BRIEF ARTICLE

Heme oxygenase-1 protects donor livers from ischemia/reperfusion injury: The role of Kupffer cells

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Abstract

AIM: To examine whether heme oxygenase (HO)-1 overexpression would exert direct or indirect effects on Kupffer cells activation, which lead to aggravation of reperfusion injury.

METHODS: Donors were pretreated with cobalt protoporphyrin (CoPP) or zinc protoporphyrin (ZnPP), HO-1 inducer and antagonist, respectively. Livers were stored at 4℃ for 24 h before transplantation. Kupffer cells were isolated and cultured for 6 h after liver reperfusion.

RESULTS: Postoperatively, serum transaminases were significantly lower and associated with less liver injury when donors were pretreated with CoPP, as compared with the ZnPP group. Production of the cytokines tumor necrosis factor-α and interleukin-6 generated by Kupffer cells decreased in the CoPP group. The CD14 expression levels (RT-PCR/Western blots) of Kupffer cells from CoPP-pretreated liver grafts reduced.

CONCLUSION: The study suggests that the potential utility of HO-1 overexpression in preventing ischemia/reperfusion injury results from inhibition of Kupffer cells activation.

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INTRODUCTION

Orthotopic liver transplantation (OLT) is an effective treatment for end-stage liver diseases[1]. However, ischemia/reperfusion (I/R) injury of the liver remains a major cause of graft injury, causing liver dysfunction and even failure posttransplantation[2,3]. The destructive effects of I/R arise from the acute generation of reactive oxygen species subsequent to reoxygenation, which inflicts direct tissue damage and initiates a cascade of deleterious

cellular responses leading to inflammation, cell death, and organ failure[4]. Endothelial cell damage results from free radicals produced from Kupffer cells and adherent polymorphonuclear leukocytes (PMNs)[5]. Ultimately, this results in loss of microvascular integrity and reduction of blood flow ('no-reflow phenomenon')[6]. Methods to protect liver grafts against I/R injury have considerable clinical consequences.

Kupffer cells, the resident macrophages of the liver, are involved in liver I/R injury through the release of cytokines such as tumor necrosis factor-α (TNF-α) and other biologically active mediators[7-9]. Multiple lines of evidence have suggested that Kupffer cells are critical to the onset of liver injury and following secretion, cytokines aggravate hepatocyte damage. The use of *in vitro* and animal models has shown that inactivation of Kupffer cells prevents liver injury[10,11]. Activation of Kupffer cells directly or indirectly by endotoxin (lipopolysaccharide, LPS) results in the release of an array of inflammatory mediators, growth factors, and reactive oxygen species.

The heme oxygenase (HO) system is the rate-limiting step in the oxidative degradation of heme into biliverdin, carbon monoxide (CO) and free iron[12]. Three HO isoforms have been identified: inducible HO-1, also known as heat shock protein 32; constitutively expressed HO-2; and a related but less well-characterized HO-3. HO-1 is induced in a variety of organs during diverse stress-related conditions and is thought to provide cytoprotection[13,14]. Upregulation of HO-1 is known to be a protective response from cellular stress, following I/R injury, radiation and inflammation[15]. Overexpression of HO-1 exerts a cytoprotective function in a number of I/R injury and liver transplant models[16-18]. Thus, HO-1 is an attractive target for anti-inflammatory therapies and potential candidate responsible for cell injury.

Immunochemical studies with specific monoclonal antibodies have revealed the distribution of HO-1 in the rat liver with distinct topographical patterns[19]. HO-1 has been shown to be expressed principally in Kupffer cells[16,20-22]. However, exact mechanisms by which HO-1 induction may lead to cytoprotection during I/R injury in organ transplantation have not been fully clarified. We designed a study to evaluate the role of HO-1-mediated cytoprotection in rat liver transplantation models. The aim was to demonstrate whether HO-1 plays a critical role in inhibiting Kupffer cells activation.

MATERIALS AND METHODS

Animals
Male Sprague-Dawley (S-D) Rats (Third Military Medical University Laboratory Animal Center, Chongqing, China) weighing 220-250 g were used. Animals were fed standard rodent chow and water *ad libitum* and cared for according to the local animal welfare guidelines. All procedures used in this study were approved by the ethics committee for the use of experimental animals at Kunming Medical College.

Synthetic metalloporphyrins
Metalloporphyrins (CoPP and ZnPP) were purchased from Sigma Chemical inc. USA. They were dissolved in 0.1 mol/L NaOH, subsequently adjusted to pH 7.4 with HCl, and diluted in 0.85% NaCl. The stock concentration was 0.5 mg/mL (CoPP) and 2 mg/mL (ZnPP).

Experimental design
S-D rats underwent ether anesthesia. The basic techniques of liver harvesting and orthotopic transplantation without hepatic arterial reconstruction were performed according to the method described previously by Kamada *et al*[23]. In the control group ($n = 8$), no drugs were applied. There were two treatment groups. In ZnPP group ($n = 8$), donors received ZnPP, an HO-1 inhibitor (20 mg/kg ip) 24 h prior to harvest. CoPP group ($n = 8$) rats received CoPP, an HO-1 inducer (5 mg/kg ip) 24 h prior to harvest. All liver grafts were harvested and stored with UW solution for 24 h at 4℃, and orthotopically transplanted into syngeneic S-D recipients. All transplant experiments in this study were performed by a single person. The anhepatic phase was 11.3 ± 0.7 min. Separate groups of rats were killed at 6 h after their vessels were unclamped, and liver samples were collected for further analysis.

Blood sample collection and serum liver enzymes
At 6 h following by liver reperfusion, blood was collected into ethylenediaminetetraacetic acid (EDTA) tubes. After centrifugation of whole blood (2000 *g*, 15 min), serum was stored at -70℃ until analysis. Serum alanine aminotransferase (ALT) and aspartate transaminase (AST) levels were measured using an automated clinical analyzer (7060 automatic analyzer, Hitachi, Japan).

Histopathology
Liver graft tissues were fixed in 10% formalin, embedded in paraffin. Serial sections of 3 μm thickness were stained with hematoxylin and eosin for routine pathological examination.

Isolation and culture of Kupffer cells
Kupffer cells were isolated from integral left liver tissue obtained from fresh specimens. The liver was perfused *in vitro* through the vena cava with 80 mL Hanks' balanced salt solution (HBSS) Ca^{2+}/Mg^{2+}-free (Hyclone, Germany) at 37℃, and transferred to a 100 mm culture dish and perfusion was continued with complete HBSS containing 0.05% collagenase IV (Sigma, USA) and 3 mmol/L Ca^{2+} at 37℃. Liver tissue was finely diced into 2 mm^3 sized pieces and the suspension incubated under constant agitation at 37℃ for 30 min. The liver homogenate was filtered through gauze mesh and the cells suspension was centrifuged at 50 *g* for 3 min at 4℃ to remove hepatocytes. The non-parenchymal cells-enriched supernatant was centrifuged at 400 *g* for 6 min. The cell pellet was resuspended in 30% Percoll (Pharmacia, Sweden) with a density of 1.040 g/mL, and this was carefully layered onto 60% Percoll with a density

Table 1 Primers used for RT-PCR

Gene	Primer sequence	Size of amplified DNA (bp)	Annealing temperature (℃)
HO-1	F 5' TGGAAGAGGAGATAGAGCGA 3'	451	53
HO-1	R 5' TGTTGAGCAGGAAGGCGGTC 3'	451	
CD14	F 5' GGACCCGATCTCAACACCT 3'	377	55
CD14	R 5' CCAGCAGTATCCCGCAGT 3'	377	
β-actin	F 5' CGGGAAATCGTGCGTGAC 3'	443	55
β-actin	R 5' TGGAAGGTGGACAGCGAGG 3'	443	

RT-PCR: Reverse-transcriptase polymerase chain reaction; F: Forward; R: Reverse.

of 1.075 g/mL. The double layer discontinuous gradient formed was overlaid with 3 mL of HBSS and centrifuged at 400 *g* for 15 min at 4℃. The opaque interface was collected, resuspended in HBSS and centrifuged at 400 *g* for 5 min at 4℃. The cells were seeded onto tissue culture plates at a density of 2×10^6/mL and cultured in RPMI 1640 medium (Gibco, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA), 100 U/mL Penicillin/Streptomycin (Hyclone, Germany) and 10 mmol/L HEPES at 37℃ with 5%CO_2. Nonadherent cells were removed after 2 h by gentle washing the plated cells and replacing the culture medium. Adherent cells were incubated for 48 h before performing uptake assays. More than 95% of adherent cells were ED2 (Serotec, UK) positive.

Reverse-transcriptase polymerase chain reaction (RT-PCR)
Total RNA was extracted from Kupffer cells at 48 h after culture or liver tissues with Trizol (Invitrogen, USA). Reverse transcription was performed on 1 μg RNA using random primers. Reverse-transcription reaction product (PrimeScript™ RT-PCR Kit, TaKaRa) was used for PCR reaction, with initial heating at 94℃ for 5 min, followed by 40 cycles of 94℃ for 30 s, 53-55℃ for 30 s, 72℃ for 1 min, and finally 72℃ for 7 min. The primers are listed in Table 1. The PCR products were subjected to electrophoresis on 1.5% agarose gel containing ethidium bromide and visualized by UV illumination. β-actin was used as an internal control for RNA integrity.

Western blotting analysis
Proteins were extracted from Kupffer cells with radioimmunoprecipitation (RIPA) containing phenylmethyl sulfonylfluoride (PMSF). Protein quantification of samples was performed using the BCA assay. Proteins (15 μg/sample) in SDS-loading buffer were heated to 100℃ for 5 min, subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane. The membrane was blocked overnight in 5% nonfat dry milk in TBST buffer at 4℃. Blots were incubated at room temperature (RT) for 2 h with anti-CD14 (dilution, 1:200, Santa Cruz Biotechnology, Inc. USA) in TBST buffer with 5% nonfat dry milk. After washing in TBST buffer three times at RT for 10 min, blots were incubated for 2 h at RT
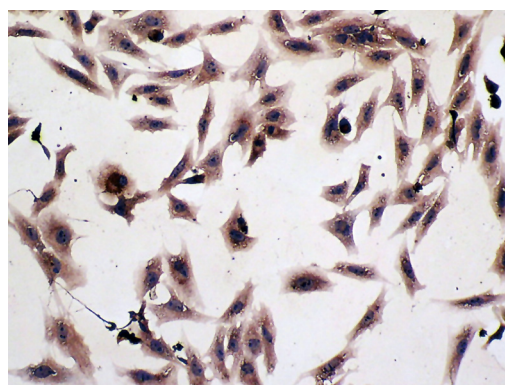

Figure 1 ED2 immunocytochemistry staining of cultured Kupffer cells. More than 95% are ED2 positive. Cells adhere to plastic and exhibit a spread out morphology and irregular shape (× 400).

with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (dilution, 1:50 000, Pierce Biotechnology) in TBST buffer with 5% nonfat dry milk. Finally, membranes were washed three times and developed with ECL (Amersham Pharmacia Biotech, Piscataway, New Jersey).

Measurement of cytokine release
The medium from Kupffer cells culture was collected and centrifuged at 1000 *g* for 5 min, and supernatant was kept -70℃ until assayed. IL-6 and TNF-α levels in the supernatant were determined with rat-specific enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc. USA). All samples, including standard and control solution, were assayed in duplicate.

Statistical analysis
Statistical analysis was performed using SPSS Version 13.0 for Windows (SPSS, Inc, Chicago, IL). All data are expressed as mean ± SE. Difference between experimental groups were analyzed using one-way analysis of variance or Student's *t* test. All differences were considered statistically significant at the *P* value of < 0.05.

RESULTS
Primary cultures of Kupffer cells
The isolated Kupffer cells were seeded onto tissue culture plates and incubated for 48 h. Cells attached rapidly to the dish surface, and spread in an irregular outline after 48 h in culture (Figure 1). More than 95% were ED2 positive on immunocytochemistry staining.

Liver HO-1 expression levels before transplantation
To assess HO-1 expression in experimental livers that were either untreated or pretreated with an HO-1 inducer or inhibitor, we performed RT-PCR analyses. A wide variation in HO-1 gene expression was detected in liver tissues that were collected before transplantation (Figure 2A). Livers harvested from donors that were pretreated with CoPP significantly up-regulated HO-1 mRNA expression levels as compared with control or the ZnPP

A

Grafts HO-1 expression levels pretransplant

B

C

	Control	ZnPP	CoPP
Suzuki's score	8.19 ± 0.89	10.28 ± 0.82	4.40 ± 0.46
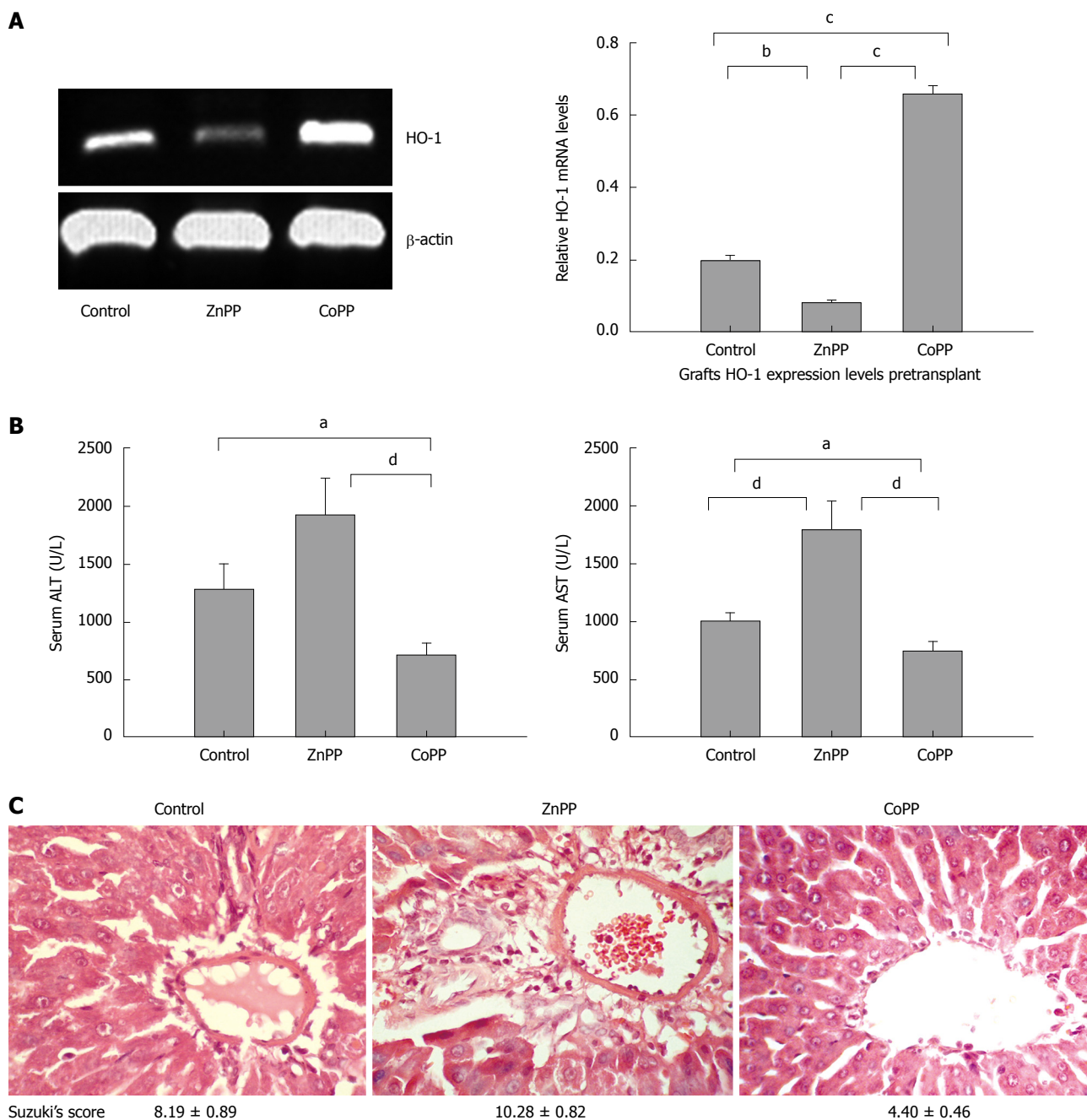
Figure 2 HO-1 overexpression ameliorates hepatic I/R injury. A: The expression of hepatic HO-1 was evaluated by RT-PCR before liver transplantation. β-actin was used as an internal control. Decreased expression of HO-1 mRNA was detected 24 h after ZnPP treatment. CoPP treatment increased HO-1 mRNA (*n* = 4/group). [b]*P* < 0.001; [c]*P* < 0.0001; B: Hepatocellular function. Serum ALT/AST levels (U/L) were higher in the ZnPP group as compared with controls. Significantly lower serum transaminase levels were seen in the CoPP group (*n* = 8/group). [a]*P* < 0.05; [d]*P* < 0.01; C: Representative photomicrographs of rat livers following 6 h reperfusion. Livers in ZnPP group show severe lobular distortion, sinusoidal congestion, ballooning, hepatocyte necrosis. Livers pretreated with CoPP exhibit good preservation of lobular architecture. Original magnification, × 400; HE stain.

group (0.658 ± 0.022, 0.198 ± 0.0165, 0.083 ± 0.008, respectively; *P* < 0.0001). ZnPP treatment decreased HO-1 mRNA as compared with control (*P* < 0.001).

HO-1 overexpression improves liver function

We analyzed the hepatocellular function in rats that underwent cold ischemia followed by 6 h of reperfusion in three animal groups. As shown in Figure 2B, pretreatment with CoPP significantly decreased serum ALT levels as compared with the control or the ZnPP group (711.37 ± 111.13, 1285.00 ± 219.46, 1932.13 ± 313.41, respectively, *P* < 0.05 *vs* control, and *P* < 0.01 *vs* ZnPP-treated control). Serum AST levels were also significantly reduced in the CoPP treatment group as compared with both the control and the ZnPP group (740.38 ± 84.71, 998.63 ± 69.45, 1795.50 ± 244.59, respectively, *P* < 0.05 *vs* control, and *P* < 0.01 *vs* ZnPP-treated control). Both ALT and AST levels were higher in the ZnPP group.
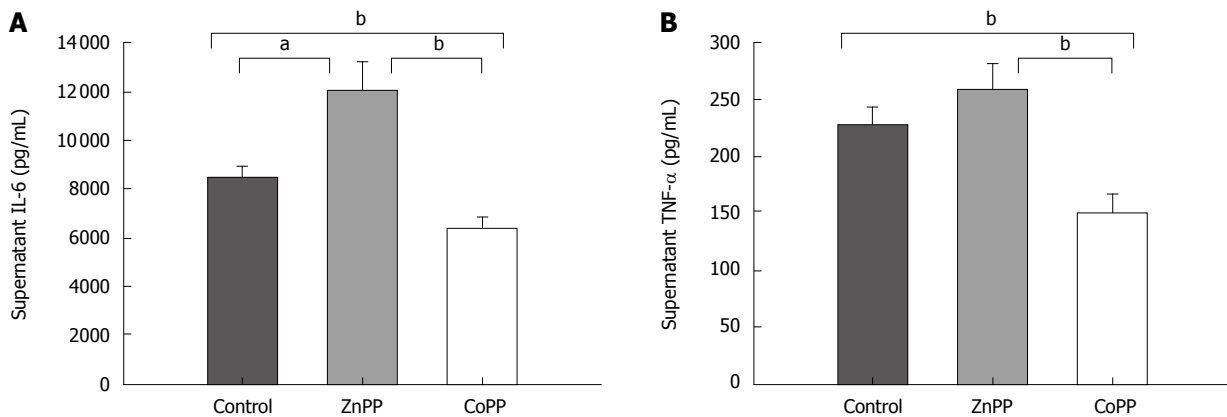
Figure 3 Concentration of IL-6 and TNF-α in cultured supernatant. Detection of cytokines at 48 h of Kupffer cell culture. ZnPP treatment increased cytokine levels of IL-6 (A) and TNF-α (B) were assayed. Significantly lower cytokine levels were detected in the CoPP group as compared with controls. The cytokine levels in cultured supernatant were measured by ELISA technique as described in Materials and methods (*n* = 5/group). [a]*P* < 0.05, [b]*P* < 0.01.

HO-1 overexpression ameliorates hepatic I/R injury

The hepatocellular damage was evaluated by liver histology. Livers in the ZnPP group showed a more severe hepatocyte necrosis, sinusoidal congestion and ballooning, as compared with controls. In contrast, livers in the CoPP group revealed almost complete preservation of lobular architecture without sinusoidal congestion, ballooning, or necrosis (Figure 2C). The hepatocellular damage was graded using a modified Suzuki's criteria[24].

Cytokines release

The cytokine levels (IL-6/TNF-α) in supernatant from the release of cultured Kupffer cells revealed a substantial increase in the ZnPP pretreated group as compared with the CoPP group (12019.25 ± 1244.89/257.75 ± 21.56 pg/mL *vs* 6360.13 ± 522.36/152.00 ± 14.66 pg/mL, respectively; *P* < 0.01). The IL-6/TNF-α levels of supernatant in CoPP pretreated group were significantly lower, when compared to the control group (8459.86 ± 453.51/228.38 ± 15.04, *P* < 0.01; Figure 3A and B).

HO-1 and CD14 expression of Kupffer cells (Effect of HO-1 on Kupffer cells activation)

HO-1 mRNA expression levels of Kupffer cells pretreated with CoPP were significantly up-regulated, and those pretreated with ZnPP were down-regulated (Figure 4A). A prominent increase of CD14 mRNA levels in Kupffer cells was detected in the ZnPP group as compared with the control group or the CoPP group (1.547 ± 0.227, 0.772 ± 0.135, 0.325 ± 0.084 pg/mL, respectively; *P* < 0.05 *vs* control, and *P* < 0.01 *vs* CoPP-pretreated group). CoPP treatment was significantly lower, when compared to the control group (*P* < 0.05). As shown in Figure 4B and consistent with mRNA data, Western blot-assessed CD14 protein levels of Kupffer cells were also significantly up-regulated in the ZnPP group as compared with the control group (1.996 ± 0.446 pg/mL *vs* 1.303 ± 0.081 pg/mL, *P* < 0.05). CoPP treatment reduced CD14 protein levels (0.771 ± 0.136, *P* < 0.05 *vs* control).

DISCUSSION

I/R injury is one of the major obstacles in liver transplantation. HO-1 overexpression exerts cytoprotection and ameliorates hepatocellular damage in a number of liver I/R injury models[25-27], and prolongs cold ischemia followed by *ex vivo* perfusion or OLT[18]. In our study, CoPP-induced HO-1 overexpression significantly decreased hepatocyte injury posttransplantation. Donor livers with enhanced HO-1 expression reduced serum ALT/AST levels of recipients, ameliorated hepatic injury and suppressed cytokine release. In the transplant model, cultured Kupffer cells from donors pretreated with CoPP down-regulated CD14 mRNA and protein expression levels and reduced cytokine release.

HO-1 has been suggested as a cytoprotective gene during liver transplantation. However, the mechanism of HO-1-cytoprotection against I/R injury remains elusive. Tsuchihashi *et al*[28] showed that basal HO-1 levels are more critical than the ability to up-regulate HO-1 in response to I/R injury. Generally, the cellular mechanisms of HO-1-derived protection included regulation of the inflammatory response, improvement of microvascular flow and the antiapoptotic effects. The beneficial effects of HO-1 may be the result of the ability of the end-products of heme degradation[18]. Kaizu *et al*[29] reported that exogenous CO treatment suppressed early proinflammatory gene expression and neutrophil infiltration, and efficiently ameliorated hepatic I/R injury. CO has vasodilatating effects, thereby maintaining microvascular hepatic blood flow[18,30]. Furthermore, Tomiyama *et al*[31] recently demonstrated that CO ameliorated hepatic I/R injury and *in vitro* rat primary Kupffer cells culture also showed significant down-regulation of LPS-induced inflammatory responses. Biliverdin and the subsequently formed bilirubin possess potent antioxidant effects.

The mechanism of hepatic I/R injury is largely attributed to Kupffer cell activation and release of proinflammatory mediators such as reactive oxygen species and cytokines. During the initial stages of reperfusion Kupffer cells are activated. Activated Kupffer cells release a large amount of proinflammatory cytokines (TNF-α, IL-6, and IL-1)[32-34], which lead to aggravation of I/R injury. Liver I/R injury can be attenuated by the suppression of Kupffer cells[35]. Therefore modulation

A

B
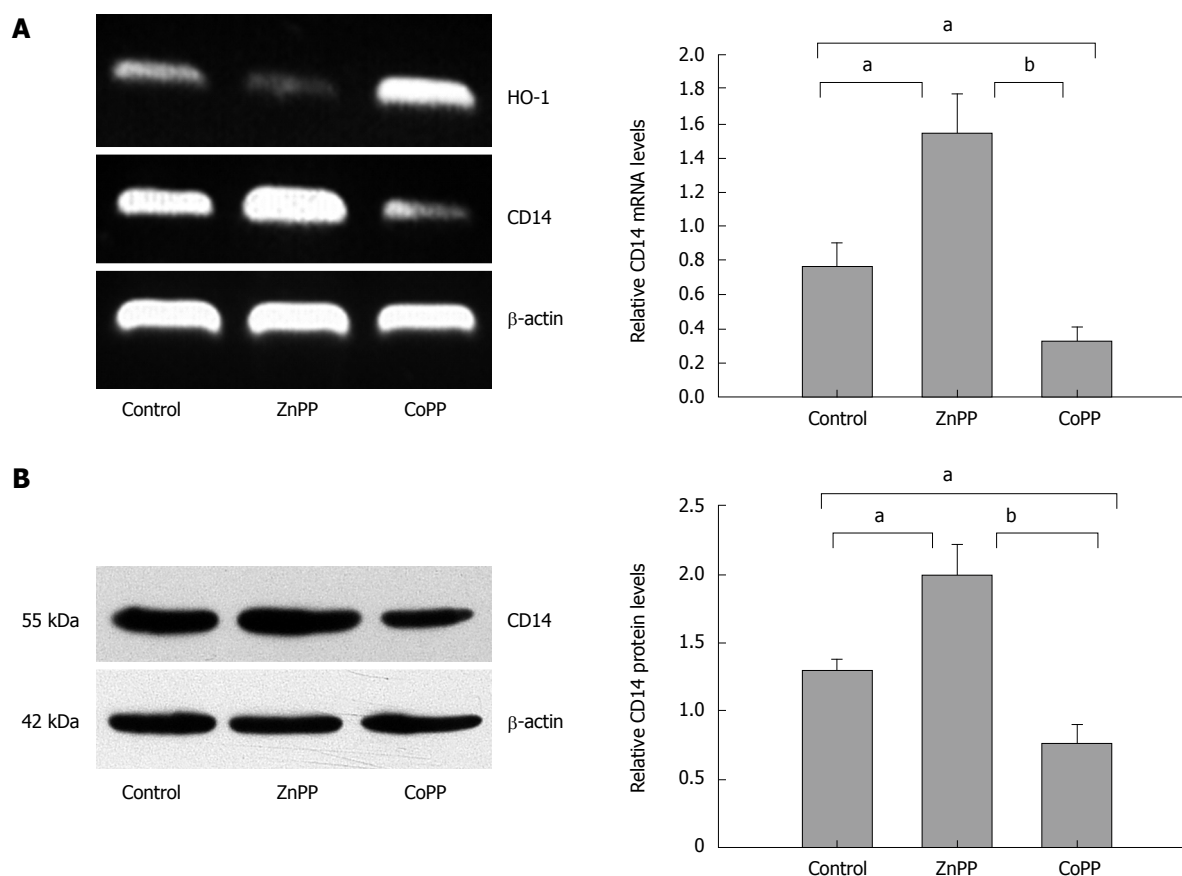
Figure 4 HO-1 and CD14 expression levels of cultured Kupffer cells. A: HO-1 mRNA expression levels of Kupffer cells were significantly downregulated in the ZnPP group and upregulated in the CoPP group. ZnPP treatment CD14 mRNA expression was expressed as relative increase compared with control. CoPP pretreated was lower (*n* = 4/group); B: Western blot analysis for CD14 protein of Kupffer cells. β-actin was used as internal control. CD14 protein expression levels increased in the ZnPP group and decreased in the CoPP group (*n* = 4/group). $^{a}P < 0.05$, $^{b}P < 0.01$.

of Kupffer cell activity can attenuate I/R injury in liver transplantation models.

In this study, we observed that donor pretreatment with CoPP significantly improved liver function, ameliorated hepatic injury and reduced plasma proinflammatory mediators. The elimination of oxidants from the cell is considered to be an important mechanism of HO-1-mediated protection against oxidative stress. Biliverdin (BV) and bilirubin (BR), which have been regarded as toxic metabolites of heme degradation, may serve as important mediators of nitrosative injury through a similar mechanism[36]. BV and BR were shown to scavenge peroxynitrite. Superinduction of HO-1 leads to BR-mediated reductions in oxidative stress following I/R injury and provides cytoprotection in hepatocytes that are subjected to reperfusion injury[14,18]. CO, another byproduct of HO-1, has been shown to modulate intrahepatic sinusoidal tone and improve microcirculation of the liver[29]. Kupffer cells, liver resident macrophages, are the prime source of HO-1. We monitored the circulating levels of TNF-α and IL-6, both of which are proinflammatory cytokines mainly released by activated Kupffer cells. Kupffer cells overexpressing HO-1 exhibit low levels of cytokines in supernatant. In CoPP treatment, the activation of Kupffer cells was suppressed by lower TNF-α and IL-6 levels compared with ZnPP

treatment. In many models of liver injury elevated TNF-α levels are present and correlate with injury, and inhibition of TNF-α activity can decrease liver injury[9]. As potent producers of inflammatory cytokines, Kupffer cells have been implicated in the pathway leading to liver injury[37].

Interestingly, there was an association of Kupffer cell HO-1 levels with lower levels of CD14 mRNA and protein seen in the CoPP treatment group as compared with the ZnPP treatment group. Kupffer cells have relatively low baseline expression of CD14[38]. Some studies showed that activation of Kupffer cells upregulated expression of CD14 protein and its gene[39-41]. Interpretations of studies in liver injury should take into account the relative contributions of Kupffer cells and hepatocyte CD14[9]. Thus, increased baseline HO-1 levels as seen in CoPP treatment may represent a diminished ability to activate Kupffer cells. Kupffer cells are the major site of expression of hepatic HO-1. It is well known that liver I/R injury results in Kupffer cell activation and subsequent cytokines release, leading to localized hepatic damage. However, Devey *et al*[42] reported that Kupffer cells depletion resulted in loss of HO-1 expression and increased susceptibility to hepatic I/R injury. Therefore, HO-1 expression levels of Kupffer cells are likely to play a crucial role in liver I/R injury.

In conclusion, CoPP-induced HO-1 overexpression

ameliorates liver I/R injury and suppresses cytokine release by inhibiting activation of Kupffer cells. Our data provides evidence for a novel mechanism of HO-1 dependent cytoprotection in liver I/R injury.

ACKNOWLEDGMENTS

COMMENTS

Background

Orthotopic liver transplantation (OLT) is an effective treatment for end-stage liver diseases. However, ischemia/reperfusion (I/R) injury of the liver remains a major cause of graft injury, causing liver dysfunction and even failure posttransplantation. Overexpression of heme oxygenase (HO)-1 exerts a cytoprotective function in a number of I/R injury and liver transplant models. Studies of the mechanism by which HO-1 protects donor livers against I/R injury contribute to the development of liver transplantation.

Research frontiers

The mechanism of HO-1-mediated cytoprotection is complicated. Kupffer cells are critical to the onset of liver injury and following secretion, cytokines aggravate hepatocyte damage. HO-1 is principally distributed in Kupffer cells. Recently, it has been reported that Kupffer cells depletion resulted in loss of HO-1 expression and increased susceptibility to hepatic I/R injury.

Innovations and breakthroughs

Since the mechanism of HO-1-mediated cytoprotection against I/R injury in organ transplantation remains unclear, upregulation of HO-1 is known to be involved in regulation of inflammatory response, improvement of microvascular flow and antiapoptotic effects. Liver I/R injury can be attenuated by the suppression of Kupffer cells. This study is an attempt to evaluate whether activation of Kupffer cells is related to HO-1 expression levels.

Applications

HO-1 can inhibit Kupffer cell activation and reduce release of proinflammatory mediators. This study showed that HO-1 is an attractive target against I/R injury in liver transplantation.

Terminology

Kupffer cells are the resident macrophages of the liver and involved in I/R injury through the release of cytokines and other biologically active mediators. Modulation of Kupffer cells activity can attenuate I/R injury in liver transplantation models.

Peer review

The paper deals with the role of heme oxygenase-1 in connection with ischemia/reperfusion injury in liver. It is important to find methods to prevent or reduce the injury that may follow ischemia/reperfusion, and the results presented may be useful for further research in the field. All the different parts of the paper are concise and clear.

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