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Preconditioning with tin-protoporphyrin IX attenuates ischemia/reperfusion injury in the rat kidney

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Preconditioning with tin-protoporphyrin IX attenuates ischemia/reperfusion injury in the rat kidney.

Background. Heme oxygenase (HO)-1 is induced as a unique stress response and leads to a transient resistance against oxidative damage, including ischemia and reperfusion (I/R) injury. In the present study, we examined whether HO-1 induction may confer a protection against I/R injury in the rat kidney.

Methods. Lewis rats were divided into four groups as follows: (*1*) vehicle group; (*2*) group treated with ferri-protoporphyrin IX (hemin), an inducer of HO; (*3*) group treated with low-dose tin-protoporphyrin IX (SnPP), an inhibitor of HO; and (*4*) group treated with high-dose SnPP. Renal warm ischemia for 60 minutes was performed 24 hours after each treatment.

Results. At 24 hours after treatment, hemin induced a significant increase in renal HO activity, but failed to induce HO-1 protein synthesis. Although both low- and high-dose SnPP reduced HO activity, a marked HO-1 expression was observed only in the high-dose SnPP-treated kidney. Hemin exacerbated the renal function after reperfusion, while high-dose SnPP significantly suppressed the intercellular adhesion molecule (ICAM)-1 expression, the infiltration of ED-1–positive macrophages and the expression of activated caspase-3, which resulted in attenuation of apoptotic cell death and ameliorated I/R injury.

Conclusion. These results suggest that prior induction of HO-1 protein by high-dose SnPP may lead to anti-inflammatory and antiapoptotic effects on warm renal I/R injury independently of its enzyme activity, and that HO enzyme activation may not always act as an antioxidant, especially under I/R-induced oxidative stress.

Renal ischemia followed by reperfusion results in tubular epithelial cell injury mediated by oxidative stress [1]. Ischemia and reperfusion (I/R) injury may be exacerbated by the release of proinflammatory cytokines, the expression of adhesion molecules, and cellular infiltration [2–4]. Extended exposure to ischemia causes irreversible consequences leading to cellular death, while a number of mechanisms are involved in cellular protection during a recovery phase.

In general, oxidative stress such as chemotherapy and hypoxia may enhance intracellular accumulation of heme molecules, which participate in renal cell damage [5]. Under these circumstances, inducible heme oxygenase (HO)-1 may be rapidly induced after stressful stimuli to protect renal cells from further degeneration. On the other hand, HO-2 is the constitutive form, which is induced only by glucocorticoids [6]. The protective mechanisms of inducible HO-1 have been implicated in the removal of potentially toxic heme molecules and a lipid-soluble iron from intracellular space, in generation of antioxidant bile pigments and vasodilative carbon monoxide, and in concomitant induction of ferritin biosynthesis, which sequesters redox-active iron [7–9]. Therefore, protective effect of HO-1 induction seems to be dependent on its enzymatic action.

Although it has been reported that prior induction of HO-1 protects the renal cells from subsequent stressfull stimuli [10–13], the beneficial effect of HO-1 has not been identified on renal I/R injury [10, 11]. Paradoxically, it has been suggested that the defense mechanism of HO-1 might be mediated by increased induction of HO-1 protein independently of HO enzyme activity [14]. Furthermore, it has been reported that up-regulation of HO activity worsened cytotoxicity in some experimental models [15, 16].

HO enzyme activity is up-regulated by its substrate ferri-protoporphyrin IX (hemin) [7], whereas tin-protoporphyrin IX (SnPP), a potent competitive inhibitor of HO, regulates HO by a dual control mechanism; potently inhibiting the HO enzyme activity while enhancing the synthesis of HO enzyme protein [17]. Since SnPP completely blocks the activity of both preformed and newly synthesized enzyme, it acts as an inhibitor of HO in vivo. Thus, we compared these metalloporphyrins in the present study, which was designed to examine whether inducible HO-1 may protect against warm renal I/R injury.

Key words: heme oxygenase-1, tin-protoporphyrin IX, ischemia and reperfusion injury, kidney.

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METHODS

The present study was carried out according to the guidelines for care and use of experimental animals, as determined by the committee of the Research Institute for Artificial Organs, Transplantation and Gene Therapy, Hokuyu Hospital and Kitasato University School of Medicine.

Animals

Inbred male Lewis rats (190 to 250 g) were bred at our laboratory, housed in a climate-controlled room (22 ± 1°C), with a 12 hours light/dark cycle, and given tap water and standard rat chow (CE-2, CLEA Japan Co., Tokyo, Japan).

Experimental design

The animals were divided into four groups as follows: (*1*) vehicle group, which received 4 ml/kg of 0.9% NaCl solution as a vehicle control; (*2*) ferri-protoporphyrin IX chloride (hemin) group, which received 30 μmol/kg hemin (Sigma Chemical Co., St. Louis, MO, USA) as a potent inducer of HO; (*3*) low-dose tin-protoporphyrin IX (SnPP) group, which received 2 μmol/kg SnPP (Porphyrin Products, Logan, UT, USA) as a competitive inhibitor of HO enzyme activity; and (*4*) high-dose SnPP group, which received 20 μmol/kg SnPP. Hemin or SnPP solution was prepared under subdued light by dissolving the compound in 1 mL 0.2 N NaCl, adjusting the pH from 7.40 to 7.80 with 1 N HCl, and diluting the solution to the final volume with 0.9% NaCl. The solution was kept in the dark and used within 1 hour. Doses of hemin and SnPP used in the present study were selected according to previous reports [12, 17, 18]. Fresh kidneys taken from untreated animals were used as a fresh control. For examination of protective genes induction (HO-1, ferritin, and HSP 70), and HO activity, kidney tissue specimens (N = six per group) were harvested 24 hours after the treatments and stored at −80°C. In the high-dose SnPP groups, the rats were sacrificed at the time points indicated in the figures after injection to examine the time courses of HO-1 mRNA and protein induction. In addition, for the examination of inhibitory effect of an inhibitor of protein synthesis on HO-1 induction at translational level, 2 mg/kg cycloheximide was administered intraperitoneally 2 hours before high-dose SnPP treatment.

Renal model of warm I/R

The animals were anesthetized with ether 24 hours after the treatment. Body temperature was maintained at 37°C by a heating lamp until animals recovered from anesthesia. The right renal artery was isolated and clamped with a nontraumatic microvascular clip for 60 minutes, and a contralateral nephrectomy was immediately performed. After 60 minutes of ischemia, the clip was removed allowing the kidney to reperfuse, and the wound was closed with 3-0 silk. Sham-operated animals underwent isolation of the right renal artery without occlusions, and a left nephrectomy was performed.

For the measurement of the serum creatinine level after reperfusion, 0.5 mL blood sample was collected via the tail vein under the anesthetized condition on days 0 (preischemia), 1, 3, 5, and 7 (postischemia), and measured by automatic analyzer (7150; Hitachi, Tokyo, Japan). The kidney tissue samples (N = six per group) were taken 24 hours after reperfusion to examine the intercellular adhesion molecule (ICAM)-1 expression and the infiltration of ED-1–positive macrophages. Apoptosis was evaluated with terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick end labeling (TUNEL) assay and activated caspase-3 immunohistochemistry.

Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA was extracted from the kidney tissue specimens by the guanidinium isothiocyanate method as previously described [19]. In brief, 5 μg total RNA was reverse transcripted in cDNA using a commercially available SuperScript preamplification system (GIBCO, Life Technologies, Tokyo, Japan). The resulting cDNA (1 μg) was amplified for 30 cycles using primers 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) as an internal control was obtained from an MPCR kit for rat apoptosis genes set-2 (Maxim Biotech, Inc., San Francisco, CA, USA). The amplification product length was 451 bp. The polymerase chain reaction (PCR) products were separated on a 2% agarose gel containing ethidium bromide, and photographed. Digital photographs were assessed using image analysis software (Luminous Imager, AISIN COSMOS R&D Co., Aichi, Japan) and mRNA expression was assessed by the band intensity ratio of HO-1 to GAPDH.

Western blot analysis

The kidney tissues were homogenized in ice-cold lysis buffer containing protease inhibitors as described previously [20]. The homogenates were centrifuged at 10,000 *g* at 4°C for 20 minutes. The resulting supernatants were mixed with sample buffer and boiled for 5 minutes. Samples containing 100 μg protein were separated by electrophoresis on 12.5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and then transferred to polyvinylidine difluoride transfer membranes. The membranes were incubated with either rabbit anti-rat HO-1 polyclonal antibodies, rabbit anti-rat HO-2 polyclonal antibodies (1:1000 dilution for HO-1 and 1:2000 dilution for HO-2) (Stress-Gen Biotech, Victoria, BC), rabbit antihuman ferritin

antibodies (1:1000 dilution) (DAKO A/S, Glostrup, Denmark), rabbit anti-rat heat shock protein (HSP) 70 polyclonal antibodies (1:5000 dilution) (StressGen Biotech, Victoria, BC, Canada) or mouse anti-β-tubulin monoclonal antibodies (1:500 dilution) (Pharmingen, San Diego, CA, USA) as an internal control. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated goat antirabbit (1:2000 dilution) Dako Co., Carpinteria, CA, USA) or goat antimouse (1:5000 dilution) (Pierce Chemical Co., Rockford, IL, USA) secondary antibodies. Immunoreactive bands were visualized by an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England, UK). The band intensities of ferritin and HSP 70 were measured by NIH Image Analysis software and presented in comparison to β-tubulin expression.

HO enzyme activity in the whole kidney tissue

The kidney tissues were homogenized and lysed in 0.25 mol/L sucrose lysing buffer containing 0.1 mol/L phenylmethylsulfonyl fluoride. The microsomal fraction was prepared and used for measurement of the HO enzyme activity as detailed previously [21]. The reaction mixture contained the following in a final volume of 300 μL; 1 mmol/L glucose 6-phosphate, 0.167 U/mL glucose 6-phosphate dehydrogenase, 0.8 mmol/L nicotinamide adenine dinucleotide phophate (NADP), 15 mmol/L hemin, 2 mmol/L $MgCl_2$, 0.01 mg/mL NADPH-cytochrome P450 reductase, 3.3 mg protein/mL rat liver cytosol, potassium phosphate buffer (pH 7.4), and the supernatant from the tissue specimens (100 μL). Incubation was carried out at 37°C for 60 minutes in the dark. An equal volume of chloroform was added to the reaction mixture, and amount of bilirubin, which was extracted into the chloroform fraction, was determined by the measurement of Δ optical density (OD) between 464 and 530 nm. Enzyme activity was expressed as pmol of bilirubin per mg of tissue protein generated over 60 minutes.

Immunohistochemistry

The kidney tissues were embedded in OCT compounds (Tissue-Tek, Sakura Finetek Inc., CA) and quick-frozen in liquid nitrogen. Cryostat sections (9 μm thick) were mounted on glass slides. The tissue sections were exposed to polyclonal rabbit anti-rat HO-1 antibodies (1:500 dilution), monoclonal mouse anti-rat ICAM-1 antibodies (1:200 dilution, Pharmingen Int., Becton Dickinson Co., USA) and monoclonal mouse anti-rat ED-1 antibodies (1:500 dilution, Serotec Ltd., Oxon, UK) for 60 minutes at room temperature. After washing, the sections were overlaid with peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Dako Envision kit/HRP, DAB, Dako Co., CA) for 30 minutes. Finally, the sections were applied with 0.1 mg/ml of 3,3′-diaminobenzidine tetrahydrochloride for 2 minutes and counterstained with methylgreen for 20 minutes. The negative control was prepared by omission of the primary antibodies. ED-1–positive cells in the outer medulla were counted and expressed as the number of cells per field of view at a magnification of ×400.

Assessment of apoptosis

TUNEL Assay. Apoptosis was determined using the in situ end-labeling technique. Formalin-fixed paraffin-embedded sections (5 μm) of kidney were investigated using the ApopTag® Peroxidase Kit (Intergen Co., Purchase, NY, USA). The specimens were deparaffinized and treated according to the prescription with the provided kit components. The peroxidase activity was visualized with 3-amino-9-ethylcarbazole (AEC) substrate, yielding a brown-red oxidation product. Hematoxylin was used as counterstain.

Immunohistochemistry of activated caspase-3. To further assess the involvement of apoptosis, activated caspase-3 expression was evaluated by using anti-cleaved caspase-3 polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA), which detects caspase-3 activation, a final step of the caspase activation cascade. The sections were incubated with cleaved caspase-3 antibodies (1:100 dilution), and subsequently incubated with biotinylated goat antirabbit immunoglobulins (1:200 dilution) (DAKO A/S., Denmark). Then, after the incubation with a preformed avidin and biotinylated horseradish peroxidase complex reagent, the immune complex was visualized with AEC substrate. The sections were counterstained with hematoxylin.

The number of TUNEL- and activated caspase-3–positive cells in the outer medulla were counted and expressed as the number of cells per field of view at a magnification of ×200.

Statistical analysis

All data were expressed as mean ± SEM. Differences were analyzed by one-way analysis of variance (ANOVA). Statistical calculations were performed on a Macintosh personal computer using the Statview II Statistical Package (Abacus Concepts, Berkeley, CA, USA). Significant difference was taken as $P < 0.05$.

RESULTS

Expression of HO isoforms in the kidney tissues

First, we compared the expression of HO-1 and HO-2 in the kidney tissues 24 hours after each treatment. Western blot analysis indicated that the injection of high-dose SnPP significantly induced HO-1 expression in the kidney, while there was no effect of hemin and SnPP treatment on HO-2 expression (Fig. 1A). In addition, the HO-1 expression was completely inhibited by adminis-

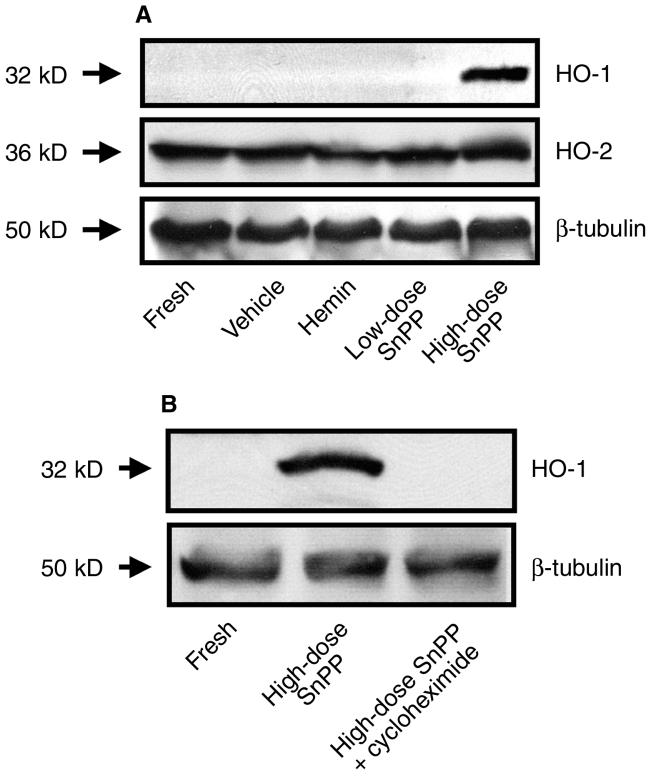

Fig. 1. Expression of heme oxygenase (HO) isoforms in the kidney tissues 24 hours after each treatment. Expression of HO-1and HO-2 protein in kidney tissues was evaluated by Western blot analysis after injection of vehicle, hemin, low- and high-dose tin-protoporphyrin IX (SnPP) (*A*). β-tubulin was used as an internal control. A prominent expression of HO-1 protein was detected in the high-dose SnPP-treated kidney alone, although there was no significant increase in HO-2 expression (N = six in each group). The HO-1 expression was completely inhibited by administration of cycloheximide (2 mg/kg) 2 hours before high-dose SnPP injection (*B*).

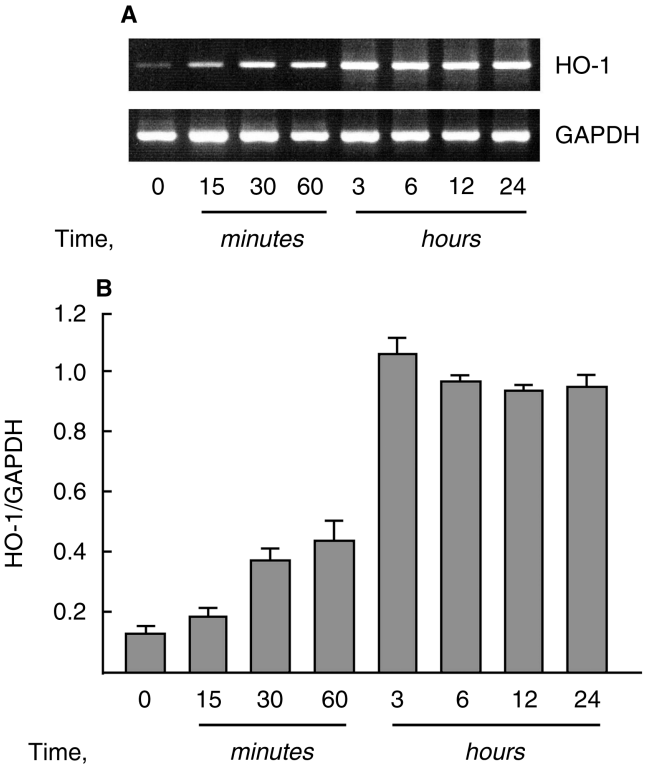

Fig. 2. Time course of heme oxygenase (HO-1) mRNA expression in the kidney tissues after high-dose tin-protoporphyrin IX (SnPP) (20 μmol/kg) injection. Expression of HO-1 mRNA in the kidney tissues was evaluated by reverse transcription-polymerase chain reaction (RT-PCR) analysis (*A*), and the results were expressed as HO-1/glyceraldehydes-3-phosphate dehydrogenase (GAPDH) ratios (*B*) (N = four in each time). GAPDH was used as an internal control. The expression of HO-1 mRNA peaked at 3 hours after injection and reached near to the plateau with further small decreases thereafter until 24 hours later.

tration of cycloheximide (2 mg/kg) 2 hours before SnPP injection (Fig. 1B). This result suggests that induction of HO-1 requires new protein synthesis.

Second, we examined the time course of HO-1 gene and protein expression in the kidney tissues after high-dose SnPP injection. The expression of HO-1 mRNA after injection occurred as early as 30 minutes, peaked at 3 hours, and reached near to a plateau with further small decreases thereafter until 24 hours (Fig. 2). The expression of HO-1 protein was initially observed 6 hours after injection, and enhanced during the periods between 12 and 24 hours (Fig. 3).

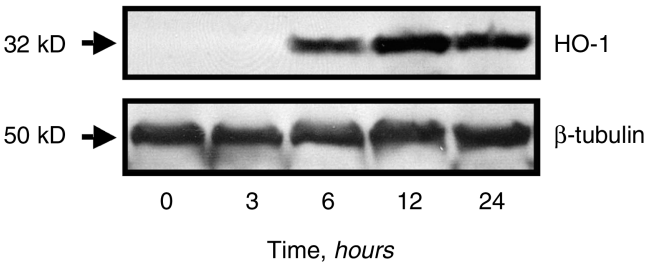

Fig. 3. Time course of heme oxygenase (HO-1) protein synthesis in the kidney tissues after high-dose tin-protoporphyrin IX (SnPP) (20 μmol/kg) injection. Expression of HO-1 protein was evaluated by Western blot analysis (N = four in each time). β-tubulin was used as an internal control. The expression of HO-1 protein was initially observed 6 hours after injection, and enhanced between 12 and 24 hours.

HO enzyme activity in the whole kidney tissues

HO enzyme activity was measured in the whole kidneys 24 hours after each treatment (Fig. 4). Hemin treatment caused a significant increase in the renal HO activity compared with vehicle treatment ($P < 0.05$), while either low-dose or high-dose SnPP treatment markedly reduced the HO activity below the fresh control level ($P < 0.01$).

Localization of HO-1 protein expression in the kidney tissues

Cell-type and spatial expression pattern of HO-1 immunoreactive protein was determined by immunohistochemical examination. A marked expression of HO-1 was observed in the proximal and distal tubuli in the cortex and prominently in the outer strip region of the

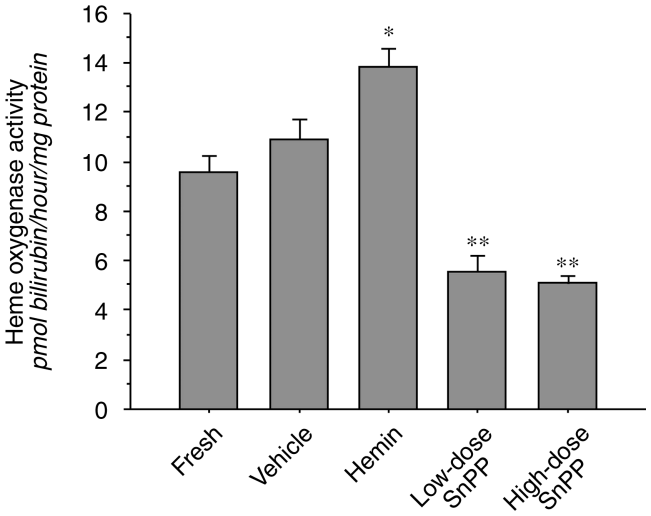

Fig. 4. Heme oxygenase (HO) enzyme activity in the whole kidney tissues 24 hours after each treatment. Hemin treatment caused a significant increase in renal HO activity compared with vehicle treatment (*$P < 0.05$), while either low-dose or high-dose tin-protoporphyrin IX (SnPP) treatment markedly reduced HO activity below the fresh control level (**$P < 0.01$) (N = six in each group).

outer medulla 24 hours after the high-dose SnPP treatment (Fig. 5D), while it was not detected in the tissues from other groups (Fig. 5 A to C).

Expression of ferritin and HSP 70 in the kidney tissues

We compared the expression of other protective genes such as ferritin and HSP 70 in the kidney tissues 24 hours after each treatment. Western blot analysis indicated that there was no significant increase in ferritin and HSP 70 expression (Fig. 6).

Alterations of serum creatinine levels after reperfusion

The serial alterations of the serum creatinine levels were monitored as a sensitive marker of cellular damage of reperfused kidneys (Fig. 7). All animals survived for 7 days after reperfusion. The serum creatinine levels of vehicle-treated animals peaked 24 hours after 60 minutes of warm I/R and declined thereafter to near the basal values until 7 days after reperfusion. Hemin significantly increased the serum creatinine levels at 1 and 3 days after reperfusion when compared with vehicle control ($P < 0.05$), whereas high-dose SnPP significantly decreased the peak levels of serum creatinine ($P < 0.05$; vehicle vs. high-dose SnPP). There was no obvious drug-induced cytotoxicity as assessed by the serum creatinine levels in animals free from warm ischemic insult during the period of 7 days after the treatments with 30 to 90 μmol/kg of hemin or with 2 to 40 μmol/kg of SnPP (data not shown).

Immunohistochemical expression of ICAM-1 in the kidney tissues after reperfusion

In immunohistochemistry of vehicle-, hemin- or low-dose SnPP-treated kidney tissues, ICAM-1–positive materials were dense in endothelial cells of peritubular capillaries within the outer medulla 24 hours after 60 minutes of warm I/R (Fig. 8 B to D), while few positive ICAM-1 stainings were observed in the sham-operated animals (Fig. 8A). High-dose SnPP markedly suppressed the renal ICAM-1 expression (Fig. 8E).

Assessment of ED-1–positive cells infiltration in the kidney tissues after reperfusion

In vehicle-, hemin- or low-dose SnPP-treated kidney tissues, a pronounced infiltration of ED-1–positive macrophages was observed in the outer medulla 24 hours after warm I/R compared with the sham-operated group (Fig. 8 F to I), while high-dose SnPP inhibited the infiltration of macrophages (Fig. 8J). The number of infiltrating macrophages in the outer medulla 24 hours after reperfusion was significantly suppressed to 30.8 ± 4.5/field of high-dose SnPP-treated kidney tissues ($P < 0.05$ vs. vehicle), when compared with 43.2 ± 2.5, 55.4 ± 2.0, and 47.4 ± 2.9/field of those treated with vehicle, hemin or low-dose SnPP, respectively, synchronizing the alterations of the serum creatinine levels after reperfusion (Table 1). A significant increase in infiltrating macrophages was observed in the hemin-treated group compared with the vehicle group ($P < 0.05$).

Antiapoptotic effect of high-dose SnPP pretreatment on renal I/R injury

TUNEL assay and immunohistochemical staining of activated caspase-3 were carried out to examine whether high-dose SnPP pretreatment has an antiapoptotic effect on renal I/R injury. At 24 hours after reperfusion, activated caspase-3 expression was observed in tubular epithelial cells within the outer medulla in vehicle-, hemin- or low-dose SnPP-treated kidney (Fig. 9 B to D), while high-dose SnPP treatment significantly decreased the number of activated caspase-3–positive cells compared with vehicle group (Fig. 9E and Table 2). The number of TUNEL-positive cells was also markedly decreased in high-dose SnPP group, while hemin treatment significantly increased TUNEL-positive cells compared with vehicle group (Table 2).

DISCUSSION

This study aimed to examine mechanisms by which the increased expression of HO-1 may attenuate inflammatory responses after reperfusion of warm ischemically injured kidney. We found that HO-1 protein synthesis was markedly up-regulated in kidney tissues after prior administration with high-dose SnPP, which may be asso-

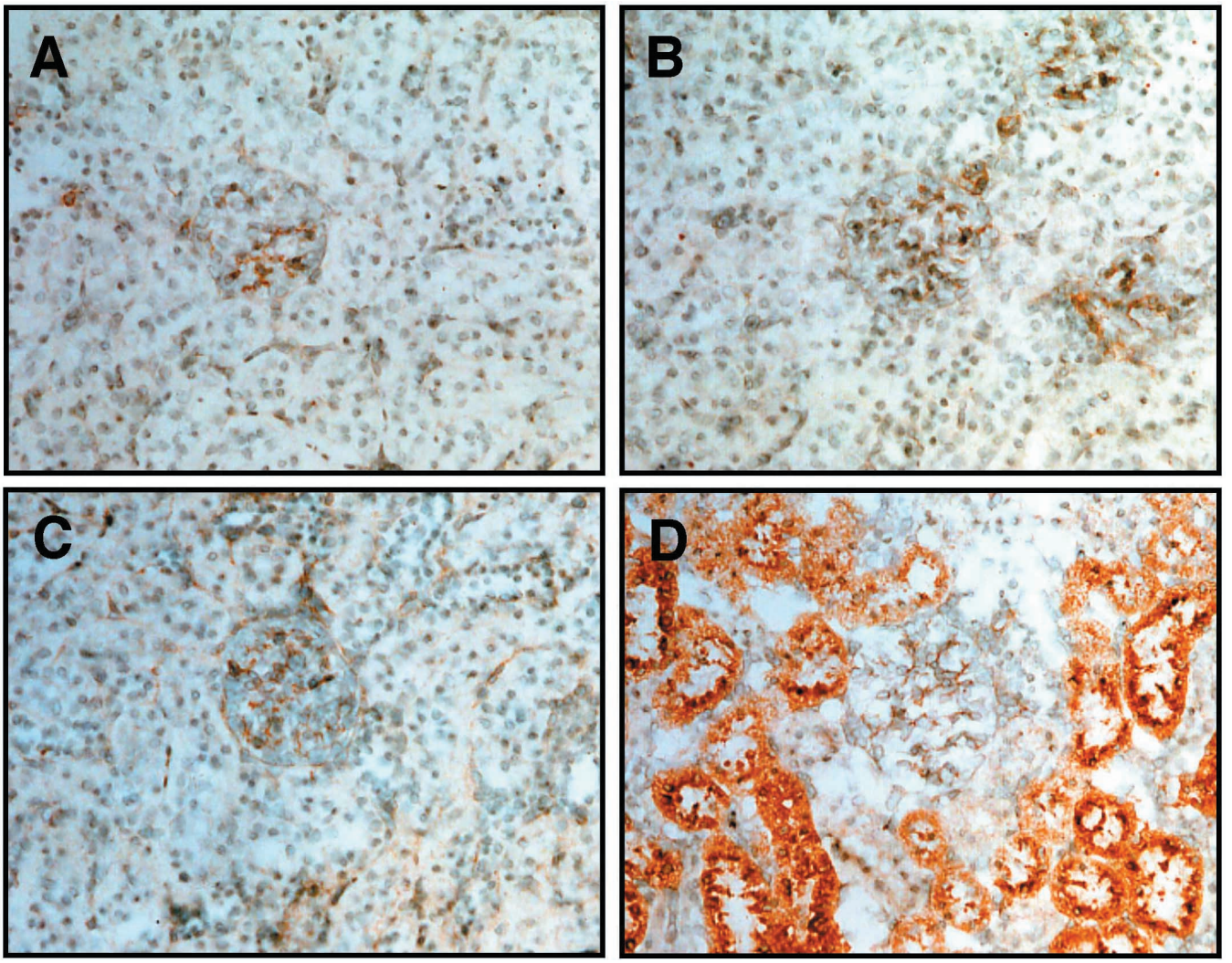

Fig. 5. Localization of heme oxygenase (HO-1)–positive materials in the kidney tissues. HO-1–positive materials were not detected in vehicle- (*A*), hemin- (*B*), or low-dose tin-protoporphyrin IX (SnPP)-treated kidney tissues (*C*), although a marked expression of HO-1 was observed in proximal and distal tubuli in the cortex and outer medulla 24 hours after high-dose SnPP treatment (*D*). Original magnification, ×200.

ciated with an earlier recovery from warm ischemic injury. In addition, the expression of ICAM-1, the accumulation of macrophages, and apoptotic cell death were reduced in reperfused kidney tissues pretreated with high-dose SnPP. Because SnPP is a potent competitive inhibitor of HO enzyme, the beneficial effect of SnPP may be mediated via unidentified mechanisms independently of HO activity.

In the present experiment, high-dose SnPP caused significant increases in renal HO-1 mRNA and protein synthesis despite the reduction of HO enzyme activity. Several investigators also have shown this paradoxic observation in lung or liver of SnPP-treated rat [14, 17]. Inhibition of HO activity by SnPP is so pronounced that, despite the marked increase in synthesis of new HO-1 protein, SnPP completely blocks the activity of both preformed and newly synthesized enzyme because of its uniquely potent capacity for binding to the catalytic site of HO [17]. The molecular mechanisms by which SnPP stimulates the expression of HO-1 in the kidney tissues remain unclear. Since the induction of HO-1 by heme or heavy metals is believed to occur through a complex mechanism involving the activation of transcription factor such as activator protein-1, SnPP may induce HO-1 at both the transcriptional and translational levels [22].

There has been much evidence that HO-1 is protective against oxidative injury in various organs, such as the heart, liver, lung, or kidney [14, 23–26]. In an in vivo rodent model of glycerol-induced acute renal failure or cisplatin-induced nephropathy, a considerable amount of HO-1 mRNA and protein synthesis were induced with an increased HO activity following administration of these pharmacologic drugs, while the inhibition of HO enzyme activity by SnPP worsened nephrotoxicity [10, 27]. HO-1 may be

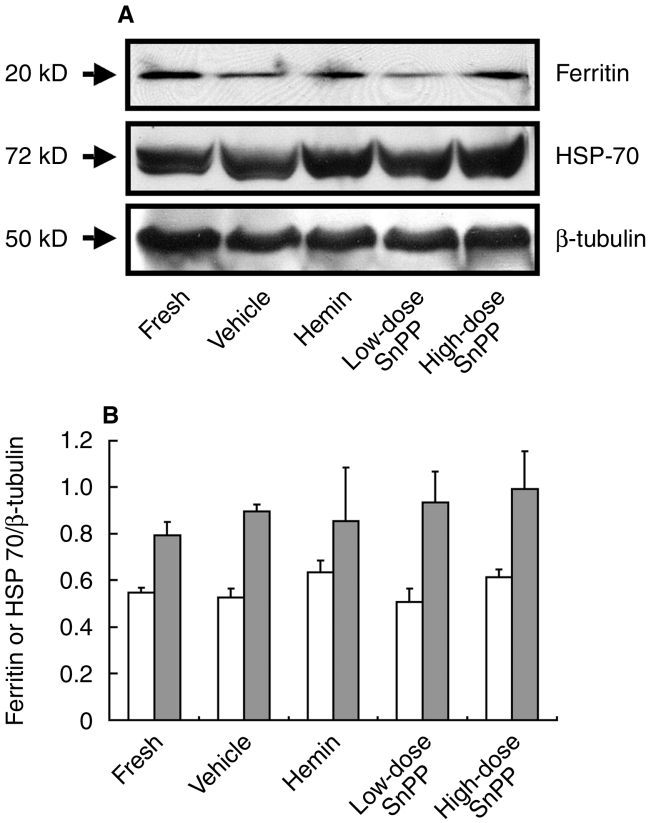

Fig. 6. Expression of ferritin and heat shock protein (HSP) 70 in the kidney tissues 24 hours after each treatment. Expression of ferritin and HSP 70 protein in kidney tissues was evaluated by Western blot analysis (*A*), and the results were expressed as ferritin (□) or HSP 70 (■)/β-tubulin ratios (B) (N = six in each group). There was no significant increase in ferritin and HSP 70 expression after injection of vehicle, hemin, low- and high-dose tin-protoporphyrin IX (SnPP).

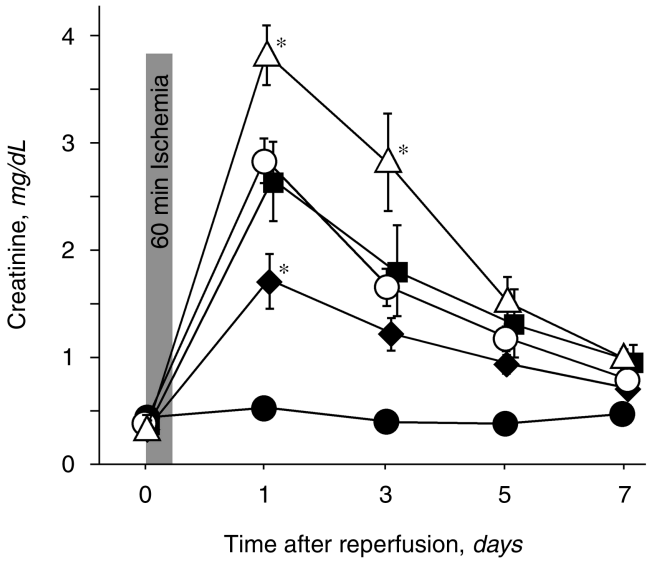

Fig. 7. Renal function indicated by serum creatinine levels after warm ischemia and reperfusion (I/R). Mean serum creatinine level in sham-operated (●), vehicle (○), hemin (△), low-dose tin-protoporphyrin IX (SnPP) (■), or high-dose SnPP group (◆) was presented (N = 10 in each group). High-dose SnPP treatment significantly attenuated an increment in serum creatinine levels after reperfusion compared with vehicle group, while hemin treatment exacerbated renal injury. *$P <$ 0.05 vs. vehicle control.

a critical protectant against acute glycerol- or cisplatin-induced nephrotoxicity. Conceivably, end products of HO enzymatic action might account for the protective effect of HO-1. However, the inhibition of HO activity does not exacerbate renal function in a model of gentamycin-induced nephrotoxicity and HO enzyme activation does not protect against acute renal failure in a rat postischemic model [10, 11, 27]. Additionally, in fibroblasts derived from HO-1–deficient mice, serum deprivation–induced apoptosis was markedly accentuated as compared with wild-type fibroblasts, whereas incubation of HO-1–deficient fibroblasts with a cyclic guanosine monophosphate (GMP) analogue, to mimic production of carbon monoxide, or with bilirubin failed to protect against the cell death [9], which indicated that none of the products of HO-1 activity account for HO-1's cytoprotective effect. And further, a previous study has demonstrated that intratracheal administration of hemoglobin induces HO-1 in the rat lung and protects against hyperoxic lung injury; interestingly, the protection is not mediated by increased HO enzyme activity because SnPP did not inhibit the protective effect [14]. In the present study, pretreatment of high-dose SnPP attenuated renal function following warm I/R despite the decreased HO enzyme activity. These findings indicate that HO enzymatic action is not always associated with beneficial effects of HO-1 and strongly support the presence of cytoprotective mechanisms that are independent of HO enzyme activity.

As demonstrated in the present study, hemin pretreatment enhanced HO activation but further worsened warm ischemically damaged renal function when compared with vehicle control. In general, free heme is a pro-oxidant and the toxic effect of heme has been well documented [28]. The removal of these harmful molecules from cells has been considered to be one of the protective mechanisms of HO-1 induction. However, it has also been suggested that products of heme catabolism, but not heme itself, may be responsible for the oxidant activity [29]. Indeed, HO-generated iron release accounts for heme protein–triggered proximal tubular injury, and HO inhibition by SnPP almost completely protects proximal tubular cells against myoglobin cytotoxicity [30, 31]. In addition, the intracellular iron release by HO contributes to microsomal lipid peroxidation, while SnPP inhibits this pro-oxidant action [32]. Thus, the present study suggested that pro-oxidant end products generated via HO activation might account for the cytotoxic effect of hemin. In addition to renal expression

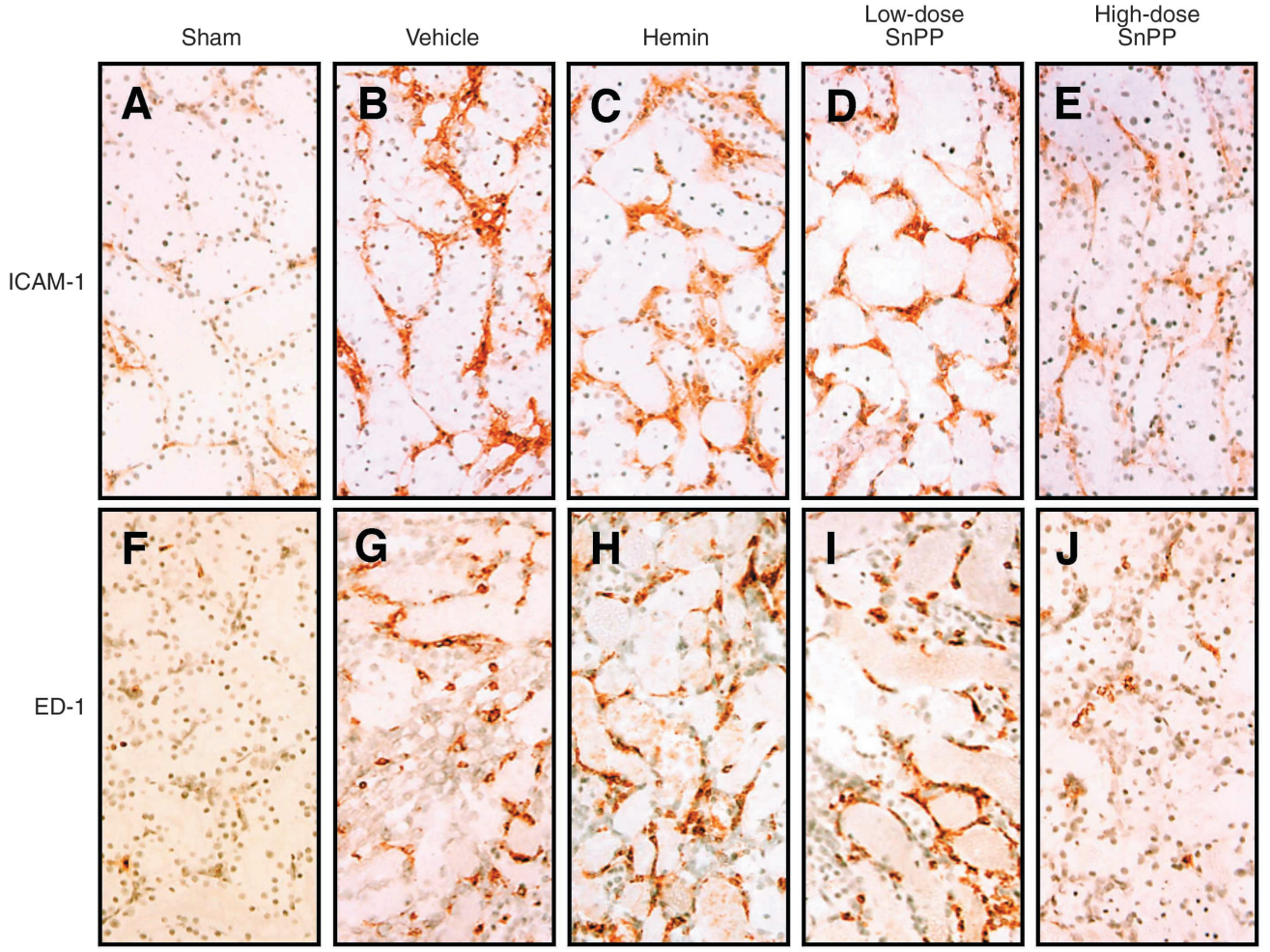

Fig. 8. Immunohistochemical expression of intercellular adhesion molecule-1 (ICAM-1) (*A* to *E*) and infiltration of ED-1–positive macrophages (*F* to *J*) in the outer medulla after warm ischemia and reperfusion (I/R). In immunohistochemistry of vehicle (B), hemin (C), or low-dose tin-protoporphyrin IX (SnPP) group (D), ICAM-1-positive materials were dense in endothelial cells of peritubular capillaries 24 hours after warm I/R compared with the sham-operated group (A), while high-dose SnPP markedly suppressed the expression of ICAM-1 (E). A pronounced infiltration of ED-1–positive macrophages was observed in the outer medulla 24 hours after warm I/R in vehicle (G), hemin (H), or low-dose SnPP group (I), compared with the sham-operated group (F), while high-dose SnPP obviously inhibited the infiltration of macrophages (J). Original magnification, ×200.

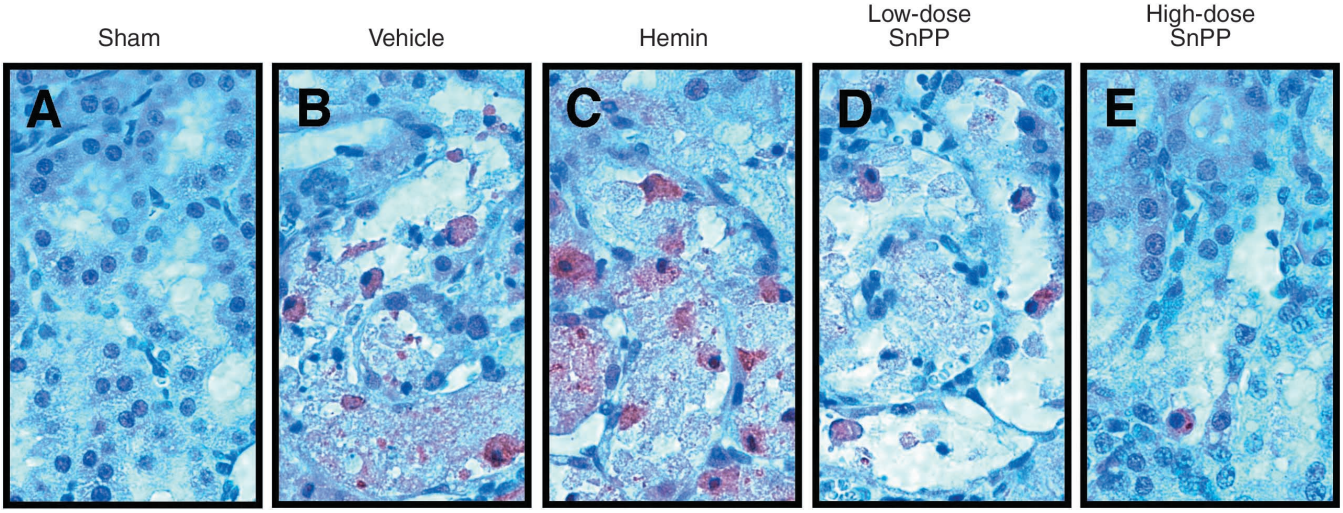

Fig. 9. Activated caspase-3 immunostaining in the outer medulla after warm ischemia and reperfusion (I/R). Although there is no activated caspase-3–positive cell in sham group (*A*) at 24 hours after reperfusion, activated caspase-3 expression was observed in tubular epithelial cells within the outer medulla in vehicle (*B*), hemin (*C*) and low-dose tin-protoporphyrin IX (SnPP) group (*D*). High-dose SnPP treatment significantly decreased the number of activated caspase-3–positive cells (*E*). Original magnification, ×400.

Table 1. Infiltration of ED-1–positive monuclear cells in outer medulla 24 hours after reperfusion

Sham	Vehicle	Hemin	Low-dose SnPP	High-dose SnPP
1.8 ± 0.5	43.2 ± 2.5	55.4 ± 2.0[a]	47.4 ± 2.9	30.8 ± 4.5[a]

SnPP is tin-protoporphyrin IX. Infiltration of ED-1–positive cells were expressed as the number of cells per field of view at a magnification of ×400.
[a] $P < 0.05$ vs. vehicle group

Table 2. The number of TUNEL- and activated caspase-3–positive cells in outer medulla 24 hours after reperfusion

	Sham	Vehicle	Hemin	Low-dose SnPP	High-dose SnPP
TUNEL	0.3 ± 0.3	16.8 ± 1.3	28.5 ± 2.4[a]	14.3 ± 1.9	6.5 ± 1.0[a]
Caspase-3	0.5 ± 0.3	13.8 ± 1.7	20.8 ± 2.5	9.5 ± 2.1	1.3 ± 0.5[a]

Abbreviations are: TUNEL, terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick end labeling; SnPP, tin-protoporphyrin IX. The positive stainings of TUNEL and activated caspase-3 were expressed as the number of positive cells per field of view at a magnification of ×200.
[a] $P < 0.01$ vs. vehicle group

of the protective gene HO-1, the inhibition of HO activity may be associated with the protective effect of high-dose SnPP treatment on renal I/R injury.

Most of the metalloporphyrin tested as HO inhibitors have been found to be rather nonspecific [33]. Indeed, zinc protoporphyrin (ZnPP), a widely used HO inhibitor, has been found to inhibit other heme-containing enzymes, such as guanylate cyclase and nitric oxide synthase in some systems [34, 35], and further shown to abolish vascular relaxation in the rat isolated blood vessels despite light-exposed ZnPP, which does not inhibit HO enzyme [33]. Therefore, we cannot exclude the possibility that the protective effect of high-dose SnPP treatment in our renal I/R model may not be dependent on induced HO-1 protein but only associated with nonspecific effects of SnPP. According to a number of studies, however, SnPP has been found to be rather specific compared with other HO inhibitors [36, 37]. Further biochemical studies will be required to elucidate the cytoprotective mechanisms of high-dose SnPP treatment against renal I/R injury.

Under several physiologic stresses, HO-1 is concomitantly induced with other cytoprotective gene expressions such as ferritin or HSP 70 [10, 14, 38, 39]. The ferritin synthesis is regulated by iron released from heme degradation and provides a mechanism for coping with the potentially toxic nature of iron [40]. The coupling of ferritin synthesis to HO-1 induction has been reported to prevent renal failure in rat rhabdomyolysis [10]. HSP 70 is also considered to have an antioxidant effect on I/R injury [41, 42]. In the present study, no significant induction of ferritin or HSP 70 was observed in the kidney tissues following the administration with hemin or high-dose SnPP. Therefore, it is possible that HO-1 expression produces protective effects on renal I/R injury in the absence of other antioxidant protein inductions.

The expression of ICAM-1 and the infiltration of ED-1–positive macrophages were observed in the outer medulla of the vehicle-treated group after renal I/R. Following high-dose SnPP pretreatment, HO-1–positive materials were prominently expressed in nearly the same region, which may be most vulnerable to oxidative damage such as warm I/R [25]. Recent studies have reported that HO-1 highly expressed in the endothelial cells inhibits the expression of adhesion molecules such as P-selectin, E-selectin, and ICAM-1 [43–45], and further reported that HO enzyme activation in rat macrophages stimulates the carbon monoxide/guanylate cyclase pathway and contributes to the synthesis of proinflammatory genes such as tumor necrosis factor-α and interleukin-6, while SnPP inhibits the production of these cytokines [46]. In addition, there is evidence that high-dose SnPP decreases the inflammatory cell number in the exudates in the pleural cavity 24 hours after injection of carrageenin [47]. Although it has been speculated that the decrease in inflammatory cell number might result from a toxic effect of high-dose SnPP, the doses of up to 40 μmol/kg SnPP had no toxic effect on the renal function of stress-free rats in the present study (data not shown). Therefore, it is possible that HO-1 protein synthesis induced by high-dose SnPP may be associated with attenuation of ICAM-1 expression and inhibition of macrophage infiltration, whereas HO enzyme activation enhanced by hemin may lead to infiltration of activated macrophages after reperfusion.

Apoptosis and inflammation are considered to be central mechanisms leading to organ damage in the course of renal I/R. Therefore, the inhibition of apoptosis by a caspase inhibitor has been reported to prevent renal I/R injury [48]. Recently, cellular overexpression of HO-1 has been shown to confer marked resistance to apoptosis [49], and moreover, cardioselective overexpression of HO-1 has been reported to prevent I/R-induced cardiac dysfunction and apoptosis [50]. In the present study, high-dose SnPP pretreatment suppressed the caspase-3 activation and decreased the apoptotic cell death. Our results suggest that overexpression of renal HO-1 contributed to antiapoptotic effects of high-dose SnPP pretreatment, which resulted in attenuation of renal I/R injury.

CONCLUSION

In conclusion, relatively high-dose SnPP administration significantly induces HO-1 protein expression in kidney tissues, which leads to attenuation of renal warm I/R injury; the protection seems not to be mediated by heme-degradation pathways, while it may be related to the down-regulation of proinflammatory cytokines, adhesion molecules, and apoptotic cell death independently of HO

enzyme activation. Thus, less-toxic SnPP would be potentially applicable to donor preconditioning or pharmacologic treatment for pathologic conditions that lead to oxidative insults including I/R.

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