

Role of heme oxygenase-1 in protection of the kidney after hemorrhagic shock

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Abstract. Hemorrhagic shock followed by resuscitation (HSR) causes oxidative stress, which results in multiple organ damage. The kidney is one of the target organs of HSR-mediated oxidative tissue injury. Heme oxygenase (HO)-1, the rate-limiting enzyme in heme catabolism, is induced by oxidative stress; it protects against oxidative tissue injuries. The aim of the present study was to examine the role of renal HO-1 induction after HSR. Rats were subjected to hemorrhagic shock to achieve a mean arterial pressure of 30 mmHg for 60 min, followed by resuscitation with the shed blood. HSR resulted in a significant increase in functional HO-1 protein in the tubular epithelial cells of the kidney, whereas HSR resulted in only a slight increase in gene expression of tumor necrosis factor (TNF)- α and inducible nitric oxide synthase (iNOS), and in protein expression of activated caspase-3 solely in renal cells where HO-1 expression was absent. HSR also resulted in a significant increase in Bcl-2 gene expression. Pretreatment of HSR animals with tin-mesoporphyrin (0.5 μ mol/kg), a specific competitive inhibitor of HO activity, resulted in a significant decrease in HO activity and exacerbated tissue inflammation and apoptotic cell death as judged by the marked increase in expression of TNF- α and iNOS, and in activated caspase-3-positive cells, and the significant reduction in Bcl-2 expression, respectively. These findings indicate that HO-1 induction is an adaptive response to HSR-induced oxidative stress and is essential for protecting tubular epithelial cells from oxidative damage through its anti-inflammatory and anti-apoptotic properties.

Introduction

Heme oxygenase (HO)-1 is the rate-limiting enzyme in heme catabolism; it is also known as a heat shock protein (HSP 32)

and a stress-inducible protein (1,2). HO-1 is induced, not only by its substrate heme, but also by various oxidative stresses (1,2). It is thought to play an important protective role against oxidative tissue injuries because of its anti-inflammatory and anti-apoptotic properties (1,2). Hemorrhagic shock followed by resuscitation (HSR) induces oxidative stress that leads to multiple organ damage (3,4), and the kidney is one of the target organs of HSR-mediated oxidative tissue injury (5). We previously reported that HO-1 mRNA was highly induced in the kidney after HSR in a rat model of HSR (6). However, the role of renal HO-1 induction following HSR remains elusive. In light of this, we examined the effect of HSR on functional HO-1 protein expression and its inhibition on tissue inflammation and injury in the kidney. The degree of tissue inflammation and injury was assessed by the mRNA levels of tumor necrosis factor (TNF)- α and inducible nitric oxide synthase (iNOS); expression of activated caspase-3, the key executioner of apoptosis (7); and the level of Bcl-2 mRNA, an anti-apoptotic gene (8). We here report that functional HO-1 protein is markedly induced in tubular epithelial cells of the kidney, which are the target cells of ischemic renal injury, after HSR. Apoptotic cell death was observed only in the cells in which HO-1 was not expressed. To note, pretreatment of rats with tin-mesoporphyrin (SnMP), a specific competitive inhibitor of HO activity (9), markedly inhibited HO-1 activity, aggravated apoptotic cell death and exacerbated tissue inflammation. Thus, HO-1 induction appears to be critical for protecting renal cells from oxidative stress caused by HSR.

Materials and methods

Animals. The studies reported herein conform to the Guidelines for the Care and Use of Laboratory Animals established by the Animal Use and Care Committee of the Okayama University Medical School. Male Sprague-Dawley rats weighing 380-420 g were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). They were housed in a temperature-controlled (25°C) room with alternating 12-h light/12-h dark cycles and were allowed free access to water and a chow diet until the start of the experiments.

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Hemorrhagic shock protocol. Rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg) and

subjected to sham or HSR, as described previously (6,10,11). In brief, the left femoral artery and vein were dissected using aseptic techniques and cannulated with a heparinized polyethylene tube. Catheters were inserted into the left femoral artery and vein to measure blood pressure and induce hemorrhage, respectively. After measuring baseline blood pressure, hemorrhage was initiated by bleeding into a heparinized syringe (10 units/ml) for 15 min to achieve a mean arterial blood pressure of 30 mmHg. This blood pressure level (30 ± 5 mmHg) was maintained for 60 min by further blood withdrawal or by reinfusing the shed blood. At this point, animals were resuscitated for 15 min by re-administering all the shed blood, followed by injection of sterile saline as necessary until the blood pressure was restored to the baseline level. The sham group underwent all instrumentation procedures, but blood was not collected. The animals were allowed to breathe spontaneously throughout the experiment. To maintain body temperature within the physiological range, all procedures were performed over a heating pad with continuous monitoring of rectal body temperature. Electrocardiography was also monitored continuously.

Experimental design. HSR-treated rats were randomly assigned to the following two groups: pretreatment with SnMP before HSR (SnMP/HSR group) and pretreatment with vehicle before HSR (Vehicle/HSR group). SnMP ($0.5 \mu\text{mol/kg}$; Frontier Scientific, Logan, UT, USA) or vehicle was injected through the tail vein 1 h before the onset of HSR. SnMP was prepared immediately before use as described below. SnMP was dissolved in a small volume of 0.1 N NaOH solution, and the pH was adjusted to 7.6 with 0.01 M sodium phosphate buffer (12). Sham animals were used as a control (Sham group). Under light anesthesia with ethyl ether, the animals were sacrificed by decapitation 3 h after HSR. The kidneys were excised and frozen immediately in liquid nitrogen and stored at -80°C until needed for RNA preparation. To determine HO activity, the kidneys were first perfused *in situ* with physiological saline until the venous effluent became clear and were then removed for microsome preparation.

cDNA probes. Template cDNA for HO-1, TNF- α , iNOS and Bcl-2 were prepared as described previously (10,13). All probes used for Northern blot analysis were [α - ^{32}P]dCTP-labeled cDNA probes prepared using a random primer DNA labeling system (both from Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions.

RNA isolation and Northern blot analysis. Total RNA was isolated from rat tissues using Tri-ReagentTM (Sigma Chemical, St. Louis, MO, USA) according to the manufacturer's protocol. Northern blotting was performed as described previously (10,11). Total RNA ($20 \mu\text{g}$) was subjected to electrophoresis on 1.2% (w/v) agarose gels containing 6.5% (v/v) formaldehyde. After blotting on a sheet of Bio-Rad Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, CA, USA), RNA samples were hybridized with the [α - ^{32}P]dCTP-labeled cDNA probes, followed by washing under stringent conditions. The membrane was exposed to a

sheet of Fuji Medical radiograph film with an intensifying screen at -70°C , and autoradiographs and 18S ribosomal RNA were quantified using an image scanner (GelPrintTM 2000i; Genomic Solutions, Ann Arbor, MI, USA) and computerized image analysis software (Basic QuantifierTM version 3.0, Genomic Solutions). The relative amounts of radiolabeled cDNA that hybridized to the blots were normalized to 18S ribosomal RNA levels to correct for loading errors.

Immunohistochemistry. Immunohistochemical analysis was performed using the indirect immunofluorescence method. Renal tissue was fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at a thickness of 4–6 μm . Following antigen retrieval in citrate buffer (0.01 M, pH 6.0) with heat treatment by autoclaving, nonspecific binding sites were blocked with 5% normal donkey serum for 30 min. Slides were then incubated at 4°C overnight with a monoclonal mouse HO-1 antibody (StressGen Biotechnologies, Victoria, BC, Canada) and a polyclonal rabbit cleaved caspase-3 antibody (Cell Signaling Technology, Beverly, MA, USA) at a dilution of 1:100 in 1X phosphate-buffered saline (PBS) containing 0.3% Triton X-100. For fluorescent visualization of the bound primary antibody, the slides were further incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (ChemiconTM International, Temecula, CA, USA) with a green fluorescent label and Cy3-conjugated donkey anti-rabbit IgG (Chemicon International) with a red fluorescent label for 90 min. Normal mouse and rabbit serum were used as the control for nonspecific staining. Images were taken with a Zeiss confocal laser scanning microscope model LSM510 (Zeiss, Jena, Germany).

HO activity. Renal HO activity was measured as described previously (12). Tissue was homogenized in three volumes of 0.05 M Tris-HCl (pH 7.8) containing 0.25 M sucrose, 20% (w/v) glycerol, 3 units/ml heparin, and a protease inhibitor (Complete, Roche Diagnostics GmbH, Mannheim, Germany) and centrifuged at $10,000 \times g$ for 15 min at 4°C . The supernatant was collected and centrifuged at $105,000 \times g$ for 60 min at 4°C . After centrifugation, the microsomal pellet was collected and resuspended in 20 mM Tris-HCl (pH 7.4) containing 1.15% KCl and a protease inhibitor and used to measure HO activity spectrophotometrically, as described previously. The cytosolic fraction prepared from the liver of adult untreated rats served as a source of biliverdin reductase in the HO assay. HO activity was expressed as picomoles of bilirubin formed per milligram of protein per 60 min.

Statistical analysis. The results are expressed as means \pm standard deviations. Statistical analysis was performed using an analysis of variance. A probability level of $p < 0.05$ was considered statistically significant.

Results

HO-1 protein expression. We previously reported that the HO-1 mRNA level increased markedly following HSR in the rat kidney (6). Thus, we examined the effect of HSR on HO-1 protein expression in the kidney by fluorescent

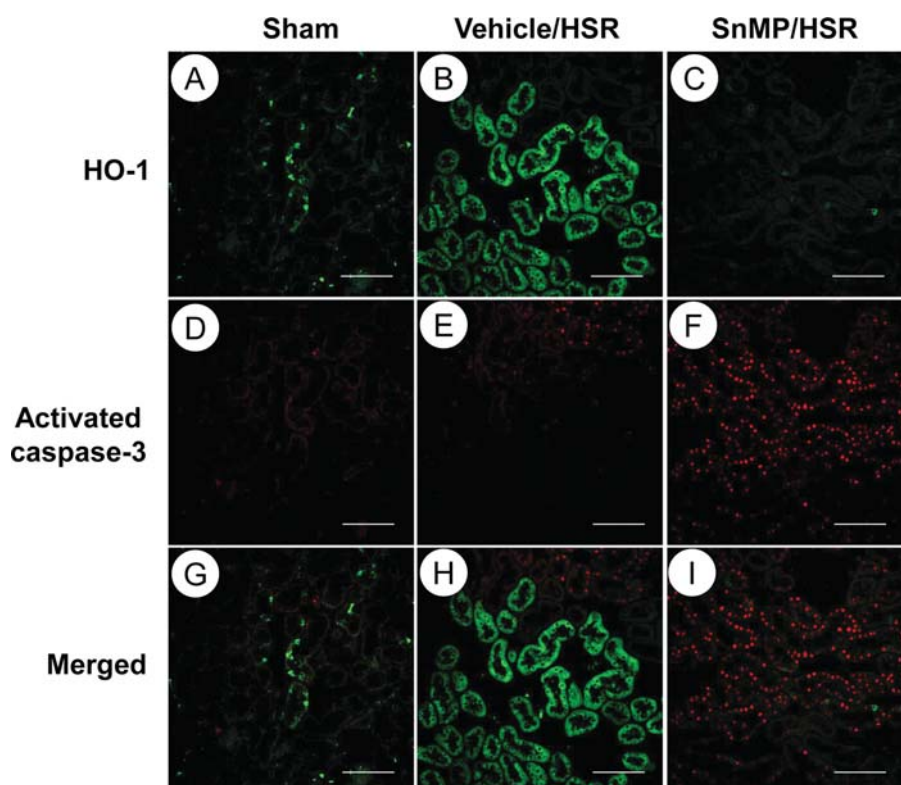


Figure 1. Effect of hemorrhagic shock followed by resuscitation (HSR) on heme oxygenase-1 (HO-1) and activated caspase-3 expression in the kidney and their modulation by tin-mesoporphyrin (SnMP) administration. Vehicle or SnMP (0.5 $\mu\text{mol/kg}$) was intravenously administered to rats 1 h before HSR. Sections from kidneys 12 h after HSR were subjected to fluorescent immunohistochemical analysis of HO-1 (green signals) and activated caspase-3 (red signals), as described in Materials and methods. Top row: HO-1-positive cells in Sham (A), Vehicle/HSR (B) and SnMP/HSR (C); Middle row: activated caspase-3-positive cells in Sham (D), Vehicle/HSR (E) and SnMP/HSR (F); Bottom row: merged images of HO-1 and activated caspase-3 in Sham (G), Vehicle/HSR (H) and SnMP/HSR (I). Sham, sham-operated control animals; Vehicle/HSR, vehicle-treated HSR animals; SnMP/HSR, SnMP-treated HSR animals. Bars, 100 μm . Original magnification, $\times 200$.

immunohistochemical analysis and by measuring HO activity 12 h after HSR. HO-1-positive cells were negligibly detectable in the sham-operated control animals (Fig. 1A and G). However, strong positive signals (green color) for the HO-1 protein were predominantly observed in the tubular epithelial cells of the kidney in the HSR animals (Fig. 1B and H). Consistent with HO-1 protein expression, HSR increased HO activity in the kidney of the HSR animals by ~ 3 -fold compared to that of the sham-operated control animals (Fig. 2). In contrast, administering SnMP, a specific competitive inhibitor of HO, to the HSR animals quenched HO-1 signals in the tubular epithelial cells (Fig. 1C and I) and decreased HO activity almost to the same level as that in the sham-operated control animals (Fig. 2). These results revealed that HSR treatment markedly increased functional HO-1 protein in the tubular epithelial cells of the kidney and that SnMP administration completely inhibited the HSR-mediated increase in HO activity.

TNF- α and iNOS gene expression. Since HO-1 has an anti-inflammatory property, we examined the effect of HSR on the gene expression of pro-inflammatory mediators, such as TNF- α and iNOS, in the kidney and its modulation by SnMP. While TNF- α and iNOS mRNA was barely detectable in the kidney of the sham-operated animals, these mRNA levels only slightly increased after HSR (Fig. 3). These data were consistent with our previous observation (6) and suggested

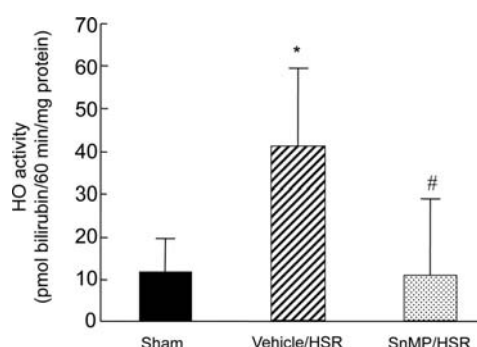


Figure 2. Effect of HSR on HO activity in the kidney and its modulation by SnMP administration. Vehicle or SnMP (0.5 $\mu\text{mol/kg}$) was intravenously administered to rats 1 h before HSR. The kidney was removed 12 h after HSR, and HO activity was measured as described in Materials and methods. Sham, sham-operated control animals; Vehicle/HSR, vehicle-treated HSR animals; SnMP/HSR, SnMP-treated HSR animals. Data are presented as means \pm SD ($n=5$). * $p<0.05$ vs. Sham; # $p<0.05$ vs. Vehicle/HSR.

that HO-1 may suppress HSR-induced activation of these pro-inflammatory genes. In support of our hypothesis, when animals were treated with SnMP before inducing hemorrhagic shock, the levels of TNF- α and iNOS mRNA increased markedly compared to those of the vehicle-treated HSR animals (Fig. 3). The average levels of TNF- α and iNOS mRNA in the SnMP-pretreated animals were ~ 5 - and 4-fold

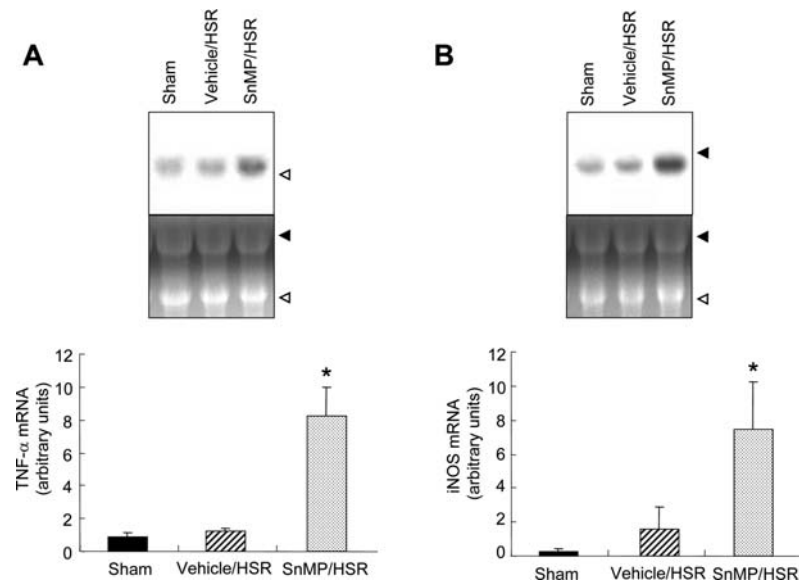


Figure 3. Effect of HSR on tumor necrosis factor (TNF)- α and inducible nitric oxide synthase (iNOS) gene expression in the kidney and their modulation by SnMP administration. Vehicle or SnMP (0.5 μ mol/kg) was intravenously administered to rats 1 h before HSR. Total RNA was extracted from the kidney 3 h after HSR, and TNF- α (A) and iNOS (B) gene expression was analyzed by Northern blot analysis. Top, autoradiographic signals of the RNA blot hybridized with [α - 32 P]dCTP-labeled TNF- α and iNOS cDNA with ethidium bromide-stained gels. Open and closed arrowheads next to the autoradiogram indicate the positions of 18S and 28S ribosomal RNA corresponding to the ethidium bromide-stained gels, respectively. Sham, sham-operated control animals; Vehicle/HSR, vehicle-treated HSR animals; and SnMP/HSR, SnMP-treated HSR animals. Bottom, levels of TNF- α and iNOS mRNA are expressed as arbitrary units. Data are presented as means \pm SD (n=5). *p<0.05 vs. Sham or Vehicle/HSR.

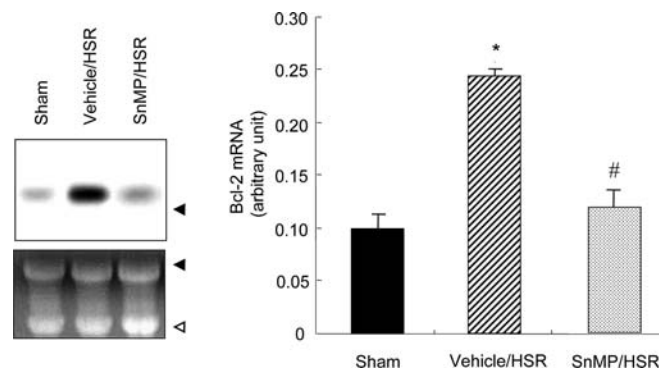


Figure 4. Effect of HSR on Bcl-2 gene expression in the kidney and its modulation by SnMP administration. Vehicle or SnMP (0.5 μ mol/kg) was intravenously administered to rats 1 h before HSR. Total RNA was extracted from the kidney 3 h after HSR, and Bcl-2 gene expression was analyzed by Northern blot analysis. Left, autoradiographic signals of the RNA blot hybridized with [α - 32 P]dCTP-labeled Bcl-2 cDNA with the ethidium bromide-stained gels. Open and closed arrowheads indicate the positions of 18S and 28S ribosomal RNA corresponding to the ethidium bromide-stained gels, respectively. Sham, Sham-operated control animals; Vehicle/HSR, Vehicle-treated HSR animals; SnMP/HSR, SnMP-treated HSR animals. Right, Bcl-2 mRNA levels are expressed as arbitrary units. Data are presented as means \pm SD (n=5). *p<0.05 vs. Sham; #p < 0.05 vs. Vehicle/HSR.

higher than those of the vehicle-treated HSR animals, respectively. These results indicate that tissue inflammation was markedly enhanced by HO-1 inhibition.

Activated caspase-3 and Bcl-2 expression. Since tissue inflammation promotes oxidative stress which leads to apoptotic cell death, and HO-1 has anti-apoptotic property (14), we examined the effect of HSR on apoptotic cell death and its modulation by SnMP. Apoptotic cell death was specifically assessed using fluorescent immunohistochemical staining of activated caspase-3, the key executioner of apoptosis, 12 h after HSR (8) and by Northern blot analysis of Bcl-2 mRNA, the key anti-apoptotic gene (7), 3 h after

HSR. While activated caspase-3 signals were hardly detectable in the kidneys of sham-operated animals (Fig. 1D and G), the kidney sections from the vehicle-treated HSR animals only marginally expressed positive signals (Fig. 1E). Of note, these signals were merely observed in the renal tubular epithelial cells where HO-1 was not expressed (Fig. 1H). When HSR animals were pretreated with SnMP, the positive signals of activated caspase-3 were obviously increased and distributed homogeneously in the kidney (Fig. 1F and I). Bcl-2 mRNA was significantly expressed in the sham-operated control animals; however, its level was further increased in the vehicle-treated HSR animals (Fig. 4). In contrast, SnMP pretreatment decreased Bcl-2 mRNA expression, which

reached a level similar to that in the sham animals (Fig. 4). Thus, these findings suggest that apoptosis increased significantly by inhibiting HO activity by SnMP treatment.

Discussion

We demonstrated that HSR induced functional HO-1 protein in the tubular epithelial cells of the kidney, while there was only a marginal increase in gene expression of pro-inflammatory mediators such as TNF- α and iNOS and a significant increase in the gene expression of Bcl-2, an anti-apoptotic gene, in the kidney after HSR. Although activated caspase-3 was slightly expressed in the kidney after HSR, its expression was only observed in the renal cells where HO-1 was not expressed. Of note, our findings also demonstrated that inhibition of HO activity by SnMP markedly aggravated tissue inflammation and apoptotic cell death induced by HSR. These findings suggest that the maintenance of induced HO activity is essential for protecting the kidney from HSR-induced oxidative tissue injury.

HSR incites an inflammatory response characterized by the upregulation of pro-inflammatory mediators, which are responsible for highly complex cascading events leading to oxygen radical generation (3). Reactive oxygen species induce apoptotic cell death, ultimately resulting in tissue injury (15,16). Conversely, oxidative stresses provoke cellular protective responses, principally involving transcriptional activation of genes encoding proteins that participate in the defense against oxidative tissue injuries (17). HO-1 is induced by oxidative stress and is thought to protect against oxidative tissue injuries (1,2). Recent studies, including those from our laboratory, strongly indicate that the protective effect of HO-1 against oxidative stress can be attributed to its anti-inflammatory and anti-apoptotic properties (18,19). In support of this notion, as shown in Fig. 1, following HSR, functional HO-1 protein was highly induced in the tubular epithelial cells of the kidney, which are the target cells of HSR-induced renal injury. In contrast to enhanced HO-1 protein expression, mRNA levels of the pro-inflammatory mediators TNF- α and iNOS showed only marginal increases after HSR (Fig. 3). Moreover, although positive signals of activated caspase-3 were slightly detectable after HSR, they were only observed in the tubular epithelial cells where HO-1 was not expressed (Fig. 1). The anti-inflammatory and anti-apoptotic properties of renal HO-1 induced by HSR were further confirmed by the fact that inhibiting HO-1 by SnMP treatment resulted in a marked elevation of mRNA levels of TNF- α and iNOS (Fig. 3), a further increase in activated caspase-3-positive cells (Fig. 1), and a reduction in Bcl-2 mRNA levels, which were increased by HSR (Fig. 4). In particular, the increase in iNOS mRNA level by inhibiting HO activity is of great importance as numerous *in vivo* and *in vitro* investigations have demonstrated that inhibiting the expression of iNOS activity or the absence of iNOS itself can ameliorate or prevent renal ischemia/reperfusion injury (20).

The precise mechanism by which increased HO-1 activity produces anti-inflammatory and anti-apoptotic effects is yet to be demonstrated definitively. HO-1 oxidatively cleaves heme and yields three metabolites: iron, biliverdin and carbon monoxide. In doing so, HO-1 reduces cellular concentrations

of free heme, a pro-oxidant and tissue inflammation activator (21,22). In good agreement with this notion, we previously demonstrated that inducing renal HO-1 in a rat model of ischemic acute renal failure degrades an excess amount of free heme, presumably derived from degenerated cytochrome P450 by ischemia to protect tubular epithelial cells from further damage (23). In addition to heme removal, HO-1 metabolites also have significant biological properties. Although excess free iron is cytotoxic because it catalyzes the Fenton reaction to generate free radicals (24), iron produced by HO-1 is directly sequestered by ferritin, leading to additional anti-oxidant (25) and anti-apoptotic effects (26). Biliverdin is rapidly converted to bilirubin by bilirubin reductase, and both biliverdin and bilirubin function as potent endogenous anti-oxidants (27). Bilirubin itself is oxidized to biliverdin and then recycled back to bilirubin by biliverdin reductase, suggesting further amplification of their anti-oxidant effects (28). In fact, bilirubin treatment has recently been shown to improve renal vascular resistance, urine output, glomerular filtration rate, tubular function, and mitochondrial integrity after renal ischemia/reperfusion (29). CO also exerts anti-inflammatory and anti-apoptotic activities that are thought to be mediated, at least in part, by activating the p38 mitogen-activated protein kinase signaling pathway (14,30). In this regard, it has been reported that in a rat model of transplant-induced renal I/R injury, administration of 250 ppm CO gas to rats resulted in a significant improvement in graft renal function associated with significant reduction in tubular epithelial cell apoptosis and decrease in pro-inflammatory mediators (31). Accordingly, HO-1 reactions in the kidney may provide a fundamental protective milieu for tubular epithelial cells to cope with HSR-induced oxidative tissue injury by favoring the removal of the pro-oxidant heme and increasing the amount of anti-oxidative, anti-inflammatory, and anti-apoptotic heme metabolites. While it remains unclear as to what extent each mechanism may contribute to this process, these findings may all be important in the host cellular defense against oxidative tissue injuries.

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