ injury. Our findings agree with those of previous studies.

HO-1 is a ubiquitous heat shock protein that is highly induced by diverse stress-related conditions [25]. Many researchers have shown that upregulation of HO-1 expression in some tissues provides endogenous protection against a variety of oxidant-induced cell and tissue injuries [17–19,26]. Over-expression of HO-1 via different paths is identified to have a protective role against I/R injury in various organs such as the heart [27], liver [28] and kidneys [18,29], which is also found to provide protection against intestinal I/R injury [30]. Our previous study indicates that administration of PEP-1-HO-1 fusion protein has a protective role against intestinal I/R injury [20]. In addition, upregulation of HO-1 has been shown to have a protective effect on remote organ injury after intestinal I/R [31,32]. In the present study, we found that treatment with PEP-1-HO-1 fusion protein attenuated the histological injury in the lungs and livers caused by intestinal I/R. Our findings are consistent with those of the above studies. Furthermore, we also found that treatment with PEP-1-HO-1 fusion protein reduced MDA levels in lung and liver tissues. MDA, an end-product of lipid peroxidation caused by ROS, is considered as a reliable marker of oxidative damage. Therefore, our results also suggest that oxidative stress is involved in remote organ damage after intestinal I/R.

In this study, we found that intestinal I/R led to the increase in serum TNF-α and IL-6 concentrations. Administration of PEP-1-HO-1 fusion protein abrogated the elevation in serum TNF-α and IL-6 concentrations. Previous studies have suggested that these released cytokines are responsible for remote organ injury after intestinal I/R [33]. The concentration of TNF-α in serum is considered as a predictor of mortality following severe intestinal I/R injury [34]. Rocourt et al. demonstrated that intestinal I/R increased cytokine expression both locally and in

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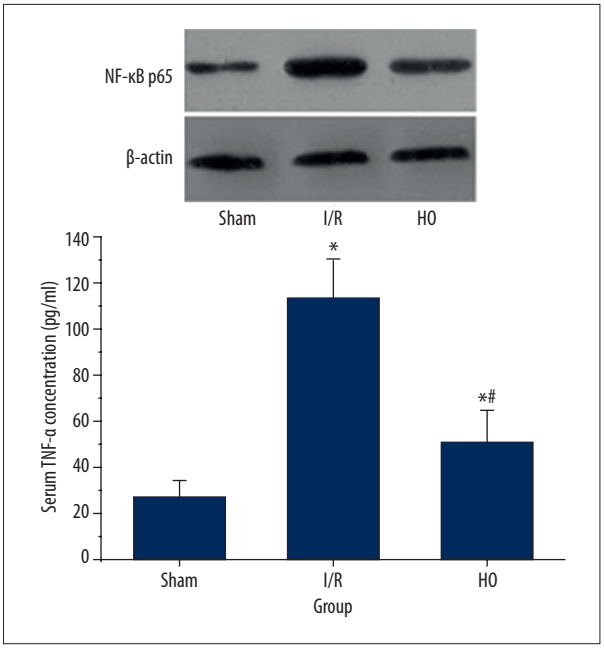

Figure 6. Effect of PEP-1-HO-1 on NF-κB expression in intestinal tissues. Data are expressed as means ±SD. * *P*<0.05 *vs.* Sham group. # *P*<0.05 *vs.* I/R group. n=6 rats/group.

the systemic circulation. Treatment with HB-EGF significantly reduced I/R-induced cytokine expression *in vivo* [35]. Our results are in line with those of previous studies. Systemic produced cytokines are closely involved in remote organ damage. However, the sources of the systemic cytokines are not entirely clear; various tissues such as reperfused intestine, liver, and endothelial cells are possible sources of the systemic cytokines [36]. However, in the present study, we did not investigate the source of inflammatory cytokines.

In addition, our results showed that intestinal I/R resulted in increased NF-κB expression in intestinal tissues, which paralleled the increase of serum TNF-α and IL-6 levels and lung and liver histological injury scores. In contrast, treatment with PEP-1-HO-1 fusion protein decreased the NF-κB expression in intestinal tissues, serum TNF-α, and IL-6 levels and lung and liver histological injury scores were attenuated. NF-κB is a transcription factor that participates in the induction of numerous immunoregulatory genes, including proinflammatory cytokines, growth factors, adhesion molecules, and chemokines. Many studies indicate that activation of NF-κB mediates I/R injury [14]. Giakoustidis et al. [37] found that administration of EGCG inhibited liver I/R-induced apoptosis and protected the liver by down-regulating NF-κB pathway. Our previous study shows that I/R increased NF-κB expression in myocardial tissues. Administration of PEP-1-HO-1 fusion protein markedly reduced elevated myocardial tissue NF-κB induced by I/R, which decreased myocardial infarct size and levels of serum TNF-α and IL-6 [15]. In addition, NF-κB plays an important role in the generation and resolution of intestinal I/R injury through various activation pathways. Intestinal I/R injury may be alleviated by inhibiting NF-κB activation [38–40]. Our results are consistent with these studies. However, the protective effects of PEP-1-HO-1 fusion protein on remote organ injury after intestinal I/R cannot be completely attributed to the decrease of circulating proinflammatory cytokines via inhibiting NF-κB activation. According to our preliminary experiment and results described by Kim et al. [41], some fusion proteins could be delivered into various cells or organs, including the lung and liver, which might provide a direct protective role against remote organ injury induced by intestinal I/R. In addition, although it has been shown that PEP-1 peptide does not exhibit tissue protective function in other animal models, it would be better to include a group of animals treated with PEP-1 alone to rule out the possibility that the beneficial effects we observed were not from PEP-1.

Conclusions

In conclusion, the present study demonstrates that administration of PEP-1-HO-1 fusion protein reduces remote organ damage caused by intestinal I/R via inhibiting the production of proinflammatory cytokines regulated by NF-κB. These results further support the potential use of PEP-1-HO-1 fusion protein as therapeutic approaches in some clinical settings mediated by intestinal I/R.

Conflicts of interest

There are no conflicts of interest for any of the authors.

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