Transduced PEP-1–heme oxygenase-1 fusion protein protects against intestinal ischemia/reperfusion injury

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1. Introduction

Among the internal organs, the intestine is probably one of the most susceptible organs to ischemia/reperfusion (I/R) injury. I/R injury is a common consequence of acute mesenteric ischemia, resuscitation, hemorrhagic, traumatic or septic shock, thoracoabdominal aneurysm repair, severe burns, or some surgical procedures including small bowel transplantation and abdominal aortic surgery [1]. The underlying mechanisms of intestinal I/R injury are complex. Many clinical and experimental research have demonstrated that oxidative stress, neutrophils, endothelial factors, cytokines, and apoptosis are involved in this process [2].
Heme oxygenase-1 (HO-1), also known as heat shock protein 32, is a rate-limiting enzyme in the heme catabolization to free iron, carbon monoxide, and biliverdin. Biliverdin is subsequently converted to bilirubin via the action of biliverdin reductase, and free iron is promptly sequestered into ferritin [3,4]. Accumulating studies indicate that HO-1 has antioxidant, anti-inflammatory, and anti-apoptotic functions [5]. HO-1 upregulation by pharmacologic means or via genetic engineering provides a protective role against I/R-induced injury in many organs including liver [6,7], heart [5,8], and kidney [9]. Conversely, hearts from HO-1 knockout mice have greater susceptibility to I/R injury [10]. Although some studies suggest that HO-1 plays an important role in intestinal I/R injury [11–14], little is known about its protective effect in intestinal I/R injury via genetic engineering.

Cell-penetrating peptides (CPPs) are promising delivery vectors for delivering biologically active molecules into cells in an active form. CPPs have been shown to efficiently improve intracellular delivery of various proteins into a large number of cells and tissues [15]. Among these CPPs, due to its outstanding delivery rates and mechanism of membrane translocation, PEP-1 is a CPP of particular interest [16]. PEP-1 has been successfully used to deliver various PEP-1 fusion proteins into a large variety of cells as well as tissues, such as skin cells [17], macrophage Raw 264.7 cells [18,19], astrocyte and neuronal cells [20], and myocardium [21]. However, it is unknown whether PEP-1 can be used to transduce some protective molecules into intestinal tissues.

In the present study, we hypothesized that HO-1 could be transduced into intestinal tissues by PEP-1 and which had a protective role against intestinal I/R injury. Thus, we generated a fusion protein composed of HO-1 and PEP-1 proteins, also known as PEP-1-HO-1 fusion protein, and investigated the effects of PEP-1-HO-1 fusion protein on intestinal injury following I/R in vivo in rats.

2. Materials and methods

2.1. Production of PEP-1-HO-1 fusion protein

PEP-1-HO-1 fusion protein were produced and purified according to the method described by Zhang et al. [21]. The protein concentration was estimated with the Bradford method before the start of the experiments.

2.2. Animals

Male Sprague-Dawley rats weighing 210–260 g (6–8 wk) were purchased from the Department of Laboratory Animal Center of Wuhan University and kept under standardized conditions of food, water, light, and temperature. All animals were fasted for 12 h before 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 U.S. National Institute of Health (NIH Publication No. 85-23, revised 1996) and approved by the Research Committee on Ethics of the Zhongnan Hospital of Wuhan University.

2.3. Serum HO-1 levels

To determine the optimal dose of PEP-1-HO-1 fusion protein, 18 rats were randomly divided into three treatment groups: 0.25, 0.5, and 0.75 mg. To determine an appropriate time point for treatment, 24 rats were randomly divided into four times (0.5, 1, 3, and 6 h) after the injection of PEP-1-HO-1 fusion protein. The animals were anesthetized with sodium pentobarbital (50 mg/kg); PEP-1-HO-1 fusion protein was administered through the left iliac vein. Blood samples at the end of the experimental procedure in all groups were collected from the right carotid artery and centrifuged at 3000 g and stored at −20°C until they could be assayed. Serum HO-1 levels were measured by the use of enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Values were expressed as pg/mL.

2.4. Groups

Twenty-four animals were randomly divided into three groups (n = 8) which included: sham, intestinal I/R (II/R), and II/R + PEP-1-HO-1 fusion protein (HO).

2.5. Surgical procedure

The model of intestinal I/R injury was prepared as previously described by Yildiz et al. [22]. Animals were first anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). The right carotid artery was cannulated to monitor mean arterial blood pressure and heart rate. Additional doses of sodium pentobarbital were given during the procedure as needed to maintain anesthesia according to the hemodynamics. Under sterile technique, animals underwent a midline laparotomy. The small intestine was exteriorized and the superior mesenteric artery (SMA) was separated carefully from the surrounding tissue and occluded with an atraumatic microvascular clamp (ischemia) for 45 min. Next, the intestine was placed back into the abdominal cavity, and the incision was closed. After 45 min of ischemia, the abdominal cavity was reopened. The occluding clamp was removed and the intestine was returned to the peritoneal cavity; the abdomen was reclosed and reperfusion occurred for 120 min. Rats in the sham group underwent an identical surgical procedure but did not receive I/R injury. Half of 1 mg of PEP-1-HO-1 fusion protein was given via the left iliac vein 30 min before ischemia in HO group. The same volume of physiological saline instead of fusion protein. The animals were anesthetized with sodium pentobarbital. The small intestines, beginning from the duodenum to the terminal ileum, were excised for the determination of enzyme activity and histopathologic analysis.

2.6. Wet-to-dry weight ratio

At 2 cm proximal to the cecum, 5 cm intestinal tissue samples were removed and rinsed with saline. The wet weight of the intestine 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. The ratio of the wet-to-dry (W/D) weight was calculated to provide an assessment of the extent of intestinal edema.

2.7. Histologic injury scoring
Intestinal samples were immersed in 10% formalin for at least 24 h. Tissue was embedded in paraffin, cut into 5-μm sections, and placed on glass microscope slides. Sections were stained with hematoxylin and eosin and then examined under a light microscope by two pathologists in a blinded manner. The histologic injury was graded using a system described by Chiu et al. [23]: grade 0 = normal mucosa, grade 1 = subepithelial space developing at the apex of the villus, grade 2 = extension of the subepithelial space with moderate lifting of epithelial layer from the lamina propria, grade 3 = massive epithelial lifting down the sides of villi, some tips may be denuded, grade 4 = denuded villi with lamina propria and dilated capillaries exposed, and grade 5 = hemorrhage, ulceration, and disintegrated lamina propria.

2.8. Western blot analysis
The recombinant plasmid containing the gene for the PEP-1-HO-1 fusion protein was constructed using a pET expression vector containing a His-probe. The His-probe protein allows for the detection of PEP-1-HO-1 fusion protein by Western blot analysis. Intestinal samples were homogenized and centrifuged at 12,000g for 10 min at 4°C. The protein concentrations in the supernatant were determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, China). Equal amounts of lysate were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were incubated with primary rabbit anti-His-probe (1:500, SC-804) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The membranes were washed in phosphate-buffered saline-0.05% Tween-20 and incubated for 1 h in horseradish peroxidase–conjugated secondary antibody (Jackson ImmunoResearch Laboratories, PA). After washed in phosphate-buffered saline-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 (Bio-Rad, Hercules, CA).

2.9. Assay of myeloperoxidase activity
Myeloperoxidase (MPO) activity as an index of polymorphonuclear neutrophil accumulation was assessed spectrophotometrically. After weighing, intestinal tissue samples were homogenized on ice using a homogenizer. MPO activity was measured quantitatively according to the manufacturer’s instructions (Jiancheng Biologic Project Company, Nanjing, China). One unit of MPO activity was defined as that which converted 1 μmol hydrogen peroxide to water per minute at 37°C. MPO activity was expressed as U/g wet weight.

2.10. Detection of lipid peroxidation and superoxide dismutase activity in intestinal tissues
Intestinal tissues were homogenized on ice in saline using a homogenizer. The homogenates were then centrifuged at 4000g for 10 min at 4°C. The supernatants obtained assayed for levels of lipid peroxidation product malondialdehyde (MDA) and superoxide dismutase (SOD) activity. MDA levels and SOD activity in the supernatants were determined according to the manufacturer’s instructions by using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China). Each measurement was performed in duplicate. The amount of MDA was expressed as nmol/mg protein; SOD activity was expressed as U/mg protein.

2.11. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay
To determine the possible role of cell death in intestinal injury, TUNEL staining was performed. Paraffin-embedded sections were deparaffinized, and in situ detection of cells with DNA-strand breaks was performed by the TUNEL-labeling method using an in situ cell death detection kit (Roche, Mannheim, Germany) following the manufacturer’s protocol. The number of TUNEL-positive cells was counted in four randomly chosen fields for each coded slide. Assays were performed in a blinded manner.

2.12. Statistical analysis
Statistical analysis was performed using SPSS statistical software (SPSS for Windows, Version 11.5; SPSS, Inc, Chicago, IL). All results are expressed as mean ± standard deviation. Differences among the groups were analyzed by one-way analysis of variance and Student-Newman-Keul test. Differences were considered significant when P < 0.05.

3. Results
3.1. Serum HO-1 levels after administration of PEP-1-HO-1
In this experiments, the influence of PEP-1-HO-1 fusion protein on serum HO-1 levels was examined. Results from this experiment indicated that serum HO-1 levels were dose-dependent (Fig. 1A). And time course studies showed that serum HO-1 was first detected 0.5 h after administration of PEP-1-HO-1; whereas peak serum HO-1 levels were reached within 2–3 h after administration of PEP-1-HO-1 (Fig. 1B). In the present study, we chose the model of 120 min reperfusion after 45 min ischemia according to the model described by Yildiz et al. [22]. In view of the model and the results of Zhang et al. [21] and our above experiments, we chose 0.5 mg as the therapeutic dose and 0.5 h before ischemia as the administration time point for all subsequent studies.
3.2. Effects of PEP-1-HO-1 fusion protein on intestinal W/D weight ratio

W/D weight ratio was used as an indicator of intestinal tissue damage after I/R. Compared with the sham group, the intestinal W/D weight ratio in II/R and HO groups were increased significantly ($P < 0.05$). Compared with the II/R group, the intestinal W/D weight ratio in HO group were significantly decreased ($P < 0.05$) (Fig. 2).

3.3. Effects of PEP-1-HO-1 fusion protein on histologic injury

Representative hematoxylin- and eosin-stained sections from sham, IIR, and HO groups are depicted in Figure 3. The animals in the sham group exhibited normal mucosal architecture and the animals in the IIR injury group exhibited significant histologic injury to the mucosa with denuded villi, disintegration of the lamina propria, and the appearance of exposed capillaries. The animals in the HO group exhibited only capillary congestion and moderate epithelial lifting from the lamina propria. The histologic injury scoring of the intestines from sham, IIR, and HO groups are shown in Figure 4. Rats in the sham group presented normal histology; the injury scoring was low. I/R aggravated the histologic injury; the injury scoring (grade 4.19 ± 0.48) was significantly greater than sham-operated animals (grade 0.15 ± 0.08). However, the injury scoring of PEP-1-HO-1–transduced animals was decreased significantly (grade 2.75 ± 0.39).

3.4. Transduction of PEP-1-HO-1 fusion protein into intestinal tissues

The presence of PEP-1-HO-1 fusion protein was identified by determining the expression of His-probe protein analyzed by Western blot analysis. Western blot analysis found that protein extracted from these PEP-1-HO-1–transduced intestinal tissues contained high concentrations of the His-probe protein, whereas no His-probe protein was detected in saline-transduced intestinal tissues (Fig. 5). This provided an indication that PEP-1-HO-1 fusion protein was successfully delivered into the intestines.

3.5. Effects of PEP-1-HO-1 fusion protein on MPO activity

The values of the tissue MPO activity and statistical differences of these measurements are shown in Figure 6. MPO activity in the IIR injury and HO groups was significantly higher than that in the sham group ($P < 0.05$). Compared with the IIR injury group, MPO activity in HO group was markedly
reduced ($P < 0.05$) but still higher than that in the sham group ($P < 0.05$).

### 3.6. Effects of PEP-1-HO-1 fusion protein on MDA levels and SOD activity

The values of the tissue MDA levels, SOD activity, and statistical differences of these measurements are shown in Figure 7. Intestinal I/R significantly increased the tissue MDA levels ($P < 0.05$) and decreased the antioxidant enzyme SOD activity ($P < 0.05$). Treatment with PEP-1-HO-1 significantly decreased the elevated tissue MDA levels ($P < 0.05$) and increased the reduced SOD activity in the tissues ($P < 0.05$).

### 3.7. Effects of PEP-1-HO-1 fusion protein on apoptotic cells

To determine the possible role of cell death in the intestinal injury, TUNEL assay was performed. There were few apoptotic cells in the sham group (Fig. 8A). A significant increase in TUNEL-positive cells along the villi was found in the IIR injury group (Fig. 8B). The number of apoptotic cells in the IIR injury group markedly increased compared with the sham-operated group ($P < 0.05$) (Fig. 8D). When rats were treated with PEP-1-HO-1 fusion protein, fewer TUNEL-positive cells were seen, and the number of apoptotic cells significantly decreased compared with the IIR injury group ($P < 0.05$) (Fig. 8C and D).

### 4. Discussion

In the present study, we demonstrated for the first time that intravenous transduction of the protective protein HO-1 into intestinal tissues by PEP-1 before injury confers intestinal protection from I/R injury, indicating that PEP-1 is an effective vector for delivering protective proteins into intestinal tissues, and HO-1 is an effective therapeutic target for intestinal protection from I/R injury.

Although the crucial function of cell membranes is to act as the barriers between the intracellular and extracellular environments, which protect a variety of cells and organs against the injuries caused by external factors, the membrane also acts as a barrier for the delivery of various exogenous therapeutic substances from entering cells. To overcome the cellular barrier and successfully deliver exogenous therapeutic substances into cells, a novel peptide carrier, PEP-1, was designed [16]. In the present study, we produced the PEP-1-HO-1 fusion protein and found that the level of serum HO-1 was dose- and time-dependent. And we found that PEP-1-HO-1 fusion protein was successfully transduced into intestinal tissues by determining the expression of His-probe protein. To date, many researchers have demonstrated that some antioxidant molecules have been successfully delivered via PEP-1 to protect against I/R injury, such as SOD1 [21,24], CAT [24,25], HSP27 [20], and metallothionein-III [26].

In the present study, we found that intestinal I/R led to the increase of MDA levels and MPO activity and was accompanied by a decrease of SOD activity. Although the exact mechanisms for the dissemination of intestinal I/R injury...
have not been fully elucidated, it is generally believed that polymorphonuclear neutrophils and mediators generated in the setting of oxidative stress, such as reactive oxygen species (ROS), play important roles [2,27]. Our results are in agreement with the conclusions. For attenuating the damage brought about by neutrophils and oxidative stress, many agents by different approaches are used to prevent intestinal I/R injury [11,28–30]. In the present study, the choice of HO-1 as a therapeutic agent is based on its excellent characteristics that a variety of studies have clearly revealed that HO-1 overexpression through different approaches play critical protective roles in some organ damage induced by I/R [6–9]. Simultaneously, accumulating experimental evidences suggest that induction of endogenous HO-1 through various pharmacologic or nonpharmacologic ways have been confirmed to have the protective role against intestinal I/R injury [11,31,32]. Although HO-1 gene transfer could be a specific and promising approach, which have been used to prevent the damage induced by I/R, such as heart [5,33] and liver [34], HO-1 gene transfer as a therapeutic method for intestinal I/R injury has not been reported. It is well known that there appears to be a critical time point of 6–8 h after infarction, and beyond this point, the damage to the intestine will be irreversible [29]. However, I/R induces HO-1 expression at 12 and 24 h after reperfusion in rats [32]. Therefore, for some sudden onset of ischemic intestinal diseases, it may be not timely and inadequate via various approaches with induction of endogenous HO-1 expression. Conversely, it is effective to use protein transduction technology to transduce HO-1 protein into the intestine for prevention or treatment of I/R injury. Our data showed that before I/R, the administration of PEP-1-HO-1 partially reversed the tissue damage, decreased intestinal W/D weight ratio and histologic injury scoring. These were accompanied by the decreases of MDA levels and MPO activity.

In this study, TUNEL staining was used as a marker for detection of apoptosis. We demonstrated that 45-min occlusion of the SMA followed by 120 min of reperfusion caused a significant increase in the number of TUNEL-positive cells in a rat model, and the intestinal apoptosis was accompanied by the dramatic increase of histologic injury. Previous studies have demonstrated that apoptosis is a major mode of cell death in the intestinal damage induced by I/R [35,36]. In previous studies dealing with I/R injury, erythropoietin enhanced functional and morphologic tissue recovery, mainly through its anti-apoptotic action [37]. In the present study, an increased number of TUNEL-positive cells in the intestinal tissue subjected to I/R was also reduced by PEP-1-HO-1, accompanied by the decrease of histologic injury. Oxidative stress is a major apoptotic stimulus in many diseases. Kojima et al. [38] have reported that oxidative stress after I/R plays an important role in induction of apoptosis in the intestine. In the present study, we found that treatment with PEP-1-HO-1...
attenuated enterocyte apoptosis and histologic injury scoring caused by intestinal I/R, accompanied by a decrease in MDA levels. MDA, an end product of lipid peroxidation caused by ROS, is considered as a reliable marker of intestinal oxidative damage. Therefore, our results demonstrate that the inhibitory effect of PEP-1-HO-1 on the I/R-induced ROS production may be the underlying mechanism for its protective effect against apoptosis.

In conclusion, our data show that transduction with a recombinant HO-1 protein fused to the PEP-1 confers cytoprotection against I/R injury in an I/R rodent model. Transduction with PEP-1-HO-1 fusion protein might provide a new strategy for protecting against intestinal insult resulting from ischemic intestinal damage.

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Conflict of interest: None declared.

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