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Induction of specific stress response increases resistance of rat liver allografts to cold ischemia and reperfusion injury

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Abstract Heme oxygenase-1 (HO-1) has been shown to increase cellular resistance against oxidative injury, but the functional significance of this is currently obscure. We investigated the protective role of HO-1, induced by tin-protoporphyrin IX (SnPP), in attenuating liver transplantation injury. Lewis rats were intraperitoneally treated with saline as control, 50 μmol/kg of SnPP, or 2 mg/kg of cycloheximide (CHX) before SnPP injection. Gene expression of HO-1 was induced after either treatment with SnPP- or CHX + SnPP instead of saline, whereas HO-1 protein synthesis was enhanced in Kupffer-like dendritic cells of the SnPP-treated group. Following reperfusion of liver grafts preserved for 30 h, there were fewer intercellular adhesion molecule-1-positive cells in SnPP-treated livers, significantly reduced numbers of dead cells, and enhanced graft viability. The present data suggest that increased synthesis of HO-1 protein by SnPP pre-conditioning is linked to the improved liver graft viability through inhibition of inflammatory adhesion molecules.

Keywords Heme oxygenase-1 · Cold ischemia and reperfusion injury · Tin-protoporphyrin · Intercellular adhesion molecule-1

Introduction

Despite the success of liver transplantation, ischemia and reperfusion (I/R) injury remains a serious problem that affects the outcome in the treatment of patients following hepatectomy and liver transplantation. Although there has been significant effort to identify the mechanisms of reperfusion injury following extended hepatic ischemia, there is no clinically proven means except for the avoidance of warm ischemia and the reduction of cold preservation time. Donor pre-conditioning by ischemic or pharmacological maneuvers has been recently shown to attenuate post-ischemic reperfusion injury in cardiac, renal, and hepatic animal models [15, 17, 30, 34]. The underlying mechanisms may involve the generation of vaso-active molecules such as nitric oxide and adenosine or the expression of stress-response genes including superoxide dismutase and heat shock proteins (HSPs) [20, 23, 26, 38].

One of the unique HSPs, heme oxygenase-1 (HO-1), is easily induced by a wide variety of stressful stimuli, including structurally unrelated chemical agents and environmental stresses such as heme, heavy metals, heat shock, hypoxia, glutathione depletion and ultraviolet irradiation [1, 5, 27]. The induction of HO-1 was originally proposed as a sensitive indicator of oxidative stress but has recently been reported to be an adaptive cellular response [1, 19]. The protective HO-1-mediated mechanism may be dependent on its enzyme activity, which includes the removal of a potentially toxic heme molecule and lipid-soluble iron from cells, as well as the generation of anti-oxidative bile pigments or vaso-active carbon monoxide [9]. Tin-protoporphyrin IX (SnPP), a synthetic metalloporphyrin known to be a potent

inhibitor of HO-1 enzyme activity, has been reported to abrogate the beneficial effects of HO-1 induced by pharmacological agents or gene transfection, but the role of HO-1 activity-dependent end products is currently controversial [8, 14, 40]. SnPP was considered to exert a protective effect in pathological conditions such as acute proximal tubule injury [40]. In fact, the inhibition of HO enzyme activity by metalloporphyrins has been applied in models where excess heme and bilirubin accumulation is considered to be harmful [6, 7]. SnPP regulates HO-1 by a dual mechanism; potently inhibiting the enzyme activity by acting as a competitive substrate for heme while enhancing the synthesis of new enzyme protein [28]. However, the functional significance of synthesized HO-1 protein is not fully understood, and an understanding of the pre-conditioning effect of SnPP on liver I/R injury also remains elusive.

The aim of the present study was twofold. Firstly, we examined whether SnPP employed as pretreatment could stimulate induction of a specific stress–response gene such as HO-1. Secondly, we investigated whether SnPP pre-conditioning could protect the functions of isolated rat liver when the liver was subjected to prolonged hypothermic preservation and transplantation.

Materials and methods

Animals

Inbred male Lewis rats, weighing 180 to 260 g, were originally supplied by Seiwa (Tokyo, Japan) and were then bred in our laboratory. They were housed in a climate-controlled room (22 ± 1 °C) with a 12-h light and dark cycle and given tap water and standard rat chow (CE-2; CLEA Japan, Tokyo, Japan). Donor animals had access to water only, 12 h before their organs were removed. The present study was carried out according to the guidelines established by Sapporo Hokuyu Hospital for the care and use of animals in experiments.

Liver preservation and transplantation

Liver grafts were removed by standard techniques, washed out with 5 ml of lactated Ringer's solution to which 100 units of heparin had been added, flushed with 5 ml of University of Wisconsin (UW) solution at 4 °C, and preserved in the same solution at 1–2 °C for 30 h. Following storage, orthotopic transplantation was performed according to the modified techniques described by Kamada et al. [13]. After transplantation, animals received a total of 5 ml of lactated Ringer's solution with 1.5 ml of 7% bicarbonate solution.

Experiment design

To determine the effect of SnPP pre-conditioning on I/R injury, we first evaluated optimal SnPP concentration and treatment time for pre-conditioning. We examined the pharmacological toxicity of SnPP (Porphyrin Products, Logan, Utah, USA), at 5, 50 and 100 μmol/kg. No mortality and liver dysfunction was observed in any group that received SnPP. According to the experiments described by Anderson et al. [4], we selected the dose of 50 μmol/kg SnPP as optimal concentration, which markedly decreased HO enzyme activity from 4 h to 7 days, and exhibited the maximum decreased value at 24 h after injection. Based on these results, 50 μmol/kg of SnPP was used in this study. To address the possible function of HO-1 protein expression in protection against I/R injury, we administered cycloheximide (CHX; Biomol Research Laboratory, Plymouth, Pa., USA) prior to SnPP treatment. Since CHX is known as a non-specific translation inhibitor, it may suppress syntheses of a variety of proteins below a threshold level that is required to sustain organ function. Therefore, we selected 2, 4 and 10 mg/kg of CHX to examine its toxicity (each, $n = 3$). All rats that received more than 10 mg/kg of CHX died, while the rats that received 4 mg/kg of CHX survived, although some of them showed loss of body weight. Since 2 mg/kg of CHX did not affect the survival and body weight of the animals, we selected 2 mg/kg of CHX for the current experiment. The optimal time point for CHX pretreatment was chosen from the method described by Bauer et al. [5]: they showed that expression of HO-1 protein was almost completely blocked by CHX administered 1 h prior to stress induction.

The animals were divided into three groups ($n = 12$) as follows: group I, animals received intraperitoneally (i.p.) 15 ml/kg of 0.9% NaCl solution as a vehicle control; group II received 50 μmol/kg i.p. of SnPP; group III received 2 mg/kg i.p. of CHX 1 h before SnPP injection. SnPP was dissolved in 0.2 N NaOH and adjusted to pH 7.50–7.55 with 1 M HCl. CHX was dissolved in 0.9% NaCl solution. Twenty-four hours after being pre-conditioned, the livers were removed, preserved, and then grafted. In order to examine the direct hepatotoxic effects of CHX, we added another group ($n = 6$) to the initial three groups: group IV; liver grafts from animals that received 2 mg/kg of CHX alone were grafted, following 2 h preservation. All animals were monitored for survival. We also analyzed the expression of HO-1 before preservation and the expression of intercellular adhesion molecule-1 (ICAM-1) and cell viability after reperfusion. With regard to HO enzyme activity, we measured both before and after transplantation. Fresh livers taken from untreated rats were used as a fresh control.

Tissue preparation

The animals were anesthetized i.p. with 50 mg/kg of pentobarbital followed by ether. Liver specimens ($n = 6$) for HO-1 expression were taken at different points in time, snap frozen in liquid nitrogen and stored at −80 °C for early study, while those ($n = 3$) for ICAM-1 expression and cell viability were removed 2 h after reperfusion ($n = 3$).

Reverse-transcriptase polymerase chain reaction

Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis was performed as previously described [16]. In brief, 100 mg of liver tissue were homogenized in 1.3 ml of RNAzolTM B (Tel-Test, Friendswood, Tex., USA), and 0.2 ml of chloroform was added for extraction of RNA. After centrifugation, the suspension was collected and mixed with 0.5 ml of isopropanol and centrifuged again. The RNA pellets were washed with 1 ml of 75% ethanol, centrifuged, and dissolved with diethylpyrocarbonate-treated water. With a commercially available preamplification system (SUPERSCRIPTTM, Life Technologies, Tokyo, Japan) 5 μg of total RNA was reverse-transcripted in cDNA. Using primers for HO-1, we amplified the cDNA products by PCR. Primers used in PCR were as follows: HO-1 sense (5'-TGG AAG AGG AGA TAG AGC GA-3') and HO-1 antisense (5'-TGT TGA GCA GGA AGG CGG TC-3'). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an

internal control, was obtained from MPCR Kit for rat apoptosis genes set-2 (Maxim Biotech, San Francisco, Calif., USA). They were amplified in 25 μl of PCR reaction volume containing 1.8 μmol of $MgCl_2$, 25 mmol of KCl, and 10 mmol of Tris buffer using 1 mg of cDNA templates. PCR products of HO-1 were 451 base pairs. The dilutions of cDNA were amplified for 25, 27, 30 and 35 cycles of PCR. The mixture was first incubated at 94 °C for 3 min, and then cycled at 94 °C for 1 min, 59 °C for 1 min, and elongated at 72 °C for 10 min and held at 4 °C. After amplification, the samples were separated on a 3% agarose gel containing 0.05% ethidium bromide, and photographed. Digital photographs were assessed with image-analysis software (Luminous Imager, AISIN COSMOS R&D, Aichi, Japan) and mRNA expression was evaluated by the band intensity ratio of HO-1 to GAPDH and presented as %GAPDH.

Western blot analysis

Liver tissues were homogenized in an ice-cold lysis buffer (10 mmol/l sodium phosphate (pH 7.2), 150 mmol/l NaCl, 1% Nonidet P-40, 2 mmol/l EDTA, 50 mmol/l NaF) containing protease inhibitors (4 μg/ml aprotinin, 0.2 mmol/l sodium vanadate, 100 mmol/l phenylmethylsulfonyl fluoride, 2 μg/ml Leupeptin) [10]. The homogenates were centrifuged at 10,000 *g* at 4 °C for 20 min. Lysates were mixed 2:1 with triple-strength sample buffer (250 mmol/l Tris, 4% sodium dodecyl sulfate, 10% glycerol, 2% β-mercaptoethanol, 0.006% bromophenol blue [pH 6.8]), and then boiled for 5 min. The resulting supernatants were collected and subjected to 13% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Separated proteins in the gel were transferred to a polyvinylidine difluoride transfer membrane, and blocked at 4 °C overnight with 5% non-fat dry milk in phosphate-buffered solution (PBS). The following day, the membrane was incubated with rabbit anti-rat HO-1 primary polyclonal antibodies (1:500, StressGen Biotech, Victoria, BC, Canada) in PBS for 60 min, washed with PBS containing 0.05% Tween 20, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Dako, Carpinteria, Calif., USA) at 1:2000 dilution in PBS. Immunoreactive bands were visualized by an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Quantification of protein signals was performed by computer-assisted densitometry.

Immunohistochemistry

Liver tissues were embedded in OCT compounds (Tissue-Tek, Sakura Finetek, Torrance, Calif., USA) and quick-frozen in liquid nitrogen. The sections (3 μm thick) mounted on glass slides were prepared and air-dried at 4 °C overnight. Sections were fixed in −20 °C acetone–methanol (1:1) for 5 min. After being air-dried and washed with PBS, the tissue sections were treated with 20% bovine serum albumin for 30 min to block non-specific proteins. After being washed with PBS, the tissue sections were exposed to optimal dilution of polyclonal rabbit anti-rat HO-1 or monoclonal mouse anti-rat ICAM-1 antibody (PharMingen, A Becton Dickinson, San Diego, Calif., USA) for 30 min. After washing the sections with PBS, we blocked endogenous peroxidases by treating with DAKO blocking reagent at room temperature for 30 min. After being washed with PBS, the tissue sections were overlaid with goat anti-mouse IgG (Envision+, Dako, Carpinteria, Calif., USA) for 30 min. Finally, 0.1 mg/ml of 3,3'-diaminobenzidine tetrahydrochloride was applied for 10 min and the sections were counterstained with hematoxylin for 2 min. The negative control was prepared by omission of the primary antibodies.

HO enzyme activity in the whole rat livers

We determined HO enzyme activity in the liver by measuring bilirubin formation ($n=4$) [11]. Liver tissue was placed on ice and washed twice with PBS at pH 7.0. To remove insoluble fractions, we lysed the tissue specimens in 0.25 mol/l sucrose lysing buffer containing 2.5 mol/l potassium phosphate, 2 mol/l sucrose, 0.5 mol/l EDTA and 0.1 mol/l phenylmethylsulfonyl fluoride. The lysates were collected by centrifugation at 2,000 rpm for 10 min and were further centrifuged at 18,000 *g* for 20 min. The supernatants were collected by ultracentrifugation at 150,000 *g* for 1 h. The pellets, including the microsome fraction, were collected and homogenized with 1% Nonidet P-40 lysing buffer solution containing 150 mmol/l NaCl, 1 mmol/l phenylmethylsulfonyl fluoride, and 50 mmol/l Tris-HCl (pH 8.0). Finally, the supernatants were collected at 15,000 rpm for 10 min and left on ice for 30 min. The reaction mixture contained the following in a final volume of 300 ml: 1 mmol/l glucose 6-phosphate, 0.167 U/ml glucose 6-phosphate dehydrogenase, 0.8 mmol/l NADP, 15 mmol/l hemin, 2 mmol/l $MgCl_2$, 0.01 mg/ml NADPH-cytochrome P450 reductase (Gentest, Woburn, Mass., USA), rat liver cytosol (3.3 mg protein/ml), potassium phosphate buffer (pH 7.4), and the supernatant from the tissue specimens (100 ml). Incubation was carried out at 37 °C for 30 min. An equal volume of chloroform was added to the reaction mixture to stop the reaction, and the bilirubin generated was extracted into the chloroform fraction. After centrifugation at 10,000 *g* for 15 min, the amount of bilirubin was determined according to a method described previously [39]. As a positive control we used 60 μmol/kg of ferri-protoporphyrin IX chloride (Hemin, Sigma Chemical, St. Louis, Mo., USA), a potent inducer of HO-1 activity [35]. Enzyme activity was expressed as picomoles of bilirubin per milligram of tissue protein generated for 30 min.

Assessment of cell viability

Cell viability was determined according to a previously described method [15]. In brief, the portal vein was cannulated with polyethylene tubing, flushed with heparin–saline solution and perfused ex vivo with a 50% mixture of rat plasma and saline containing 0.19 mg/ml of propidium iodide (PI; Sigma Chemical) at a flow rate of 2.5 ml/min for 2 min and then washed with a 50% mixture without PI for 4 min. After perfusion, the liver was excised, embedded in the OCT compound and frozen in liquid nitrogen at −80 °C. Cryostat sections 3 μm thick were prepared for fluorescent microscopic examination.

Statistical analysis

All data were expressed as mean ± SEM. Differences were analyzed by one-way analysis of variance (ANOVA). For graft survival, the Mann–Whitney U test was used. Statistical calculations were performed on a Macintosh personal computer with the Statview II Statistical Package (Abacus Concepts, Berkeley, Calif., USA). Statistical significance was taken as $P<0.05$.

Results

Time course of HO-1 gene expression and protein synthesis in the liver tissues

HO-1 mRNA expression in the liver tissues 3, 6 and 24 h after treatment was analyzed by RT-PCR. Representative data of HO-1 mRNA expression in the three groups

is shown in Fig. 1. A substantial increase in transcript levels in the liver samples, both in the SnPP-treated and the CHX + SnPP-treated groups, over those in the time-matched saline control, was observed 3 h after the onset of each treatment and was maintained for up to 24 h. An increased level of HO-1 mRNA in SnPP-treated livers was reflected by a concomitant increase in HO-1 protein synthesis, as shown by Western blot analysis at 6 h, and augmented after 24 h, whereas HO-1 protein levels of group III were slightly increased after 6 h and showed no significant difference at 24 h compared with those of control group I, despite the increased HO-1 transcript level (Fig. 2).

Cell-type specific expression patterns of HO-1 immunoreactive proteins in liver tissues

Cell-type specific and spatial expression patterns of HO-1 protein were determined by immunohistochemical examination. Although HO-1 was barely detectable in the hepatocytes and was scattered in some Kupffer-like dendritic cells in the fresh control livers (Fig. 3A), a substantial increase was observed in non-parenchymal cells in the midzonal and pericentral regions and, most notably, in the sinusoidal-lining dendritic cells in livers that received SnPP 24 h before sampling (Fig. 3B). In contrast, no HO-1 staining could be found in CHX + SnPP-treated livers (Fig. 3C).

HO enzyme activity in the whole livers

HO activity was measured in the whole livers 6, 24 h after each treatment and 2 h after transplantation (Fig. 4). In the hemin-treated positive-control group, HO activity was significantly increased after treatment and was further enhanced 2 h after reperfusion. HO activity in SnPP-treated livers remained below the baseline level of vehicle control from 6 h after treatment up to 2 h after transplantation, although HO protein expression was significantly increased. In the SnPP + CHX-treated group, HO activity reverted to the same level as in the vehicle control.

Immunohistochemical expression of ICAM-1 after reperfusion

Enhanced adhesion molecule expression during the early periods of reperfusion mediates inflammatory response and leads to graft failure. In the immunohistochemistry of the control group, ICAM-1 positive materials were dense in the sinusoidal lining cells surrounding the

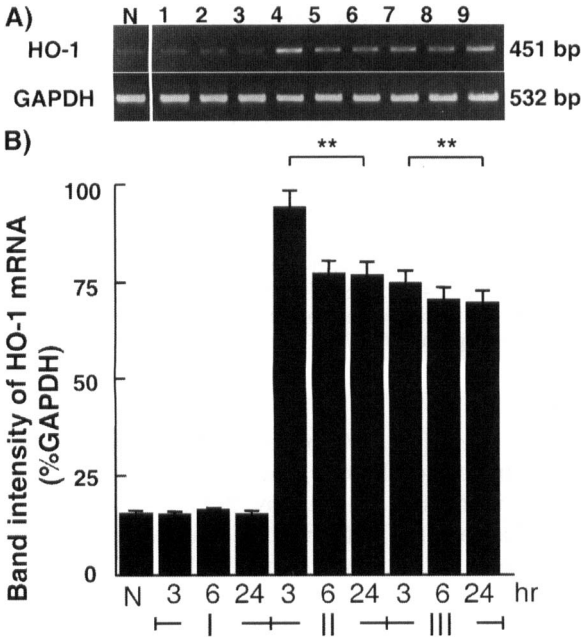

Fig. 1A, B RT-PCR analysis of HO-1 mRNA expression in the liver tissues. GAPDH was used as an internal control. **A** *Lane 1–3* from tissue samples taken 3, 6, and 24 h after intraperitoneal administration of saline; *lanes 4–6* from rats that received 50 μmol/kg of SnPP; *lanes 7–9* from rats that received 2 mg/kg of CHX followed by 50 μmol/kg of SnPP. **B**. The increase in HO-1 mRNA in groups II and III compared with those of time-matched group I and non-treated fresh liver. One representative experiment from four performed is shown. $^{**}P < 0.001$ vs group I

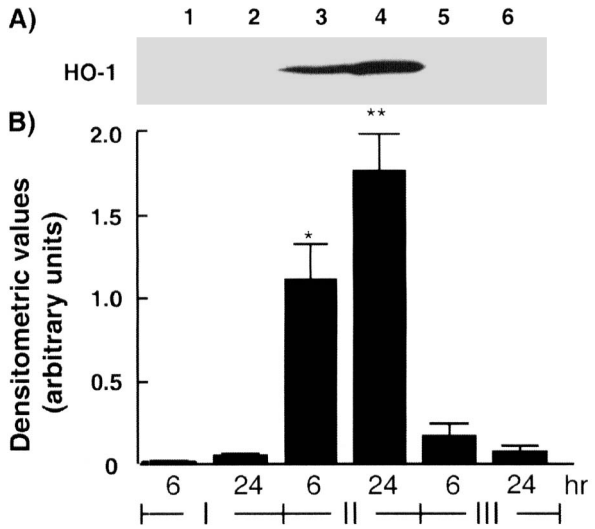

Fig. 2 **A** Western blot analysis of HO-1 protein expression in the liver tissues. Representative tissue samples taken 6 and 24 h after treatment in three experimental groups. **B** Densitometric values of HO-1 protein expressed as mean ± SEM. Significant increases in HO-1 protein were observed in group II at 6 h and 24 h after treatment compared with group I, while group III showed less induction of HO-1 protein despite increased transcript expression. Results shown are one representative experiment from four performed. $^{*}P < 0.005$, $^{**}P < 0.001$ vs group I

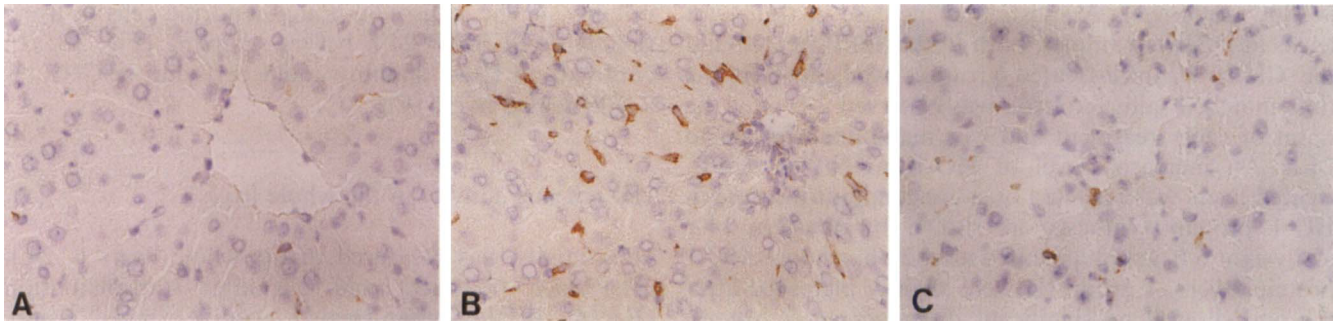

Fig. 3A–C Immunohistochemical staining for HO-1 protein. The expression of HO-1 was augmented in Kupffer-like dendritic cells of tissue specimens taken 24 h after intraperitoneal administration of SnPP (**B**), when compared with that of saline (**A**), whereas expression of HO-1 positive materials was almost completely inhibited in CHX-treated livers (**C**). Original magnification: ×400

hepatocytes, 2 h after reperfusion (Fig. 5A), whereas those in group II were markedly suppressed (Fig. 5B). In group III, ICAM-1 positive cells were moderately present in the sinusoidal lining cells after reperfusion (Fig. 5C).

Assessment of cell viability of reperfused grafts

PI-stained cells in reperfused livers indicate apoptotic dead cells. Fewer apoptotic cells were detected in group II than in groups I and III (Fig. 6A). We assessed cell viability by counting apoptotic cells, and exhibited it by histogram: PI-positive cell counts were 142.4 ± 23.5, 22.6 ± 3.5, and 109.3 ± 16.5 in groups I, II and III, respectively (Fig. 6B), indicating the excellent cell viability in group II ($P < 0.001$).

Animal survival after transplantation

Animal survival rates of more than a 2 weeks were monitored after transplantation (Table 1). The animals in group II had the best survival rate, compared with those in the other groups ($P < 0.01$). Sixteen rats in group I and nine rats in group III died the following morning because of liver failure. The direct hepatotoxic effect of CHX was considered to be negligible by the additional experiments with short-term-preserved livers, in which all rats survived.

Discussion

A recent report has demonstrated that HO-1 over-expressed by cobalt protoporphyrin confers cytoprotection in a rat-liver model of I/R injury, while inhibition of HO-1 mediated by zinc protoporphyrin abrogates the

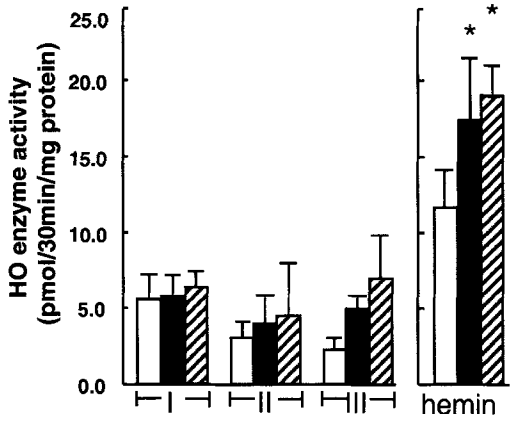

Fig. 4 HO enzyme activities of the whole-liver tissues. Respective time points for removal were: *white bar* 6 h after treatment; *black bar* 24 h after treatment; *striped bar* 2 h after transplantation. HO activity in vehicle control livers (group I) was 5.2 ± 2.1 pmol/30 min per mg protein. In the hemin-treated group, a positive control, HO activity markedly increased to 17.8 ± 4.6 pmol/30 min per mg protein 24 h after injection ($^*P < 0.005$), while those in the SnPP-treated and CHX + SnPP-treated groups (groups II and III) decreased below the baseline level to 4.6 ± 2.5 and 5.1 ± 0.5 pmol/30 min per mg protein at 24 h, respectively. Two hours after transplantation, HO activity remained decreased in all groups (vehicle control: 6.4 ± 1.6; SnPP group: 4.7 ± 3.8; SnPP + CHX: 7.9 ± 3.5 pmol/30 min per mg protein, respectively) except for the hemin-treated group (19.1 ± 2.4 pmol/30 min per mg protein $^*P < 0.005$)

beneficial effect [14]. We have previously reported that prior induction of HO-1 gene expression with a concomitant increased activity by hemin in donor livers could be associated with partial cytoprotection against hepatic cold I/R injury. Interestingly, SnPP, a potent competitive inhibitor of HO enzyme activity, did not eliminate the effects of hemin but rather conferred more beneficial effects, as indicated by histology and animal survival [35]. Since SnPP reduced HO enzyme activity below the baseline level, it was unlikely that the end products of heme degradation, including bilirubin and carbon monoxide, could be directly linked to cytoprotection against hepatic I/R injury. Thus, we tried to investigate the functional significance of SnPP on the regulation of HO-1 protein synthesis independently of

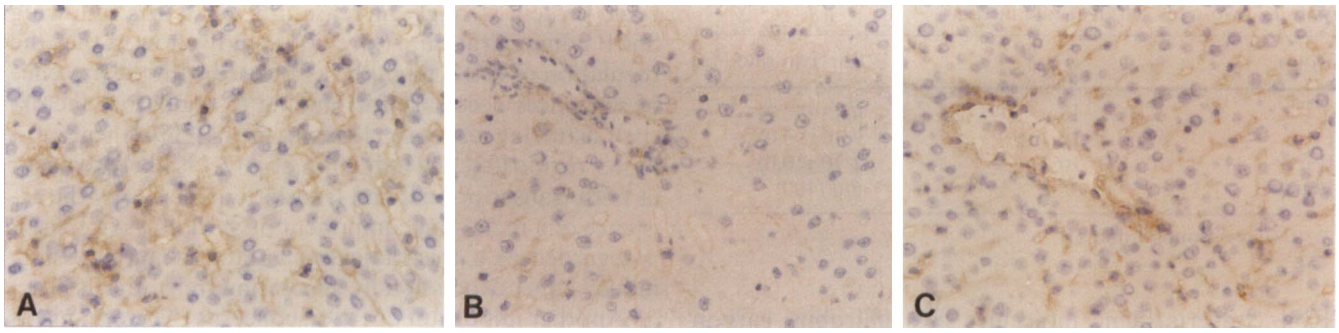

Fig. 5A–C Immunohistochemical staining for ICAM-1. ICAM-1 expression in liver tissues of group I was evident in sinusoidal lining cells around the hepatocytes 2 h after reperfusion (**A**), while highly suppressed in tissues of group II (**B**). Moderate ICAM-1 expression was seen in tissues of group III (**C**). Original magnification: ×400

enzyme activity. Furthermore, we used a potent translational inhibitor of protein synthesis, CHX, before SnPP administration. Prior administration of SnPP induced a large amount of HO-1 protein to liver grafts and appeared to confer protection against I/R injury, whereas CHX almost completely inhibited HO-1 protein synthesis and eliminated SnPP-induced cytoprotection.

The intrinsic hepatic regulation of stress-response genes such as HO-1 has been considered to be a key mechanism of cytoprotection against oxidative stress;

Fig. 6 **A** PI fluorescent staining of rat liver tissue 2 h after reperfusion. Fewer PI positive-staining cells were detected in the SnPP treated group (**b**) than in the vehicle control (**a**) and SnPP + CHX groups (**c**). In the fresh control group, PI positive-staining cells were not detected (*fresh*). Original magnification: ×400. **B** PI-positive cells in five different fields in each group. Concomitant with **A**, PI positive-staining cells in the SnPP-treated group indicated excellent cellular viability when compared with those in the control and SnPP + CHX-treated groups (**$P < 0.001$)

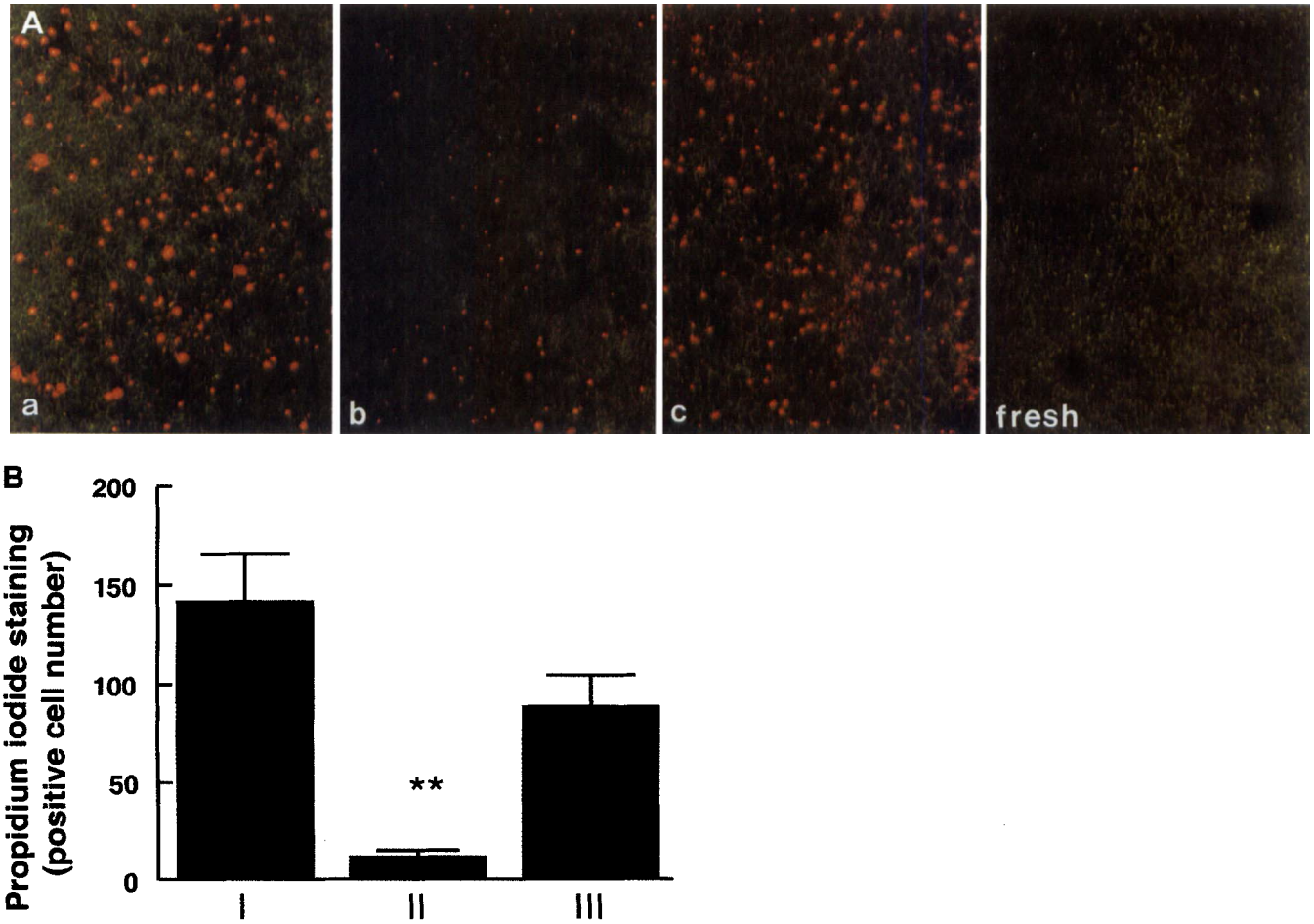

Table 1 Two-week survival rate in rats

Group	*n*	Survival rate (%)
I	18	4/18 (22.2)
II	10	9/10 (90.0)[#]
III	10	1/10 (10.0)
IV	6	6/6 (100)

[#] $P < 0.01$ vs groups I and III

HO-1 converts heme molecules into bilirubin, carbon monoxide, and free iron [1, 9]. Bilirubin is a known potent antioxidant [33]. Carbon monoxide is an important vaso-relaxing factor of hepatic sinusoidal tone via the guanylyl cyclase activating pathway [25]. Paradoxically, these heme-degraded end products are toxic if generated in excess; in addition, the release of free iron gives rise to hydroxyl radicals through the Fenton reaction [21]. Therefore, the role of HO-1-mediated end products is probably dependent on the experimental design. A recent study with the use of a HO-1-deficient cell model demonstrated that HO-1 is necessary to protect cells from oxidative stress, but none of the products of HO activity directly accounts for the protective effects of HO-1 [9]. The present study suggests that the graft viability increased by SnPP pre-conditioning is highly linked to the increased HO-1 protein synthesis and is not always associated with either intensity of HO activity or level of HO-1 mRNA expression. This protective effect may be involved in a mechanism other than the bilirubin-generating system or carbon monoxide–guanylyl cyclase pathway. Indeed, Serfass and Burstyn reported the evocative finding about the cytoprotective effect of SnPP [29]: soluble guanylyl cyclase is known as a major target for nitric oxide and carbon monoxide; interestingly SnPP may stimulate soluble guanylyl cyclase activity independently of the heme-HO-1 system.

HO-1 expression has been reported to be essential to induce long-term allo-graft or xeno-graft survival through the inactivation of endothelial cells [31], followed by the evidence that over-expression of HO-1 in cultured human umbilical-vein endothelial cells inhibited tumor necrosis factor-α or lipopolysaccharide-stimulated upregulation of adhesion molecules such as E-selectin and vascular cell adhesion molecule-1 (VCAM-1) [32]. Although the underlying mechanism is still unclear, we also noted a functional link between increased expression of HO-1 protein and attenuation of ICAM-1 positive endothelial cells following reperfusion. A steady level of HO-1 mRNA expression lacking in the protein synthesis seen in the CHX-pretreated group was not enough to keep graft viability, suggesting that the protective effect of HO-1 could be regulated at a post-transcriptional level.

One important question concerns the molecular mechanisms by which amounts of HO-1 mRNA and enzyme protein strongly increase in liver tissues following SnPP administration. A previous study showed that the regulation of HO-1 might be associated with the presence of sequences necessary for binding to regulatory factors such as metal response elements in the HO-1 gene promoter region [22]: the induction of HO-1 is likely to occur at both transcriptional and translational levels by the existence of a metal-ion component of SnPP [2]; since SnPP is regarded as a free-radical generator, it may possibly induce HO-1 expression by generating free radicals [36].

Another important question concerns the mechanisms by which the amount of HO-1 protein augmented in liver tissue downregulates the expression of adhesion molecules such as ICAM-1. An antioxidant pyrrolidine dithiocarbamate, which is currently being advocated as a treatment to retard the onset of acquired immune deficiency syndrome and for limiting neutrophil-mediated oxidant injury, not only stimulates HO-1 gene expression via the activation of transcription factor, activated protein-1, but also inhibits the activation of nuclear factor kappa B, which is an important transcription factor of pro-inflammatory cytokines and adhesion molecules [12, 18]. Inducible HO-1, highly expressed in Kupffer-like dendritic cells by SnPP administration, may be regulated by a similar mechanism. Although carbon monoxide may modulate the expression of cytokines [24], it is unlikely that a potential cytoprotection by SnPP pre-conditioning may be involved in carbon monoxide-induced anti-inflammatory response. In addition, the role of SnPP-induced HO-1 expression in hepatic I/R injury may also be attributable to the inactivation of these pro-inflammatory factors via increased production of nitric oxide, which may inhibit inflammatory response [3, 37].

Further experiments will be needed to clarify a close relationship between increased protein synthesis of HO-1 and reduced expression of pro-inflammatory adhesion molecule.

In summary, the present study demonstrates that the improved viability of liver grafts by SnPP pre-conditioning could be associated with increased synthesis of HO-1 enzyme protein independently of HO-1 mRNA expression and HO enzyme activity, suggesting an alternative protective mechanism other than the heme-degrading pathway; liver cells highly expressing HO-1 positive deposits correlate to those expressing reduced levels of ICAM-1, which was enhanced during the periods of reperfusion. Less-toxic SnPP may be potentially applicable to the pharmacological treatment in a pathological situation where excess heme or iron accumulation is considered to be harmful, for example I/R injury.

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