

Basic Study

Heme oxygenase-1 protects rat liver against warm ischemia/reperfusion injury *via* TLR2/TLR4-triggered signaling pathways

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mechanisms of induced heme oxygenase (HO)-1 in protecting liver from warm ischemia/reperfusion (I/R) injury.

METHODS: Partial warm ischemia was produced in the left and middle hepatic lobes of SD rats for 75 min, followed by 6 h of reperfusion. Rats were treated with saline, cobalt protoporphyrin (CoPP) or zinc protoporphyrin (ZnPP) at 24 h prior to the ischemia insult. Blood and samples of ischemic lobes subjected to ischemia were collected at 6 h after reperfusion. Serum transaminases level, plasma lactate dehydrogenase and myeloperoxidase activity in liver were measured. Liver histological injury and inflammatory cell infiltration were evaluated by tissue section and liver immunohistochemical analysis. We used quantitative reverse transcription polymerase chain reaction to analyze liver expression of inflammatory cytokines and chemokines. The cell lysates were subjected to immunoprecipitation with anti-Toll-IL-1R-containing adaptor inducing interferon- β (TRIF) and anti-myeloid differentiation factor 88 (MyD88), and then the immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

RESULTS: HO-1 protected livers from I/R injury, as evidenced by diminished liver enzymes and well-preserved tissue architecture. In comparison with ZnPP livers 6 h after surgery, CoPP treatment livers showed a significant increase inflammatory cell infiltration of lymphocytes, plasma cells, neutrophils and macrophages. The Toll-like receptor (TLR)-4 and TANK binding kinase 1 protein levels of rats treated with CoPP significantly reduced in TRIF-immunoprecipitated complex, as compared with ZnPP treatment. In addition, pretreatment with CoPP reduced the expression levels of TLR2, TLR4, IL-1R-associated kinase (IRAK)-1 and tumor necrosis factor receptor-associated factor 6 in MyD88-immunoprecipitated complex. The inflammatory cytokines and chemokines mRNA expression rapidly decreased in

Abstract

AIM: To investigate the efficacy and molecular

CoPP-pretreated liver, compared with the ZnPP-treated group. However, the expression of negative regulators Toll-interacting protein, suppressor of cytokine signaling-1, IRAK-M and Src homology 2 domain-containing inositol-5-phosphatase-1 in CoPP treatment rats were markedly up-regulated as compared with ZnPP-treated rats.

CONCLUSION: HO-1 protects liver against I/R injury by inhibiting TLR2/TLR4-triggered MyD88- and TRIF-dependent signaling pathways and increasing expression of negative regulators of TLR signaling in rats.

Key words: Heme oxygenase-1; Ischemia reperfusion injury; Toll-like receptor; Myeloid differentiation factor 88; Liver

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Core tip: Heme oxygenase (HO)-1, a rate-limiting enzyme in heme degradation, has been shown to provide cytoprotection in various tissue and organ injury models. There is evidence suggesting that augmented Toll-like receptor (TLR) reactivity contributes to the development of heightened systemic inflammation following severe liver injury. In this study, by inducing the expression of HO-1 in a rat liver ischemia/reperfusion injury model, we demonstrated that HO-1 suppresses activation of the TLR2/TLR4-triggered myeloid differentiation factor 88 dependent pathway and promotes expression of negative regulators of TLR signaling.

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INTRODUCTION

Heme oxygenase (HO)-1 is a stress-inducible rate-limiting enzyme in heme degradation that confers cytoprotection against oxidative injury and provides a vital function in maintaining tissue homeostasis. The primary function of HO-1 enzyme is to degrade the heme molecule to carbon monoxide (CO), free iron, and biliverdin, which is immediately reduced to bilirubin by biliverdin reductase^[1]. However, CO exerts several biological functions including antiapoptotic and anti-inflammatory properties^[2]. The release of free iron is rapidly sequestered into the iron storage protein ferritin. HO-1 is especially attractive because of its characteristic inducibility. Recent studies have shown that HO-1 may serve as a key endogenous factor in the adaptation and/or defense against oxidative and

cellular stress^[3]. HO-1 is induced in response to various noxious stimuli (such as hypoxia, ischemia, radiation, inflammation and oxidative stress)^[4,5]. In addition, induced HO-1 overexpression can protect against the systemic inflammatory response and attenuate organ ischemia/reperfusion (I/R) injury^[6-9]. Thus, HO-1 is an attractive new therapeutic strategies and potential candidate responsible for treatment of tissue or organ I/R injury.

Toll-like receptors (TLRs) were initially discovered in *Drosophila*, where they play an important role in both developmental and immunological signaling. TLRs mediate cell activation *via* engagement of myeloid differentiation factor 88 (MyD88)- and/or Toll-interleukin-1 receptor (TIR) domain-containing adaptor inducing interferon (IFN)- β (TRIF)-dependent signaling pathways. MyD88 is an adaptor protein used by all TLRs except of TLR-3^[10,11]. MyD88-dependent signaling *via* TLR-2 and TLR-4 requires the presence of TIR domain-containing adaptor protein (TIRAP)^[12,13]. Activation of MyD88/ TIRAP leads to the activation of tumor necrosis factor (TNF) receptor- associated factor 6 (TRAF6), which leads to the activation of an I κ B kinase (IKK) complex and subsequent phosphorylation and degradation of I κ B^[14,15]. TLR-4 and TLR-3 are associated with TRIF-dependent pathways, which finally result in the production of interferon and other co-stimulatory molecules by activating nuclear factor kappa B (NF- κ B) and IFN regulatory factor (IRF)3^[16]. TLR signaling pathways ultimately lead to activation of transcription factors, which regulate production of cytokines and chemokines. In contrast, the expression of Toll-interacting protein (Tollip), suppressor of cytokine signaling (SOCS)-1, interleukin (IL)-1R-associated kinase-M (IRAK-M) and Src homology 2 domain-containing inositol-5- phosphatase (SHIP)-1 inhibit TLR signaling pathways^[17-20].

TLRs have been shown to be expressed on several cell types of the liver, including Kupffer cells, hepatocytes, hepatic stellate cells, biliary epithelial cells, liver sinusoidal endothelial cells, hepatic dendritic cells and other types of immune cells in the liver^[21]. Liver I/R injury is a process triggered when the liver is transiently deprived of oxygen and reoxygenated. Augmented TLR reactivity contributes to the development of heightened systemic inflammation following severe liver injury, potentially by activating proapoptotic pathways and the release of proinflammatory cytokines^[22-25]. Our previous studies have shown that Kupffer cells from donors pretreated with cobalt protoporphyrin (CoPP) (HO-1 inducer) down-regulated CD14 mRNA and protein expression levels^[26]. CD14 was found to participate in the function of TLR-4^[22]. Whether the protective effect of HO-1 is associated with TLR signaling pathways is unknown. Therefore, this study was designed to investigate the potential effects and mechanisms of TLR pathways of induced HO-1 in rat liver I/R injury.

Table 1 Primers used for quantitative real-time polymerase chain reaction

Gene	Sense	Antisense
TNF- α	GCGTGTTCATCCGTTCTCTACC	CTTCAGCGTCTCGTGTGTTCT
IL-6	TTCCAGCCAGTTGCCTTCTT	GTCGTGTGTTGGGTATCCTC
IL-1 β	ATCCCAAACAATACCCAAAGAAG	GAACTGTGCAGACTCAAACCTCCA
IFN- γ	AACCCACAGATCCAGCACAAAAG	TACCCAGAATCAGCACCGA
CXCL-1	TAGAAGGTGTTGAGCGGGAAGA	TGAGACGAGAAGGAGCATTGGT
CXCL-2	CTTATGTAGTTGGAAGGTGATGCA	CACAGTGTGGAGGTGGTGTAGTC
HPRT	GACCGGTTCTGTCATGTCG	ACCTGGTTCATCATCACTAATCAC

TNF- α : Tumor necrosis factor- α ; IL: Interleukin; CXCL: Chemokine CXC ligand; HPRT: Hypoxanthine guanine phosphoribosyl transferase.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (220–250 g) (Kunming Medical University Laboratory Animal Center, China) were used. Animals were housed in micro-isolator cages in virus-free facilities and fed laboratory chow *ad libitum*. The experimental procedures were performed with the permission of the ethics committee for the use of experimental animals at Kunming Medical University.

Liver I/R injury model

Rats were anesthetized by sodium pentobarbital (40 mg/kg, ip). We established a 70% liver I/R model as described previously^[27]. Briefly, a midline laparotomy was performed and an atraumatic clip was used to interrupt blood supply to the hepatic arterial and portal venous branches to the lateral and median lobes of the liver. After 75 min of local ischemia, the clip was removed. Rats were divided randomly into four groups: sham group, I/R group, CoPP group and zinc protoporphyrin (ZnPP) group. Each rat was treated with saline (sham and I/R group, 0.5 mL/rat, ip), CoPP (an HO-1 inducer, 5 mg/kg ip) or ZnPP (an HO-1 inhibitor, 20 mg/kg ip) 24 h prior to the onset of ischemia. Animals were sacrificed after 6 h of reperfusion, and blood and samples of ischemic lobes subjected to ischemia were taken for future analysis. Sham-operated rats underwent the same procedure, but without vascular occlusion.

Hepatocellular damage assay

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using a clinical chemistry system (7060 automatic analyzer, Hitachi, Japan). Plasma lactate dehydrogenase (LDH) was determined spectrophotometrically by using a specific kit (Sigma).

Liver histology

Liver tissues were fixed in 10% neutral buffered formalin, processed, and then embedded in paraffin for light microscopy. Sections (4 μ m) were stained with hematoxylin and eosin for histological examination. Histological severity of I/R injury was graded using

Suzuki's criteria as described^[28,29].

Myeloperoxidase activity assay

To detect neutrophil activity, myeloperoxidase (MPO), an enzyme specific for polymorphonuclear neutrophils, was measured in the liver^[30]. Briefly, the frozen tissue was thawed and placed in 4 mL iced 0.5% hexadecyltrimethylammonium bromide and 50 mmol potassium phosphate buffer solution (pH = 5.0). Each sample was homogenized for 30 s and centrifuged at 15000 *g* for 15 min at 4 °C. Supernatants were then mixed with hydrogen peroxide-sodium acetate and tetramethylbenzidine solutions. The change in absorbance was measured spectrophotometrically at 655 nm. One absorbance unit of MPO activity was defined as the quantity of enzyme degrading 1 μ mol peroxide per minute at 25 °C per gram of tissue.

Immunohistochemistry

Liver samples fixed with 10% neutral buffered formalin were sliced at 4 μ m, deparaffinated, and hydrated. Endogenous peroxidase activity was inhibited with 3% H₂O₂. Sections were then blocked with 10% normal heat-inactivated goat serum. Primary mAbs against rat lymphocyte antigen 6G (Ly-6G), CD3, CD4 and CD11b were used (Santa Cruz, CA, United States) on liver paraffin sections. The secondary, biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA) was incubated with immunoperoxidase (ABC Kit, Vector). Positive cells were counted blindly in 10 high-power fields per section (\times 200).

Quantitative reverse-transcription polymerase chain reaction

A total of 2.5 μ g of RNA was reverse-transcribed into complementary DNA (cDNA) using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Quantitative polymerase chain reaction (PCR) was performed using the DNA Engine with Chromo 4 Detector (MJ Research, Waltham, MA). In a final reaction volume of 25 μ L, the following were added: 1 \times SuperMix (Platinum SYBR Green qPCR Kit, Invitrogen), cDNA, and 0.5 mmol/L of each primer. Amplification conditions were: 50 °C (2 min), 95 °C (5 min), followed by 50 cycles of 95 °C (15 s), 60 °C (30 s). Primers used to amplify specific gene fragments are listed in Table

1. Target gene expressions were calculated by their ratios to the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase.

Immunoprecipitation

Samples of frozen liver tissue were crushed under liquid nitrogen and then homogenized in immunoprecipitation buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% NP-40) containing protease and phosphatase inhibitors (2 μ g/mL aprotinin, 1 μ g/mL leupeptin, 1 mmol/L phenylmethyl sulfonyl fluoride (PMSF), 1 mmol/L Na_3VO_4 , 10 mmol/L NaF). In a pre-clearing phase, Sepharose G beads (GE Healthcare Europe GmbH, Munich, Germany) were incubated for 30 min with cell lysates under continuous shaking at 4 °C. Precleared lysates were incubated with the respective Ab (1-4 μ g per sample) overnight at 4 °C. Thereafter, immune complexes were captured by incubation for 4 h at 4 °C with protein G agarose (50 μ L per sample) and beads were extensively washed with ice-cold lysis buffer. Finally, immunoprecipitated proteins were subjected to SDS-PAGE protein separation followed by Western blot.

Western blot analysis

Proteins were extracted with radioimmunoprecipitation containing PMSF and subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes, as described^[26]. Cytoplasmic and nuclear proteins were isolated with a commercial kit (NE-Per Nuclear and Cytoplasmic Extraction Kit, Thermo Scientific, Waltham, MA). Proteins were then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Antibodies against HO-1, SOCS-1, IRAK-M, SHIP-1 and β -actin were from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Anti-TLR4, anti-Tollip, anti-MyD88, anti-IRAK1 were purchased from Alexis Biochemicals (San Diego, CA, United States), and antibodies against TLR2, TLR3, TRIF, TANK binding kinase (TBK)-1, TRAF6, p/t-I κ B (inhibitor of NF- κ B)- α , p/t-IRF3, p/t-p38, p/t-NF- κ B were obtained from Cell Signaling (Danvers, MA, United States). The membranes were incubated with antibody and relative quantities of proteins were determined with a densitometer and expressed as absorbance units.

Statistical analysis

Quantitative data are shown as mean \pm SE. Data were analyzed using SPSS software version 15.0 for Windows (SPSS Inc., Chicago, IL). Statistical analysis was performed using the Student's *t*-test or analysis of variance where appropriate. *P* values of < 0.05 were considered statistically significant.

RESULTS

HO-1 improves liver I/R injury

To determine whether HO-1 can provide protection against liver I/R injury in rats, we analyzed hepatocellular

function. Serum ALT, AST and LDH levels were significantly decreased at 6 h after reperfusion in rats treated with CoPP, as compared with those conditioned with ZnPP (Figure 1A-C). Furthermore, MPO assay (U/g, Figure 1D), reflecting neutrophil activity, was decreased in livers subjected to CoPP compared with ZnPP.

Rats treated with ZnPP revealed significant severe lobular edema, congestion, ballooning, and hepatocellular necrosis after hepatic ischemia followed by 6 h of reperfusion. However, animals treated with CoPP showed good preservation, with mild edema and normal hepatic architecture. HO-1 reduced liver histological injury qualitatively (Figure 2A) and quantitatively by the Suzuki's score at 6 h after reperfusion (Figure 2B).

HO-1 ameliorates liver inflammatory cell infiltration

Livers subjected to I/R injury were also enlarged and contained a mixed inflammatory infiltrate composed of lymphocytes, plasma cells, neutrophils and macrophages after surgery. To further characterize and quantify portal inflammation, we performed immunohistochemical staining for Ly-6G, CD3, CD4, and CD11b expression. In comparison with ZnPP livers 6 h after surgery, CoPP treatment livers showed a significant increase in neutrophils (Ly-6G), T lymphocytes (CD3, CD4) and macrophages (CD11b) (*P* < 0.05) (Figure 3).

HO-1 inhibits activation of TLR4-triggered TRIF dependent pathway

We sought to examine whether HO-1 overexpression changes activation of the TLR4-triggered TRIF-dependent pathway that employs the signaling axis consisting of the signaling adaptor TRIF and the kinase TBK1. As shown in Figure 4A, the cell lysates were subjected to immunoprecipitation using an anti-TRIF, and then the immunoprecipitates were analyzed by Western blot analysis using an anti-TLR2, anti-TLR3, anti-TLR4, anti-TBK1 or anti-TRIF. The TLR4 and TBK1 protein levels of rats treated with CoPP significantly reduced in TRIF-immunoprecipitated complex, as compared with ZnPP treatment. However, no statistically significant difference in the protein levels of TLR2 and TLR3 was found (*P* > 0.05).

Expression levels of HO-1, TLR2, TLR3 and TLR4 in the liver were analyzed by Western blots. Indeed, CoPP treatment increased expression levels of HO-1 (Figure 4B). In contrast, ZnPP treatment reduced HO-1 expression. We found that the expression of TLR2 and TLR4 was strongly up-regulated in the liver subjected to I/R injury, but not in the sham group. However, there was no significant difference in TLR3 expression levels among the four groups.

HO-1 suppresses activation of TLR2/TLR4-triggered MyD88 dependent pathway

We next investigated whether overexpression of HO-1 affects the activation of MyD88-dependent pathway. The cell lysates were subjected to immunoprecipitation

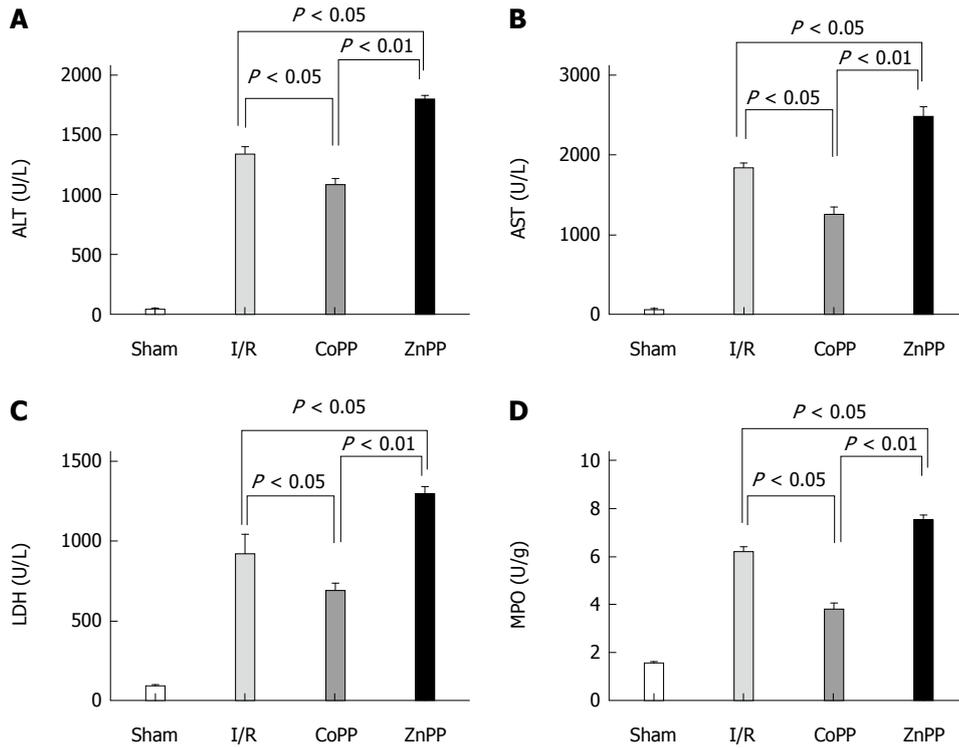
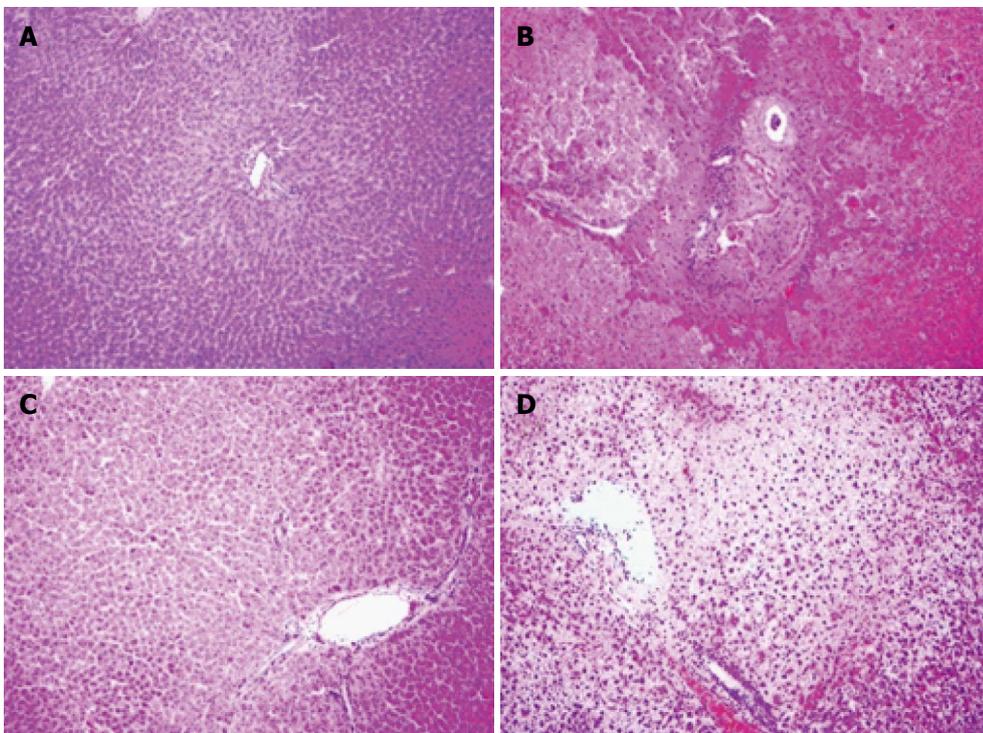


Figure 1 Serum concentrations of liver enzymes and myeloperoxidase activity. The hepatocellular function, as evidenced by serum aspartate aminotransferase (ALT) (A), aspartate aminotransferase (AST) (B) and lactate dehydrogenase (LDH) (C) levels were decreased in cobalt protoporphyrin (CoPP) treatment group, compared with zinc protoporphyrin (ZnPP) treatment. The myeloperoxidase (MPO) activity (D) was decreased in livers subjected to CoPP, compared with ZnPP. Data are shown as mean \pm SE, ($n = 4$ per group).



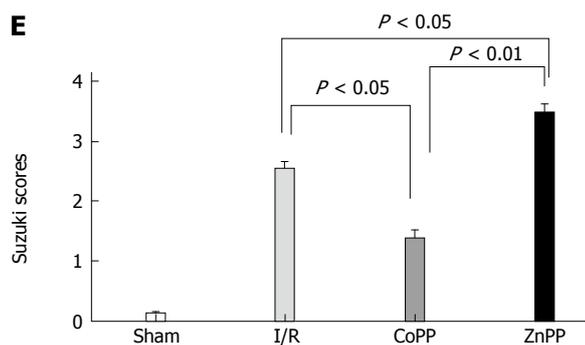


Figure 2 Representative histological findings in rat liver after 75 min of warm ischemia followed by 6 h of reperfusion. Results are representative of four to six rats per group. A: Sham; B: Ischemia/reperfusion (I/R); C: Cobalt protoporphyrin (CoPP); D: Zinc protoporphyrin (ZnPP); original magnification, $\times 100$; E: Histological injury score based on Suzuki's criteria at 6 h after reperfusion was quantified ($n = 4$ per group).

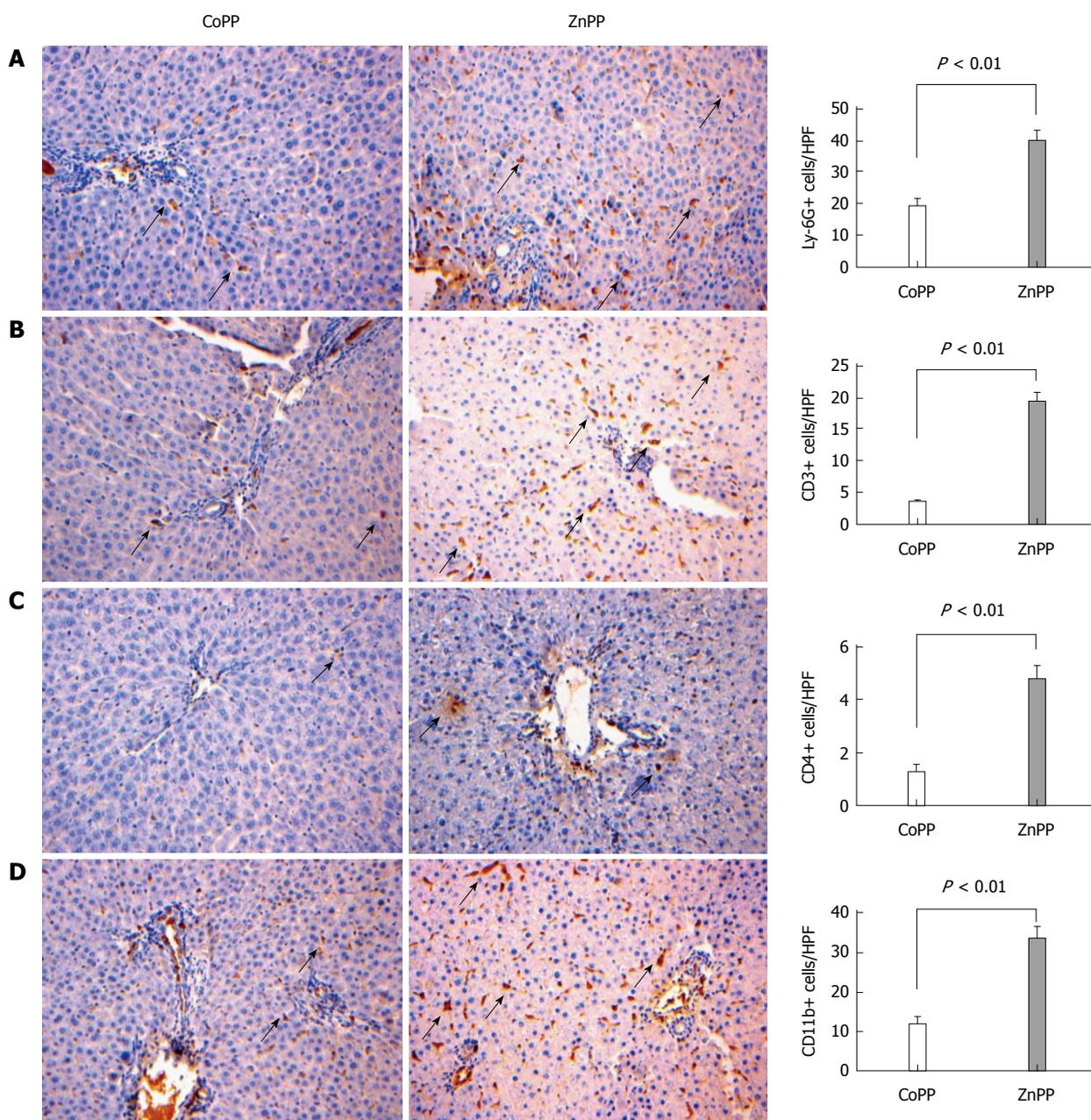


Figure 3 Accumulation of neutrophils, T cells and macrophages in ischemic liver at 6 h of reperfusion after 75 min of ischemia. A: Lymphocyte antigen 6G (Ly-6G); B: CD3; C: CD4; D: CD11b. Left panel: Representative liver sections stained by immunohistology (magnification $\times 200$); Right panel: Cell quantification/HPF ($n = 4$ per group). CoPP: Cobalt protoporphyrin; ZnPP: Zinc protoporphyrin.

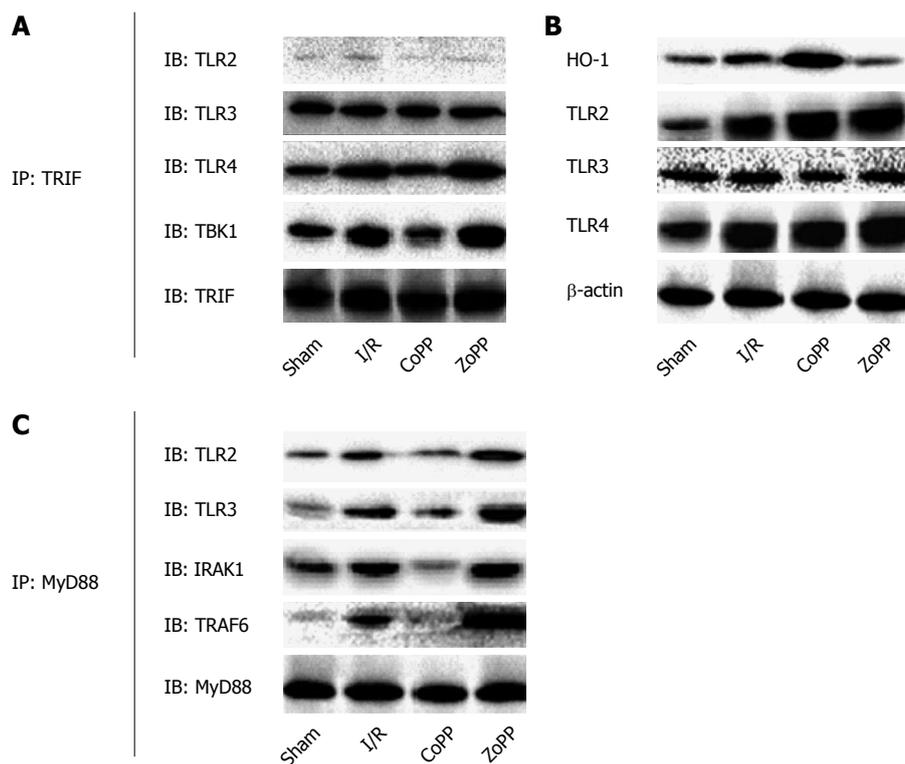


Figure 4 Heme oxygenase-1 changes activation of Toll-interleukin-1R-containing adaptor inducing interferon- β and myeloid differentiation factor 88 dependent pathways. A: Whole cell lysates were prepared, immunoprecipitated with anti-Toll-interleukin (IL)-1R-containing adaptor inducing interferon- β (TRIF), and immune complexes were analyzed by immunoblotting with the indicated antibodies. Shown are data of representative ($n = 4$) experiments; B: Expression levels of heme oxygenase (HO)-1, Toll-like receptor (TLR)-2, TLR3 and TLR4 were analyzed in whole cell lysates by immunoblotting with the respective Abs. $n = 4$ -5 per group; C: The cell lysates were subjected to immunoprecipitation with anti-myeloid differentiation factor 88 (MyD88), and then the immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies; $n = 4$ per group. TBK: TANK binding kinase; IRAK: IL-1R-associated kinase; TRAF: Tumor necrosis factor receptor-associated factor; I/R: Ischemia/ reperfusion; CoPP: Cobalt protoporphyrin; ZnPP: Zinc protoporphyrin.

using an anti-MyD88, and then followed by Western blot analyses with anti-TLR2, anti-TLR4, anti-IRAK1, anti-TRAF6 or anti-MyD88 to confirm specificity of detection. As illustrated in Figure 4C, pretreatment with CoPP reduced the expression levels of TLR2, TLR4, IRAK1 and TRAF6 in MyD88-immunoprecipitated complex. In contrast, those protein levels increased significantly in the group treated with ZnPP.

HO-1 decreases kinase phosphorylation in liver

To further confirm the activation of TRIF and/or MyD88 dependent pathway with induced HO-1 in this study, we next examined the phosphorylation of multiple kinases at 6 h after reperfusion by Western blot using cytoplasmic or nuclear protein. The phosphorylation of IRF3, p38, I κ B- α and NF- κ B significantly decreased in the CoPP-pretreated liver, compared with the ZnPP-treated group (Figure 5).

HO-1 down-regulates protein kinases-mediated cytokine/chemokine programs

We used quantitative reverse transcription polymerase chain reaction to analyze liver expression of inflammatory cytokines (TNF- α , IL-6, IL-1 β , and IFN- γ) and chemokines [chemokine CXC ligand (CXCL)-1 and CXCL-2]. In the CoPP-treated group, inflammatory

cytokines (TNF- α , IL-6, IL-1 β , and IFN- γ) and chemokines (CXCL-1 and CXCL-2) mRNA expression rapidly reduced during hepatic I/R injury at 6 h after reperfusion (Figure 6). The expression of inflammatory cytokines and chemokines was increased in ZnPP-treated group, as compared with CoPP treatment.

HO-1 promotes expression of negative regulators of TLR signaling

TLR signaling is negatively regulated at multiple levels by intermediates affecting TLR signaling cascades. Tollip, SOCS-1, IRAK-M, and SHIP-1 are negative regulators of TLR signaling implicated in interference with recruitment of MyD88 and IRAK kinases to TLR4 and IRAK-1 activation. To further examine the mechanism of HO-1 in our system, we examined the effect of HO-1 on negative regulators of TLR signaling in a rat liver warm I/R injury model. In CoPP-treated animals, the expression of Tollip, IRAK-M, SOCS-1, and SHIP-1 proteins were markedly up-regulated as compared with ZnPP-treated rats (Figure 7).

DISCUSSION

Although overexpression of HO-1 has been shown to protect against liver I/R injury^[8,9,31], its role in the liver I/R

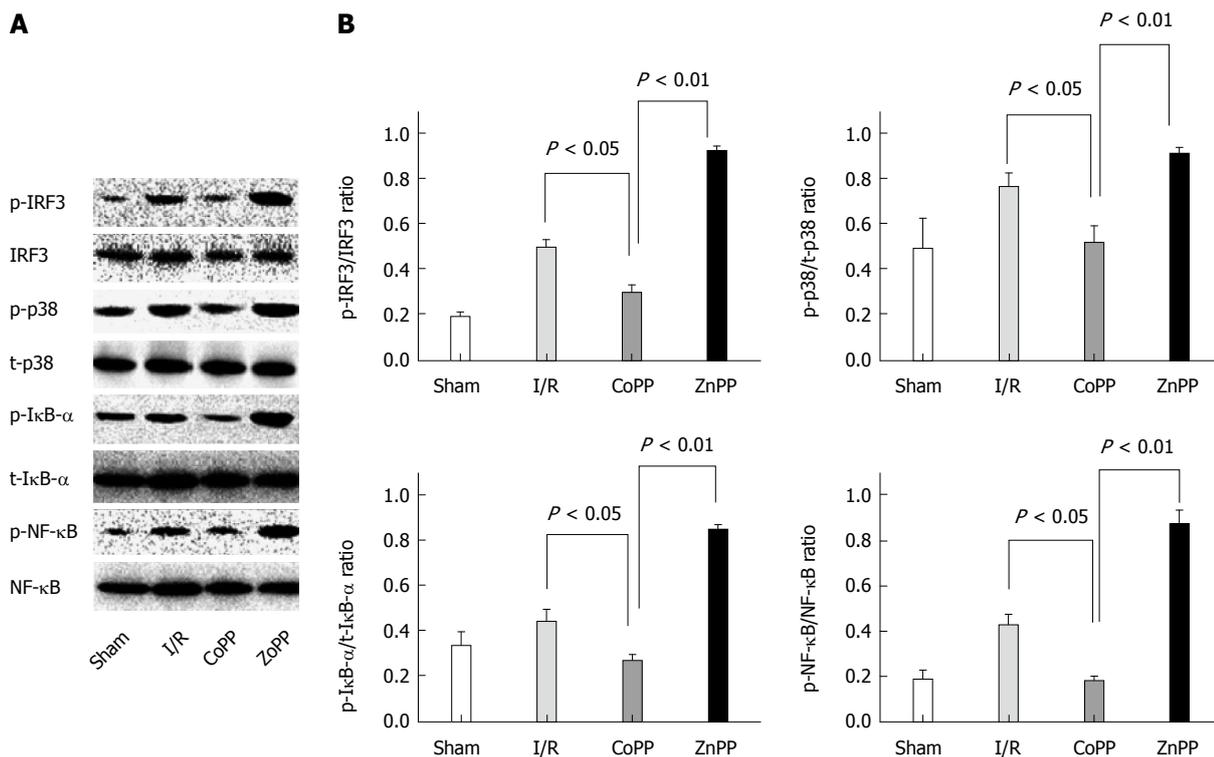


Figure 5 Phosphorylation of multiple kinases at 6 h after reperfusion. A: Phosphorylation of interferon regulatory factor (IRF)-3, p38, inhibitor of nuclear factor- κ B (NF- κ B) (I κ B)- α and NF- κ B were evaluated in Western blot using hepatic cytoplasmic or nuclear protein at 6 h after reperfusion. B: Phosphorylated (p)-to-total (t) protein kinase ratios were quantified by densitometry. Data are mean \pm SE of 4 independent experiments ($n = 5$ per group). I/R: Ischemia/reperfusion; CoPP: Cobalt protoporphyrin; ZnPP: Zinc protoporphyrin.

injury cascade, an inflammation-mediated hepatocellular injury process, has not been explored. Current results provide evidence for a novel mechanism by which overexpression of HO-1 ameliorates the hepatocellular damage. The beneficial effects were accompanied by reduced neutrophil activity/infiltration; broader suppression of the TLR-triggered MyD88 and TRIF dependent pathway; diminished kinase phosphorylation; down-regulated proinflammatory cytokine/chemokine gene programs; and enhanced expression of negative regulators of TLR signaling.

TLRs, traditionally considered innate immune receptors, signal through the adaptor proteins MyD88 and TRIF to activate NF- κ B and IRF3. Studies with TLR deficient mice have demonstrated that TLR2 and TLR4 activation in response to ischemic injury exacerbates damage^[32-34]. High mobility group protein B1 mediates injury *via* TRIF-independent TLR4 signaling in an I/R model^[35]. This study showed significant inhibition of upstream (TLR4) and downstream (TBK1) signals of TRIF in liver treated with CoPP during rat I/R injury. However, no difference was observed in TLR3 levels. These results suggest that HO-1 triggered the interaction between TLR4 and TRIF to induce downstream (TBK1) signals activation. But TLR4 and TRIF expression levels are not absolutely affected. IRF3 is phosphorylated by TBK1, dimerized and translocated to the nucleus to activate expression of TRIF-dependent genes^[36,37]. In our study, we also observed the phosphorylation of IRF3 was significantly

decreased in CoPP-pretreated liver. Therefore, we can infer the involvement of the TRIF pathway in HO-1-mediated hepatic protection.

TLRs recognize microbial components and evoke diverse responses in immune and other respiratory cells through distinct signaling cascades in which MyD88 and TRAF6 are key adaptor proteins^[38]. Thus, we further investigated the physical association of TLR2, TLR4, MyD88, IRAK1 and TRAF6. Overexpression of HO-1 decreased the expression levels of TLR2, TLR4, IRAK1 and TRAF6 in the MyD88-immunoprecipitated complex. These results demonstrated that HO-1 down-regulated the association of MyD88 with TLR2, TLR4, IRAK1 or TRAF6 and reduced activation of the corresponding downstream signaling. It is known that TRAF6-mediated activation of the IKK/I κ B- α /NF- κ B pathway is vital for the pro-inflammatory cytokine response^[39]. Phosphorylation of I κ B- α and NF- κ B was diminished in CoPP-treated rats, and was increased in ZnPP-treated rats. These observations further suggest that the TLR2/TLR4-triggered MyD88 dependent pathway plays a critical role in HO-1-mediated hepatic protection.

TLR2/TLR4-triggered MyD88 dependent pathway triggers the activation of IKK, which ultimately leads to NF- κ B translocation to the nucleus and induction of gene transcription and production of proinflammatory cytokines and chemokines^[11,40]. Activation of p38 MAPK, an important regulator in the early stages of liver I/R injury, promotes the expression of pro-inflammatory

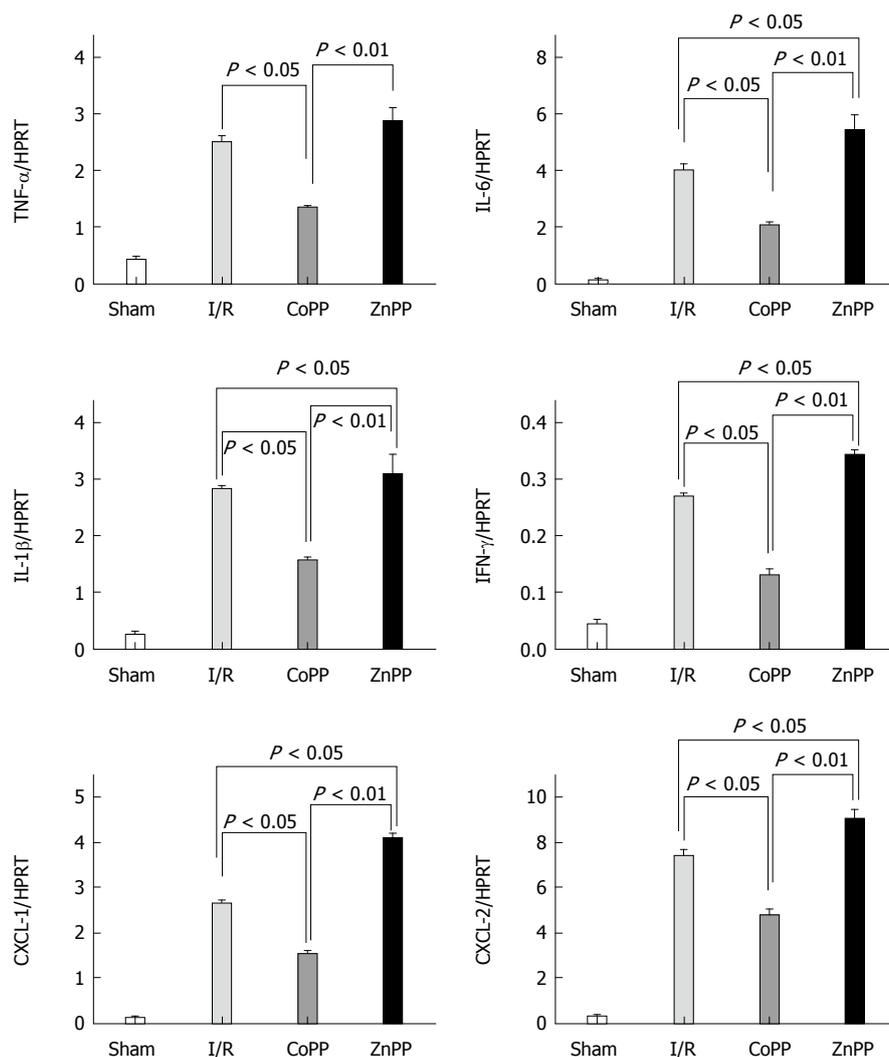


Figure 6 Quantitative reverse transcription polymerase chain reaction-assisted detection of proinflammatory and chemokines at 6 h of reperfusion after 75 min of ischemia. Data were normalized to hypoxanthine guanine phosphoribosyl transferase (*HPRT*) gene expression. Mean \pm SE are shown ($n = 5$ per group). TNF: Tumor necrosis factor; IL: Interleukin; IFN: Interferon; CXCL: Chemokine CXC ligand; I/R: Ischemia/reperfusion; CoPP: Cobalt protoporphyrin; ZnPP: Zinc protoporphyrin.

cytokines such as TNF- α and IL-1 β . These cytokines play an important role in inflammatory organ damage by promoting the recruitment of neutrophils which release reactive oxygen species and proteases^[41-43]. In this study, HO-1 decreased kinase phosphorylation of NF- κ B and p38 in association with improved hepatocellular damage. Moreover, CoPP treatment significantly decreased proinflammatory cytokines (TNF- α , IL-6, IL-1 β , and IFN- γ) and chemokines (CXCL-1 and CXCL-2) induction ratios in liver compared with ZnPP treatment, in parallel with a marked decrease in MPO activity and Ly-6G neutrophil infiltration in the ischemic liver lobes. The C-X-C chemokines, CXCL-1 and CXCL-2 (macrophage inflammatory protein-2) are potent neutrophil chemoattractants, which are important in liver I/R injury^[44,45]. Hence, HO-1 reduces the local inflammatory response, whereas treatment with ZnPP up-regulates the levels of inflammatory mediators through protein kinase phosphorylation.

The cytosolic serine/threonine kinases IRAK-1 and

IRAK-4 are recruited to the receptor intracellular TIR domains *via* the adaptor MyD88^[46]. Subsequently, TRAF6 is activated by binding to IRAK-1, resulting in the activation of MAPK cascades and NF- κ B. IRAK-M, SOCS-1, and Tollip are cytosolic molecules that inhibit intracellular IRAK activity, which leads to the suppression of NF- κ B activity^[17,47,48]. SHIP-1 inhibits LPS-mediated expression of proinflammatory cytokines and type I IFNs by affecting signaling pathways triggered by PI3K and TBK1^[49-51]. Our study provides direct evidence that up-regulation of negative regulators of TLR signaling are involved in HO-1-mediated hepatoprotective effects. But further work is needed to delineate the interaction of negative regulators.

In conclusion, HO-1 ameliorates liver I/R injury by inhibiting activation of TLR2/TLR4-triggered MyD88- and TRIF-dependent pathways, with resultant decreased phosphorylation of IRF3, p38, I κ B- α and NF- κ B and up-regulation of negative regulators of TLR signaling. This study provides evidence for a novel mechanism by

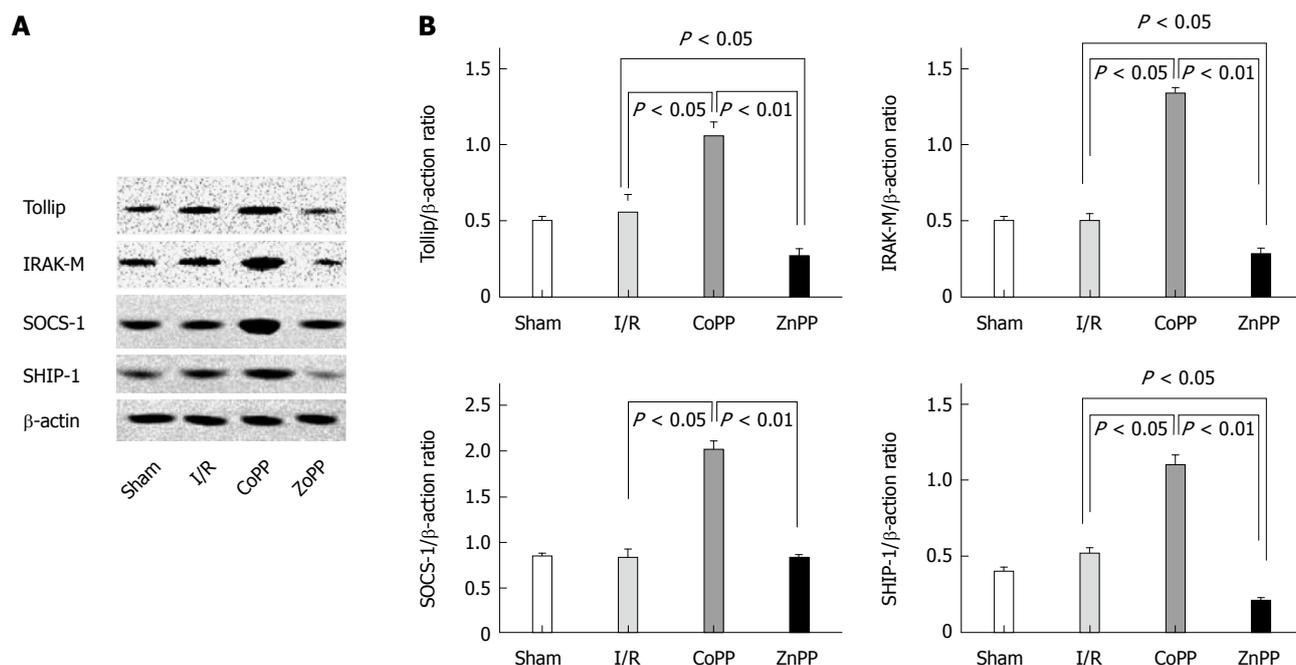


Figure 7 Expression of negative regulators of TLR signaling. A: Expression of Toll-interacting protein (Tollip), interleukin-1R-associated kinase (IRAK-M), suppressor of cytokine signaling (SOCS)-1 and Src homology 2 domain-containing inositol-5-phosphatase (SHIP-1) in liver at 6 h after reperfusion were evaluated by Western blotting assay using whole cell lysates; B: Results was quantified and expressed by the ratio to β-actin. Mean ± SE are shown (n = 4/group). I/R: Ischemia/reperfusion; CoPP: Cobalt protoporphyrin; ZnPP: Zinc protoporphyrin.

which HO-1 affects the TLR2/TLR4-triggered MyD88- and TRIF-dependent pathway during the course of liver I/R injury. Our data, combined with those of previous studies, suggest that HO-1 is a rational therapeutic target to manage hepatic inflammation and I/R injury in liver transplant recipients.

COMMENTS

Background

Heme oxygenase (HO)-1, a rate-limiting stress-responsive protein in heme catabolism, provides a potent cytoprotective defense response against oxidative injury. Augmented Toll-like receptor (TLR) reactivity contributes to the development of heightened systemic inflammation following severe liver injury. However, whether the protective effect of HO-1 is associated with TLR signaling pathways is unknown.

Research frontiers

TLR signaling can be subdivided into two categories: myeloid differentiation factor 88 (MyD88)- and Toll-interleukin (IL)-1R-containing adaptor inducing interferon-β (TRIF)-dependent. Except for TLR3, which signals via TRIF exclusively, all TLRs are intracellularly coupled to MyD88. In this study, the authors demonstrate that HO-1 affects TLR2/TLR4-triggered MyD88- and TRIF-dependent pathway during the course of liver ischemia/reperfusion (I/R) injury.

Innovations and breakthroughs

TLR signaling pathways ultimately lead to activation of transcription factors. In contrast, the expression of Toll-interacting protein, suppressor of cytokine signaling-1, IL-1R-associated kinase-M and Src homology 2 domain-containing inositol-5-phosphatase inhibit TLR signaling pathways. In addition, TLR synergy is the result of combined signaling via MyD88- and TRIF-dependent events. This study suggests that up-regulation of negative regulators of TLR signaling involved in HO-1-mediated hepatoprotective effects.

Applications

This study may represent a rational therapeutic target to manage hepatic inflammation and I/R injury in liver transplant recipients.

Peer-review

The authors investigated the potential effects and mechanisms of TLR

pathways of induced HO-1 in rat liver I/R injury. This research is well organized and of relevant interest and scientific innovation.

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