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• BRIEF REPORTS •

Heme oxygenase-1 alleviates ischemia/reperfusion injury in aged liver

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Abstract

AIM: To investigate if ischemia/reperfusion (I/R) injury in aged liver could be alleviated by heme oxygenase-1 (HO-1).

METHODS: Three groups of SD rats (16 mo old) were studied. Group 1: control donors received physiological saline 24 h before their livers were harvested; group 2: donors were pretreated with hemin 24 h before their livers were harvested; and group 3: donors received hemin 24 h before their livers were harvested and zinc protoporphyrin (ZnPP, HO-1 inhibitor) was given to recipients at reperfusion. The harvested livers were stored in University of Wisconsin solution (4 ℃) for 6 h, and then transplanted to syngeneic rats. Serum glutamic oxaloacetic transaminase (SGOT), apoptotic cells, and apoptotic gene were measured 3, 6, 12, 24, 48 h after reperfusion. We measured the apoptotic index by TUNEL, determined the expression of antiapoptotic Bcl-2 and proapoptotic (caspase-3) gene products by Western blot.

RESULTS: After 3, 6, 12, 24, and 48 h of reperfusion, the SGOT levels (584.4±85.8 u/L, 999.2±125.2 u/L, 423.4±161.3 u/L, 257.8±95.8 u/L, and 122.4±26.4 u/L) in hemin group were significantly (all $P<0.05$) lower than those in saline group (1082.2±101.2 u/L, 1775.2±328.3 u/L, 840.4±137.8 u/L, 448.6±74.3 u/L, and 306.2±49.3 u/L). Liver HO-1 enzymatic activity correlated with beneficial effects of hemin and deleterious effects of adjunctive ZnPP treatment. Markedly less apoptotic (TUNEL+) liver cells 3, 6, 12, 24, and 48 h after reperfusion (5.16±0.73, 10.2±0.67, 9.28±0.78, 7.14±1.12, and 4.78±0.65) ($P<0.05$) could be detected in hemin liver grafts, as compared to controls (7.82±1.05, 15.94±1.82, 11.67±1.59, 8.28±1.09, and 6.36±0.67). We detected the increased levels of Bcl-2 (1.5-fold) expression and compared with saline controls. These differences were most pronounced at 12 h after transplantation. In contrast, an active form of proapoptotic caspase-3 (p20) protein was found to be 2.9-fold lower at 24 h in hemin-pretreated group, as compared to saline liver transplant controls.

CONCLUSION: HO-1 overexpression can provide potent protection against cold I/R injury. This effect depends, at least in part, on HO-1-mediated inhibition of antiapoptotic mechanism.

Key words: Aged liver; Ischemia-reperfusion injury; Heme oxygenase-1

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INTRODUCTION

Orthotopic liver transplantation (OLT) has become an effective therapeutic modality for end-stage liver diseases. Advances in surgical procedures and immunosuppression protocols have considerably improved patient survival after liver transplantation. One problem associated with OLT is the disparity between the increasing number of potential recipients and the inferior number of eligible liver donors. The necessity to expand the donor population has attracted attention to the possible use of aged donor livers, which are frequently discarded because of their primary nonfunction. Ischemia/reperfusion (I/R) injury, an antigen-independent component of "harvesting" injury, is one of the most critical events leading to nonfunction or early dysfunction of aged liver grafts[1,2]. Reversible liver impairment or severe injury resulting in cell death and ultimate liver failure is dependent on the extent of liver damage caused by I/R.

Heme oxygenases (HOs) are ubiquitous enzymes that catalyze the initial and rate-limiting steps in oxidative degradation of heme to bilirubin. HOs cleave a mesocarbon of the heme molecule, producing equimolar quantities of biliverdin, iron, and carbon monoxide (CO)[3]. Biliverdin is reduced to bilirubin by bilirubin reductase, and free iron is used in intracellular metabolism or sequestered in ferritin. Three HO isoforms have been identified. HO-1, an inducible heat shock protein 32, is highly induced and confers protective effects on oxidative stress response both *in vivo* and *in vitro*. The mechanism by which HO-1 confers protection against oxidative stress has not yet been defined. It is believed that byproducts derived from the catalysis of heme by HO, namely biliverdin and ferritin accumulated from released iron, and finally CO, may mediate the physiological effects of HO-1. Both biliverdin and bilirubin possess antioxidant properties[4], whereas iron released during heme catabolism can stimulate ferritin synthesis[5]. Attention has been centered on the biological effects of reaction products that potentially possess important antioxidant, anti-inflammatory, antiapoptotic, and possible immune modulatory functions[6-11]. However, putative mechanisms by which HO-1 induction may lead to cytoprotection during I/R insults before transplantation remain unclear. Since HO-1 may play a cytoprotective role through an antiapoptotic pathway *in vitro*, we hypothesize

that one possible pathway by which HO-1 confers protection against oxidant injury is via its ability to impart antiapoptotic activity. Thus, the present study was designed to examine whether attenuation of I/R injury by prior treatment with hemin, an HO-1 inducer, might indeed involve the antiapoptotic pathway in rat hearts undergoing a cold I/R insult.

MATERIALS AND METHODS

Animals

Aged male SD rats were obtained from the Laboratory Animal Center of Jiangsu Province. Animals were fed a standard rodent diet and water according to the guidelines approved by the China Association of Laboratory Animal Care.

Study design

Syngeneic liver transplantation was performed using livers that were harvested from aged rats and stored for 6 h at 4 ℃ in University of Wisconsin solution before transplanted into recipients with revascularization without hepatic artery reconstruction[12,13]. In group 1, prospective liver donors (n = 25) were given 9 g/L saline (5 mL/kg s c) 24 h before their livers were harvested; group 2, donors (n = 25) received hemin (40 umol/kg s c) In 24 h before procurement. After a 6-h storage at 4 ℃ in University of Wisconsin solution, livers in both groups were transplanted into rats. In group 3, donors (n = 10) were treated with hemin 24 h before their livers were harvested. After a 6-h cold storage, livers were transplanted into rats, which were then infused with ZnPP (1.5 mg/kg I v), at the end of surgery after the vessels were unclamped. Separate groups of rats (n = 5 group) were killed at 3, 6, 12, 24 and 48 h after their vessels were unclamped. Serum glutamic oxaloacetic transaminase (SGOT) levels were detected and liver samples were collected for further analysis.

Enzymatic assay for HO-1 activity

To determine HO-1 activity, liver isografts were removed and homogenized on ice in Tris-HCl lysis buffer (pH 7.4) containing 5 mL/L Triton X-100 and protease inhibitors. Samples were frozen in small aliquots until use. Graft homogenates were mixed with 0.8 mmol/L reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH), 0.8 mmol/L glucose-6-phosphate, 1.0 U of G-60P dehydrogenase, 1 mmol/L $MgCl_2$, and 10 mL of purified rat liver biliverdin reductase at 4 ℃. The reaction was initiated by addition of hemin (final concentration 0.25 mmol/L). The reaction mixture was incubated at 37 ℃ in the dark for 15 min. At the end of the incubation period, all insoluble materials were removed by centrifugation, and supernatants were analyzed for bilirubin concentration. An extinction coefficient of 40 mmol/L^{-1} cm^{-1} at $A_{460\text{-}530}$ nm was used to calculate the amount of bilirubin formed. Controls included naive samples in the absence of the NADPH generating system and all the ingredients of the reaction mixture in the absence of graft homogenates. Biliverdin reductase was purified from rat livers as previously described[14].

Detection of apoptosis

Some of the specimens were immediately fixed in 4 ℃ buffered formalin and embedded in paraffin. In all cases, conventional histologic examination was performed on 4 μm thick sections of paraffin-embedded tissue. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed essentially according to the instructions of the commercial kit (ApopTag, Intergen Co., NY). Nuclear counterstaining was performed with methyl green. Negative and positive control reactions were performed for each reaction step. Negative controls were obtained by omission of terminal deoxynucleotidyl transferase. All the negative controls showed no positive signal. Positive controls included sections that were pretreated by DNAase I. All the positive controls were positive. Morphometric analysis of the cells in tissue stained by the TUNEL method was performed under the high-power magnification (×400) in a blind fashion. Thirty random fields were counted for each TUNEL-stained tissue sample. The number of hepatocytes was counted and expressed as a percentage of the total number of respective liver cells counted. Hepatocytes were clearly identified as a specific population of cells. However, sinusoidal endothelial cells (SLCs) included endothelial cells, Kupffer cells, and possibly adherent neutrophils. All nucleated cells lining the sinusoids were evaluated.

Western blot analysis of Bcl-2 and caspase-3 expressions

Protein was extracted from liver tissue samples with PBSTDS buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1 g/L sodium dodecyl sulfate [SDS], 10 g/L sodium deoxycholate, and 10 mL/L Triton X-100, pH 7.2). Proteins (30 g/sample) in SDS loading buffer (50 mmol/L Tris, pH 7.6, 100 g/L glycerol, and 10 g/L SDS) were subjected to 120 g/L SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The gel was then stained with Coomassie blue to document equal protein loading. The membrane was blocked with 3% dry milk and 1 mL/L Tween 20 (USB, Cleveland, OH) in PBS and incubated with primary antibody against Bcl-2 or caspase-3 and actin. The filters were washed and then incubated with horseradish peroxidase donkey anti-rabbit antibody (Amersham, Arlington Heights, IL). Relative quantities of protein were determined using a densitometer (Kodak Digital Science 1D Analysis Software, Rochester, NY).

Statistical analysis

Results were expressed as mean±SD. Statistical comparisons between the groups were done using unpaired two-tailed Student's t test. P values less than 0.05 were considered statistically significant.

RESULTS

Alleviation of ischemia/reperfusion injury induced by HO-1

The SGOT release was a well-established marker of hepatocellular injury after ischemia/reperfusion. We measured SGOT levels at 3, 6, 12, 24 and 48 h after reperfusion. The SGOT level in hemin group was significantly lower than that in saline and hemin±ZnPP groups at any time point after reperfusion (P<0.05, Figure 1).

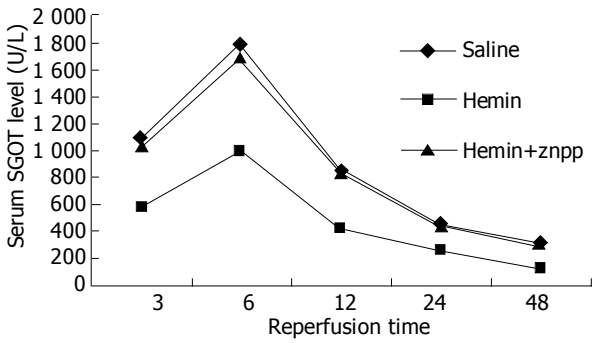

Figure 1 Comparison of serum SGOT levels in hemin group and in saline and hemin+ZnPP groups (P<0.05). there was no difference between the two groups. After a 6-h reperfusion, the serum SGOT level was the highest.

Enhancement of HO-1 enzyme activity after hemin treatment

We analyzed HO-1 enzyme activity in aged rat liver and isograft samples [nmol of bilirubin/(mg protein min)]. As shown in Table 1 and Figure 2 pretreatment with hemin significantly increased HO-1 liver activity after transplantation (2.13±0.04 *vs* 1.09±0.03, 2.17±0.04 *vs* 1.20±0.03, 2.22±0.05 *vs* 1.27±0.04, 2.03±0.04 *vs* 1.15±0.03, and 1.94±0.03 *vs* 1.05±0.03 in controls at 3, 6, 12, 24, and 48 h, respectively, $P<0.05$). Adjuvant ZnPP given during reperfusion inhibited HO-1 activity in liver isografts.

Table 1 Apoptotic index after reperfusion (mean±SD)

Group	3 h (%)	6 h (%)	12 h (%)	24 h (%)	48 h (%)
Saline	7.82±1.05	15.94±1.82	11.67±1.59	8.28±1.09	6.36±0.67
Hemin	5.16±0.73	10.2±0.67	9.28±0.78	7.14±1.12	4.78±0.65
Hemin+Znpp	7.8±0.83	15.94±1.58	11.68±1.59	8.76±0.88	6.48±0.70

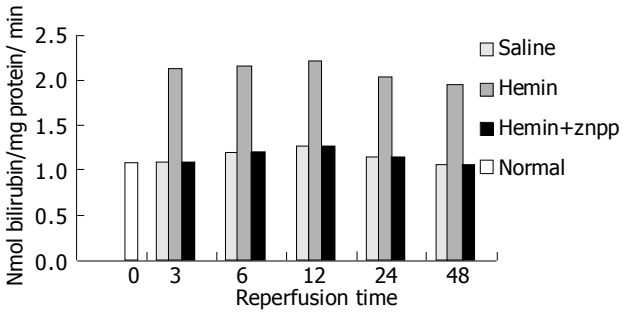

Figure 2 Enzymatic assay for HO-1 activity.

Depression of liver apoptotic cell death by HO-1 overexpression

To detect apoptotic cells, we performed TUNEL labeling in liver isografts that underwent cold I/R insults before transplantation. Classic TUNEL positivity was characterized by focal nuclear staining; the nuclear and cell membranes in apoptotic cells were intact. Results of TUNEL staining of liver isografts subjected to 3, 6, 12, 24 and 48 h of reperfusion after 24 h of cold preservation are shown in Figure 3. The frequency of TUNEL+ cells in liver myocytes was diminished in sections from rats pretreated with hemin (Figure 3A), as compared to saline-treated controls or those after adjuvant hemin plus ZnPP therapy. Consequently, an apoptotic index, calculated as the percentage of TUNEL-nuclei divided by the counterstained-nuclei, significantly diminished in the treated hemin group as compared to saline or hemin+ ZnPP-treated group.

Enhancement of Bcl-2 and depression of Caspase-3 expression by HO-1 overexpression

To evaluate whether hemin therapy affected the intragraft apoptotic networks, we assessed the expression of antiapoptotic Bcl-2 and proapoptotic (caspase-3) gene products in orthotopic liver transplants by Western blot (Figure 4). In agreement with increased HO enzymatic activity in the liver treated with hemin, we detected increased levels of Bcl-2 (1.5-fold) expression, as compared to saline controls. These differences were most pronounced at 12 h after transplantation. In contrast, an active form of the proapoptotic caspase-3 (p20) protein was found to be 2.9 folds lower at 24 h in hemin-pretreated group, as compared to saline controls.

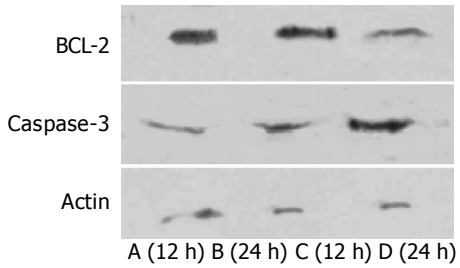

Figure 4 Western blot analyses of Bcl-2 and caspase-3 in orthotopic liver transplants at 12 h (lanes A and C) and 24 h (lanes B and D) after transplantation. At 12 and 24 h, higher levels of antiapoptotic Bcl-2 were found in hemin–treated livers (lanes A and B) as compared to saline controls (lanes C and D). Proapoptotic caspase-3 decreased in hemin–treated livers (lanes A and B) as compared to saline controls (lanes C and D).

DISCUSSION

The principal findings of this work are as follows. Hemin prevents I/R insults in aged rat livers with cold ischemia followed by reperfusion. Hemin-induced HO-1 overexpression, as determined by enzymatic assays, decreases hepatocellular apoptosis. Treatment with ZnPP, abolishes these beneficial effects, documenting the direct involvement of HO-1 in the protection against I/R injury in aged rat livers.

HO-1 activity, is considered as one of the most sensitive indicators of cellular stress[15,16]. Being analogous to heat shock regulation, HO-1 overexpression may represent an endogenous adaptive mechanism protecting cells from stress after radiation, heat shock, inflammation, and ischemia. Indeed, hemin-induced HO-1 overexpression in our present study prevented or significantly decreased aged liver injury in a clinically relevant model of 6 h cold I/R injury after syngeneic transplantation. Unlike saline-pretreated controls, which had high serum levels of SGOT, aged livers harvested from hemin-pre treated donors had low levels of SGOT. ZnPP-mediated inhibition of HO-1 activity had negative effects after hemin treatment, which endorses the hypothesis that the mechanism underlying

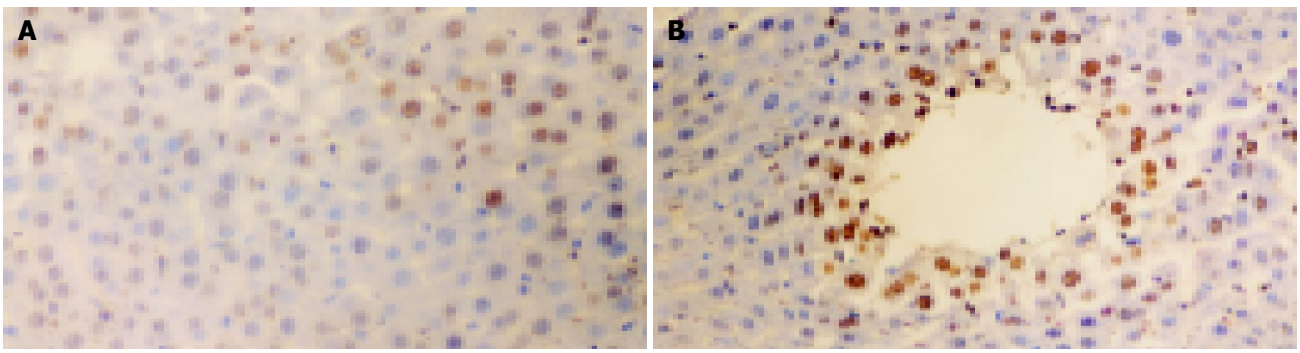

Figure 3 TUNEL-positive cells in (A) and saline-pretreated group (B) hemin-pretreated group (A) and (×200).

protection against liver I/R injury involves HO-1 induction rather than modulation of other biochemical pathways that may protect livers from oxidative injury.

Studies have shown that up-regulation of HO-1 expression could exert strikingly important adaptive antioxidant and anti-inflammatory functions to protect cells from pathophysiological conditions, including graft rejection and I/R injury[17-22]. But the mechanisms underlying the beneficial effects of HO-1 on I/R injury have to be elucidated.

The exact mechanisms underlying the fulminic I/R insults by free radicals, calcium entry, or inflammation are currently unknown, although cell apoptosis has been suggested as a key early event[23-25]. Thus, inhibition of the apoptotic pathway seems to be a rational therapeutic strategy to reduce the risk of preservation injury in transplanted organs. Up-regulation for HO-1 could inhibit apoptosis both *in vitro* and *in vivo*[10,26], consistent with our present TUNEL-based findings of the decreased frequency of apoptotic cells in hemin-pretreated liver isografts as compared to controls. The cellular and physiological mechanisms by which HO-1 exerts its cytoprotective functions against I/R injury at the graft site might involve antiapoptotic protein expression. Indeed, adjuvant treatment with ZnPP, prevented the expression of Bcl-2 and promoted the activation of caspase-3 in this study. Caspase-3 activitation is a key step in apoptosis. Bcl-2 could prevent release of apoptogenic factors such as cytochrome C and apoptosis-inducing factors from mitochondria into cytosols[27,28]. Moreover, Bcl-2 could interact with apoptosis-related family members such as Bax, and several non-family member proteins including Raf-1 and Bag-1. Bag-1 could cooperate with Bcl-2 to suppress apoptosis[29]. In this study, the up-regulation of antiapoptotic Bcl-2 occurred 3 h after the vessels were unclamped. Bcl-2 overexpression could block cell death in a caspase-independent manner, preserve mitochondrial integrity during hypoxia, and promote ATP generation even in the absence of glucose, and utilize respiratory substrates during reoxygenation[30].

The mechanism by which HO-1 influences the production of antiapoptotic proteins remains to be elucidated. It has been shown that HO-1-induced antiapoptotic effects might be mediated via CO or p38 mitogen-activated protein kinase (MAPK) signaling transduction pathways[26]. CO generation could prevents xenograft rejection via its ability to suppress endothelial cell apoptosis *in vivo*[31], whereas low CO concentrations have been identified as the key factor in HO-1-induced protection against apoptosis induced by tumor necrosis factor -induced apoptosis in cultured fibroblasts[10] as well as endothelial cells[26] *in vitro*. Moreover, animals exposed to CO *in vivo* exhibit a significant attenuation of hyperoxia-induced lung apoptosis, at least in part via the anti-inflammatory mitogen-activated protein kinase-3 (MKK3)/ p38 mitogen-activated protein kinase (p38 MAPK) pathway[32]. Three major MAPKs in cardiomyocytes subjected to I/R and the endoplasmic reticulum kinase (ERK) pathway may be critical for the survival of cells by protecting them from programed cell death caused by stress-induced activation of p38 and c-jun N-terminal kinase (JNK)[33].

In summary, HO-1 up-regulation can provide potent protection against cold ischemia and reperfusion injury in aged rat livers. This beneficial effect depends, at least in part, on HO-1 modulation of the antiapoptotic pathway. HO-1-inducing agents could be used in preventing I/R injury of marginal donors.

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