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Induction of Heme Oxygenase-1 Improves Cold Preservation Effect of Liver Graft

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Abstract—We have examined the protective effect and mechanisms of heme oxygenase-1 (HO-1) induction in rat liver model of *ex vivo* cold ischemia preservation using cobalt protoporphyrin (CoPP) as HO-1 inducer and zinc protoporphyrin (ZnPP) as HO-1 inhibitor. There was a decrease in both aspartate transaminase and lactate dehydrogenase activities and in malondialdehyde level in liver of the CoPP-treated group compared with controls ($p < 0.05$). In the CoPP-treated rats, the histological signs of reperfusion injury were much lower than in control. Up-regulation of HO-1 expression was also associated with reduced levels of tumor necrosis factor α and interleukin-6. Markedly fewer apoptotic liver cells (determined by TUNEL assay) could be detected in CoPP-treated group compared with the control group. These protective effects were prevented by administration of ZnPP. In conclusion, induction of HO-1 provides protection against liver injury during cold ischemia preservation and improves the preservation of liver graft. The mechanisms underlying these beneficial effects include reduction of oxidative injury and of inflammatory response and prevention of apoptosis.

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Key words: heme oxygenase-1, cobalt protoporphyrin, organ transplantation, cold ischemia preservation

Organ transplantation is an effective therapeutic option for end-stage organ function failure. Advances in surgical techniques, the use of anti-rejection medication, and organ typing have improved the success of organ transplantation. However, several serious problems such as the preservation of donor organ and graft chronic dysfunction still limit organ transplantation. Researchers have paid much attention to specific immunity factors, but now they consider more the importance to ischemia/reperfusion injury (IRI), which influences organ preservation and graft chronic dysfunction [1].

IRI, an antigen-independent event, plays a critical role in non-function or early dysfunction of organ graft [2]. Significant progress has been made in providing a mechanistic explanation of IRI. It includes primary microcirculatory flow disturbances caused by the production of reactive oxygen species and cytokine-mediated inflammatory damage resulting in endothelial and parenchymal cell disturbances [3]. Thus, improvement of graft quality through prevention of injury and subsequent inflammatory response may represent an attractive therapeutic strategy with a major impact on long-term graft and patient survival.

Heme oxygenases (HOs) are the rate-limiting enzymes [4] that catalyze the conversion of heme to biliverdin, carbon monoxide (CO), and iron [5]. Biliverdin is reduced to bilirubin by biliverdin reductase. Three HO isoforms have been identified: HO-1, an inducible heat shock protein 32 [6], constitutively expressed HO-2 [7], and less characterized HO-3 [8]. HO-1 is highly induced and confers protective effects in the oxidative stress response both *in vivo* and *in vitro*. Indeed, HO-1 can be induced by stimuli that have the capacity to provoke oxidative stress such as hyperthermia, hypoxia, ischemia, and radiation [9, 10]. It is believed that the byproducts derived from the catalysis of heme by HOs could mediate the physiological effects of HO-1.

Abbreviations: AST) aspartate transaminase; CoPP) cobalt protoporphyrin; HO) heme oxygenases; IL-6) interleukin-6; IRI) ischemia/reperfusion injury; LDH) lactate dehydrogenase; MDA) malondialdehyde; TNF-α) tumor necrosis factor α; TUNEL) terminal deoxynucleotide transferase-mediated dUTP nick end labeling; UW solution) University of Wisconsin solution; ZnPP) zinc protoporphyrin.

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Both biliverdin and bilirubin possess antioxidant properties [11], and CO functions as a signaling molecule [12], whereas iron released during heme catabolism can stimulate ferritin synthesis [13]. Attention has been focused on the biological effects of the reaction products that potentially possess important antioxidant, antiinflammatory, and antiapoptotic functions [14-19]. Recent studies have shown that HO-1 exerts protective effects under stimulus and disease conditions especially in a number of transplantation models [20-24]. Researchers have found that induction of HO-1 expression in donor kidney prolonged graft survival and improved long-term function after extended time of ischemia [25]. We wondered whether this beneficial effect could improve the preservation effect and function of liver graft and the success of liver transplantation.

In this study, the rat liver *ex vivo* cold ischemia preservation model [26] was set up and rats were given HO-1 inducer cobalt protoporphyrin (CoPP) in order to study the preservation effect and mechanisms of HO-1 overexpression in liver cold preservation.

MATERIALS AND METHODS

Animals. Wistar male rats weighing from 250 to 300 g were used. Animals were fed standard rodent chow and water. Animal experiments were performed in accordance with the regulation for laboratory animals and were approved by local authorities.

Synthetic metalloporphyrins. Metalloporphyrins CoPP (Sigma, USA) and ZnPP (Aldrich, USA) were dissolved in 0.2 M NaOH, subsequently adjusted to pH 7.4, and diluted in 0.85% NaCl. Stock concentration of metalloporphyrins was 1 mg/ml.

***Ex vivo* liver cold ischemia preservation model.** Nonfasted male Wistar rats underwent sodium pentobarbital anesthesia (50 mg/kg intraperitoneally) and systemic heparinization. After skeletonization of the liver, the portal vein, bile duct, and *inferior vena cava* were canulated and the liver was flushed with 20 ml of the University of Wisconsin solution (UW solution) and stored for 24 h at 4°C in UW solution. Animals were divided into three groups. Group 1, control animals, received 0.9% saline (2.5 ml/kg intraperitoneally) 48 and 24 h before liver harvesting ($n = 8$). Group 2 rats were given CoPP, the HO-1 inducer (2.5 mg/kg intraperitoneally), 48 and 24 h before liver harvesting ($n = 8$). Group 3 rats were treated with CoPP (2.5 mg/kg intraperitoneally) 48 and 24 h before liver harvesting, and in addition, ZnPP, a competitive HO-1 inhibitor, was injected into the animals (15 mg/kg intraperitoneally) 24 h before liver recovery ($n = 8$). All isolated livers were procured and stored for 24 h at 4°C in UW solution, and then were perfused on a rat liver perfusion apparatus as described [27, 28]. Perfusing solution contained Krebs–Henseleit buffer supplemented with 2.5% BSA and 20 μM sodium taurocholate and saturated with O_2/CO_2 (95%/5%). The total volume of 200 ml circulating perfusate was used. Livers were perfused *ex vivo* for 2 h at constant temperature, pH, and inflow pressure. The portal vein flow was recorded at 30 min intervals and adjusted to maintain portal pressure of 13-18 cm H_2O. Outflow samples were collected at 30 min intervals for analysis of aspartate transaminase (AST) and lactate dehydrogenase (LDH). At the end of experiment, bile production was recorded, and a portion of liver was snap frozen for analysis of malondialdehyde (MDA), tumor necrosis factor α (TNF-α), and interleukin-6 (IL-6) content and Western blot analysis of HO-1 expression. Remaining tissue samples were processed for histological examination.

Biochemical analysis of hepatocyte injury. AST and LDH levels as indicators of hepatocyte injury were measured in outflow samples obtained after reperfusion using commercial kits (Zhong Sheng Bei Kong Bio-Technology and Science Inc., China).

Assay of malondialdehyde in hepatic tissue. The relative magnitude of oxidative stress associated with liver IRI was assessed by measuring MDA using a commercially available kit (Nan Jing Jian Cheng Biological Institute, China).

Histology. Liver specimens were fixed in 10% buffered formalin solution and embedded in paraffin. Sections (5 μm thick) were stained with hematoxylin and eosin (HE). The histological severity of IRI in an *ex vivo* perfusion model was observed under the light microscope.

Transmission electron microscopy. Liver samples were immediately fixed with 2.5% glutaraldehyde, cut into 1 mm^3 cubes, and stored in 2.5% glutaraldehyde. Then the specimens were postfixed with osmium tetroxide, dehydrated, embedded, sectioned, and examined using a Philips (Japan) CM 120 electron microscope.

Western blot analysis for HO-1. Proteins were extracted from liver tissue samples with PBS-TDS buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, pH 7.2). Proteins (40 μg/sample) in SDS loading buffer were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane (Invitrogen, USA). The gel was stained with Coomassie Blue to document equal protein loading. The membrane was blocked with 5% dry milk and incubated with mouse anti-rat HO-1 monoclonal antibodies (Calbiochem, Germany). The filter was washed and then incubated with goat anti-rat IgG-AP (Sino-American Biotechnology Co., China).

ELISA for TNF-α and IL-6. TNF-α and IL-6 expression in homogenized liver samples was analyzed using ELISA kits (Boster, China) and resultant absorbance was determined using a microplate reader at 450 nm. Results were expressed as pg/mg protein.

***In situ* detection of apoptosis using the terminal deoxynucleotide transferase-mediated dUTP nick end**

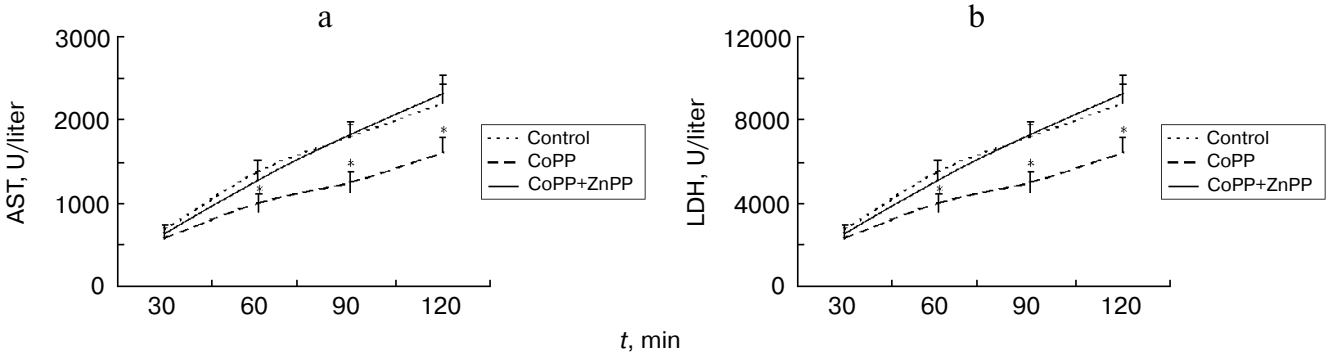

Fig. 1. Effects of IRI on AST (a) and LDH (b) release from livers cold-stored for 24 h followed by 2 h of *ex vivo* perfusion; * $p < 0.05$ vs. control.

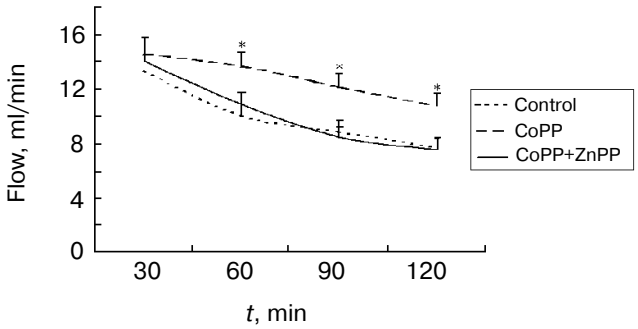

Fig. 2. Portal vein flow in isolated perfused rat liver after 24 h of cold ischemia; * $p < 0.05$ vs. control.

labeling (TUNEL) assay. Paraffin-embedded sections (5 μm) were deparaffinized by soaking twice in xylene for 10 min. Slides were successively soaked for 2 min each in 100, 95, 85, and 70% ethanol and finally in a double-distilled water for 2 min. After treatment with 3% H_2O_2 for 10 min to block endogenous peroxidases, the TUNEL assay was performed using a commercial kit (Boster). Tissues treated with DNase I served as positive controls and sections stained without terminal nucleotidyl transferase served as negative controls. The specimens were counterstained with hematoxylin to reveal cell nuclei. The apoptosis index was calculated as the percent of TUNEL+ nuclei divided by the total number of nuclei.

Statistical analysis. Results were expressed as mean ± SD. The unpaired Student's *t*-test was applied to analyze differences between groups. All differences were considered statistically significant at $p < 0.05$.

RESULTS

Effect of HO-1 induction on liver function. AST and LDH levels measured in outflow samples obtained during reperfusion were markedly reduced ($p < 0.05$) in the CoPP-treated group compared with control group (Fig. 1). There was no significant difference between control and (CoPP + ZnPP)-treated group ($p > 0.05$). Pretreatment of rats with CoPP also significantly improved ($p < 0.05$) portal flow throughout the 2 h reperfusion period (Fig. 2). As shown in Table 1, CoPP-treated livers produced more bile than control and (CoPP + ZnPP)-treated livers ($p < 0.05$).

Liver tissue MDA concentration. The MDA concentration in liver tissue after 24 h of cold storage and 2 h of *ex vivo* perfusion was 3.75 ± 0.48 nmol/mg protein. In contrast, after CoPP treatment livers had a marked decrease in MDA concentration (2.75 ± 0.32 nmol/mg protein). There was no significant difference between control and (CoPP + ZnPP)-treated group (Table 1).

Liver histology in *ex vivo* cold ischemia model followed by reperfusion. There was extensive centrilobular ballooning in control group (Fig. 3). In marked contrast, the CoPP-treated livers showed an absence of centrilobular

Table 1. Bile production, MDA concentration, and apoptotic index in isolated perfused rat liver cold-stored for 24 h followed by 2 h of *ex vivo* perfusion ($\bar{x}$ ± SD)

Group	MDA concentration, nmol/mg protein	Bile production, μl/g liver	Apoptotic index, %
Control	3.75 ± 0.48	2.6 ± 0.6	5.1 ± 1.4
CoPP-treated	2.75 ± 0.32#	10.4 ± 2.0#	2.3 ± 1.0#
(CoPP + ZnPP)-treated	3.72 ± 0.41*	3.0 ± 0.7*	4.9 ± 1.3*

# $p < 0.01$ vs. control.
* $p > 0.05$ vs. control.

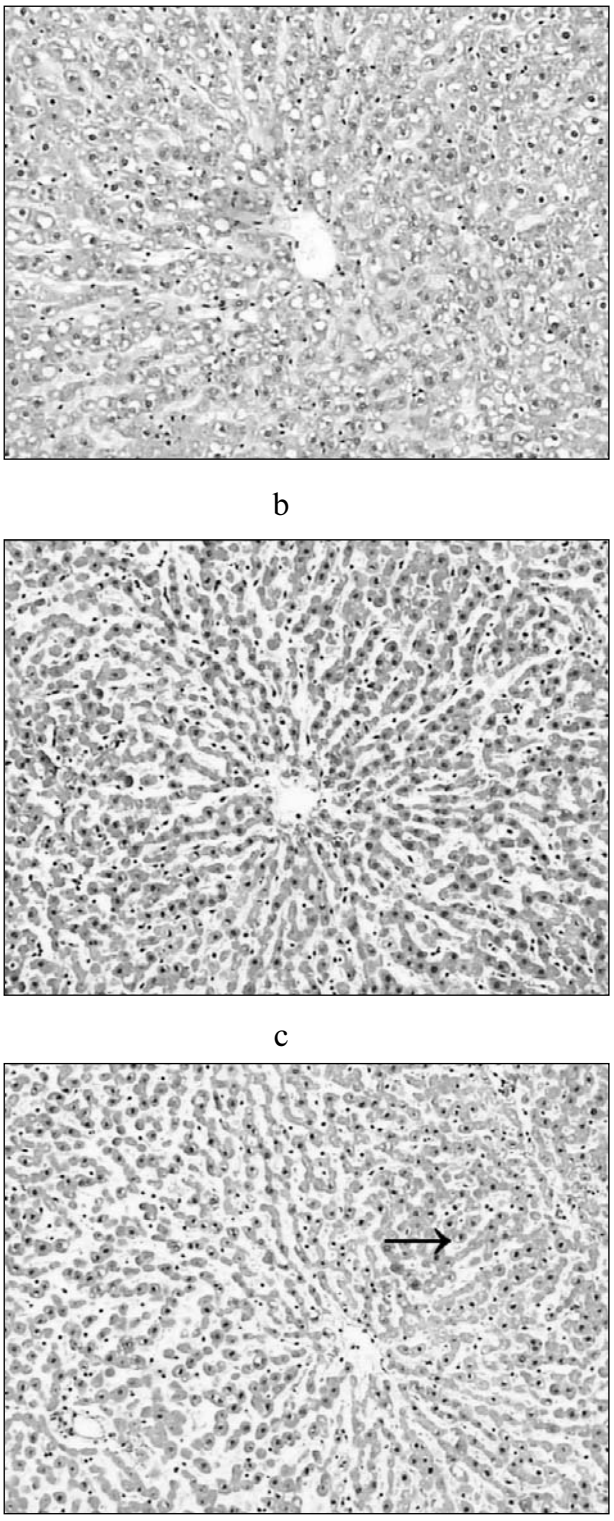

Fig. 3. Microphotographs of rat livers after 24 h cold storage followed by 2 h of *ex vivo* perfusion. a) Control group; b) CoPP-pretreated group; c) (CoPP + ZnPP)-pretreated group; patchy ballooning changes are revealed (arrow). HE ×200.

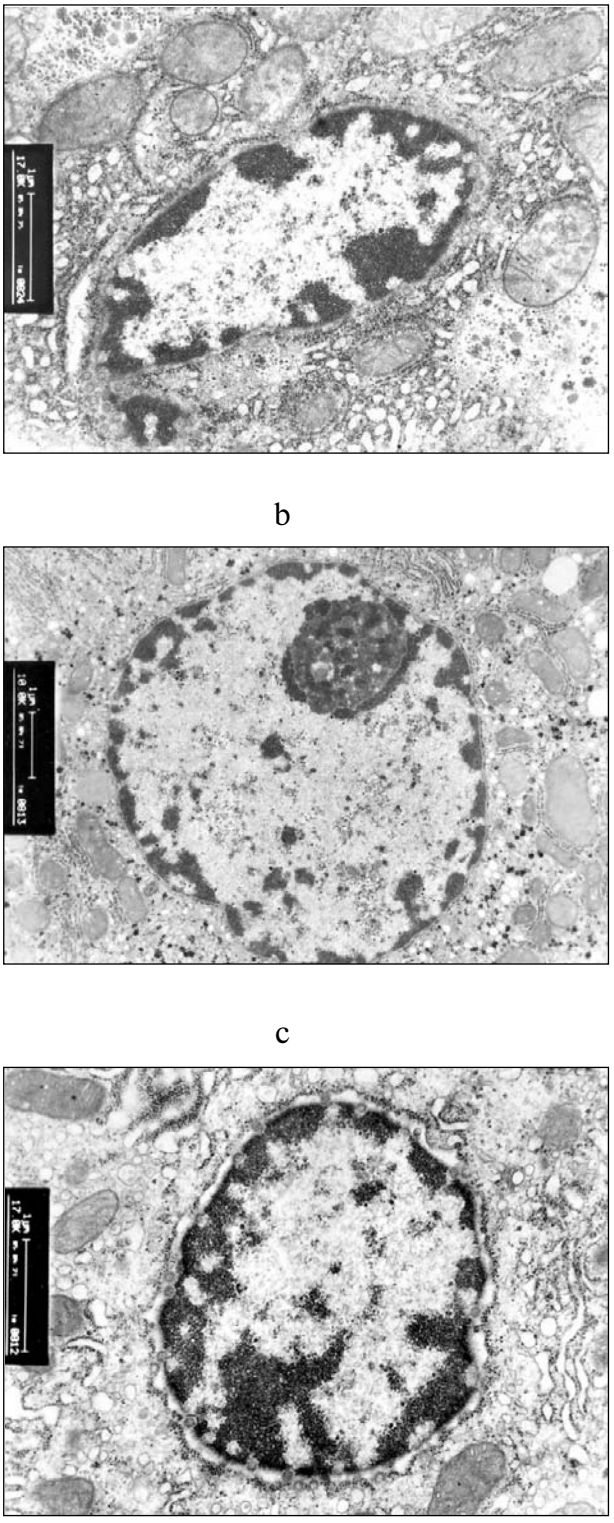

Fig. 4. Electron microscopy images of livers after 24 h cold storage followed by 2 h of *ex vivo* perfusion. a) Control group; b) CoPP-pretreated group; c) (CoPP + ZnPP)-pretreated group. Small white bars correspond to 1 μm.

Table 2. TNF-α and IL-6 expression in rat livers cold-stored for 24 h followed by 2 h of *ex vivo* perfusion ($\bar{x} \pm$ SD)

Group	TNF-α, pg/μg protein	IL-6, pg/μg protein
Control	25.1 ± 2.6	60.4 ± 4.4
CoPP-treated	19.2 ± 1.8#	52.1 ± 2.1#
(CoPP + ZnPP)-treated	25.8 ± 2.7*	61.9 ± 5.0*

# $p < 0.01$ vs. control.
* $p > 0.05$ vs. control.

ballooning. Livers treated with CoPP + ZnPP were characterized by patchy ballooning changes. These structural abnormalities were further defined by electron microscopy (Fig. 4). Cells showed nucleoli fragmentation, nuclear membrane thickening, chromatin dumping around the nuclear membrane, and mitochondrial membrane swelling in control group; the nuclear architecture appeared intact and mitochondrial membrane were swelled in CoPP-pretreated group; (CoPP + ZnPP)-pretreated group showed nuclear membrane thickening, chromatin dumping around the nuclear membrane, and mitochondrial membrane swelling.

ELISA for TNF-α and IL-6. Livers from control, CoPP-treated rats, and (CoPP + ZnPP)-treated rats were stored in cold UW solution for 24 h and perfused 2 h *ex vivo*. At the end of the perfusion, TNF-α and IL-6 expression were detected by ELISA. As shown in Table 2, CoPP pretreatment significantly reduced TNF-α and IL-6 protein expression in rat livers compared with saline-treated controls.

Western blot analysis of HO-1 in *ex vivo* liver cold ischemia preservation model. We used Western analysis to evaluate HO-1 expression in liver samples following cold ischemia at completion of the 2 h perfusion period. As shown in Fig. 5, CoPP treatment and CoPP + ZnPP treatment had enhanced HO-1 expression. In contrast, HO-1 was virtually undetectable in control.

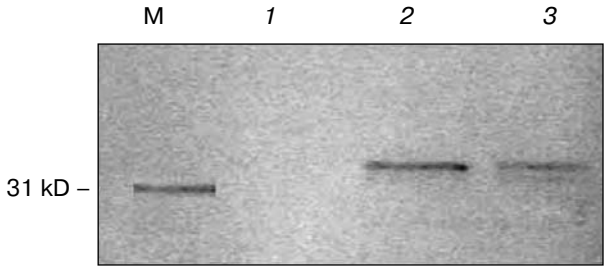

Fig. 5. Western blot analysis of HO-1 protein levels in rat livers following 24 h of cold storage and 2 h of *ex vivo* perfusion. Lanes: M) markers; *1*) control; *2*) CoPP treatment; *3*) CoPP + ZnPP treatment. HO-1 migrates as a 31-kD protein.

Effect of HO-1 induction on hepatic apoptosis. We used the TUNEL assay to determine the histological extent of apoptosis. Classic TUNEL positive signal was characterized by focal nuclear staining. The results showed that the frequency of TUNEL+ cells was reduced in sections from rats pretreated with CoPP compared with saline-treated control or those treated with CoPP + ZnPP. Consequently, an apoptotic index calculated as the percentage of TUNEL+ nuclei divided by the total counterstained nuclei was significantly lower in CoPP group than in control or (CoPP + ZnPP)-treated group (Table 1).

DISCUSSION

Almost all transplanted organs suffer a certain degree of IRI during transportation and preservation. Indeed, IRI may not only limit organs cold preservation time, but also cause posttransplantational non-function and late chronic rejection [1, 2]. Since hepatocytes have many mitochondria, they are very susceptible to IRI. The mechanism of liver injury following ischemia/reperfusion involves a complex interaction of events, which include neutrophil activation, increased expression of adhesion molecules, Kupffer cell activation, cytokine release, sinusoidal endothelial cell death, and hepatocyte injury [29]. Preservation of livers before transplantation for more than 24 h still carries the risk of compromising the function of the future graft [30, 31]. Recently, increased HO-1 expression was shown to decrease IRI of graft and to prolong kidney and small bowel survival [20-24].

In this study, an *ex vivo* liver cold ischemia preservation model was set up. According to previous results [32], rats were given 2.5 mg/kg CoPP twice to induce HO-1 overexpression.

HO-1 is expressed at low levels in liver during normal conditions. Western blot analysis showed that HO-1 was barely detectable in control tissue, whereas treatment of rats with CoPP and CoPP + ZnPP resulted in enhanced HO-1 expression in liver. The functions of reperfused liver after cold storage were significantly improved by CoPP treatment. The perfusate activities for AST and LDH were lower in the CoPP group. This protection of hepatic functions correlated with less ischemia/reperfusion damage when assessed histologically.

It is well established that the expression of HO-1 can be induced effectively by administration of CoPP [33]. In particular, high amounts of HO-1 are expressed in liver and spleen following CoPP treatment. However, one may speculate that CoPP could induce other heat shock proteins besides HO-1, and infusion of CoPP in high doses may modulate other heme enzymes such as nitric oxide synthase and guanylate cyclase [34, 35]. To determine if amelioration of hepatocyte injury in cold ischemia

preservation model was indeed mediated by the induction of HO-1, rats were pretreated with CoPP and ZnPP (a potent HO-1 inhibitor). Our results showed that there was not much difference in HO-1 expression between CoPP- and (CoPP + ZnPP)-treated groups. Although ZnPP could not inhibit CoPP-induced HO-1 overexpression, it inhibited the activity of HO-1 and abolished HO-1-mediated protective effects. These findings documented direct involvement of HO-1 in protection against cold ischemia preservation injury of liver.

The mechanism of liver protection by HO-1 overexpression remains unclear. In order to get more insight into this problem, we studied antioxidation, antiinflammation, and antiapoptosis events in liver.

Free radical-mediated lipid peroxidation is an important determinant of oxidative injury. The relative magnitude of oxidative stress associated with liver IRI could be assessed by measuring tissue levels of MDA. Our results showed that the concentration of MDA in CoPP-pretreated liver was much lower than in control. These findings suggested that HO-1 overexpression helped to decrease oxidative liver injury, and this might be related to accumulation of end products of heme degradation. Biliverdin and its reduced product bilirubin can scavenge reactive oxygen species, and they are both considered as endogenous antioxidants that may protect cells from oxidative injury. Iron, one of the HO-1 byproducts, can stimulate ferritin synthesis, and the latter protein can reduce the production of reactive oxygen species.

Expression of inflammatory cytokines in early reperfusion is the major mechanism that causes cold reperfusion injury. Upregulation of HO-1 inhibits inflammatory responses consistent with our present ELISA findings of markedly decreased TNF-α and IL-6 levels in CoPP-pretreated livers. In our ongoing RT-PCR studies, markedly diminished expression of mRNA coding for TNF-α and IL-6 was readily detectable in liver that overexpresses HO-1 despite previous IRI (unpublished data).

Recently, apoptosis has been identified as a mechanism of hepatic injury after ischemia and reperfusion and proposed to be critical in liver graft after transplantation [36]. Apoptosis differs from necrosis not only morphologically but also by mediators and mechanisms of cell death. Fragmentation of DNA is the most important feature of cell apoptosis. Sinusoidal endothelial cells are particularly vulnerable to apoptosis, and damage to endothelial cells can lead to secondary hepatocyte injury and death after liver ischemia and reperfusion. It has been shown that CO suppressed *in vitro* endothelial cell apoptosis [37]. In other studies, apoptosis was a quantitatively minor phenomenon in liver cold ischemia and reperfusion [38]. Therefore, we investigated whether HO-1 induction could prevent apoptosis in the liver cold ischemia preservation model.

We used the TUNEL assay to determine the extent of apoptosis in rat liver after cold storage followed by 2 h of *ex vivo* perfusion. The results showed that apoptotic index was significantly diminished in the CoPP-treated group compared with control or (CoPP + ZnPP)-treated groups. These findings implied that CoPP-induced HO-1 overexpression could prevent apoptosis in *ex vivo* liver preservation and perfusion. Consistent with these observations, electron microscopy images showed nucleolus fragmented and chromatin dumped around the nuclear membrane forming apoptotic cell in control group while cell apoptosis was significantly diminished in the CoPP-treated group. The exact mechanism through which HO-1 prevents apoptosis remains to be elucidated. Brouard and coauthors reported that the antiapoptotic effect of HO-1 is mediated by CO via the activation of the p38 mitogen-activated protein kinase pathway [37]. Others have shown that HO-1 may influence the production of antiapoptotic proteins [38].

In conclusion, our results using a model of *ex vivo* cold ischemia preservation of liver suggested that CoPP-mediated HO-1 overexpression, as documented by Western blot analysis, improved liver cold ischemia preservation effect and preserved hepatocyte integrity. The mechanisms involved include reduction of oxidative injury and inflammation and prevention of apoptosis. Treatment with ZnPP, the HO-1 inhibitor, abolished these beneficial effects, documenting the direct involvement of HO-1 in protection against liver cold ischemia preservation injury.

This study documents the potential utility of HO-1 induction agents in preventing IRI resulting from prolonged storage of liver transplants.

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