Heme oxygenase-1 gene ablation or expression modulates cisplatin-induced renal tubular apoptosis

FUMIE SHIRAISHI, LISA M. CURTIS, LEIGH TRUONG, KENNETH POSS, GARY A. VISNER, KIRSTEN MADSEN, HARRY S. NICK, and ANUPAM AGARWAL. Heme oxygenase-1 gene ablation or expression modulates cisplatin-induced renal tubular apoptosis. Am J Physiol Renal Physiol 278: F726–F736, 2000.—Heme oxygenase-1 (HO-1) is a 32-kDa microsomal enzyme that catalyzes the conversion of heme to biliverdin, releasing iron and carbon monoxide. Induction of HO-1 occurs as a protective response in cells/tissues exposed to a wide variety of oxidant stimuli. The chemotherapeutic effects of cis-diamminedichloroplatinum(II) (cisplatin), a commonly used anticancer drug, are limited by significant nephrotoxicity, which is characterized by varying degrees of renal tubular apoptosis and necrosis. The purpose of this study was to evaluate the functional significance of HO-1 expression in cisplatin-induced renal injury. Our studies demonstrate that transgenic mice deficient in HO-1 (−/−) develop more severe renal failure and have significantly greater renal injury compared with wild-type (+/+) mice treated with cisplatin. In vitro studies in human renal proximal tubule cells demonstrate that hemin, an inducer of HO-1, significantly attenuated cisplatin-induced apoptosis and necrosis, whereas inhibition of HO-1 enzyme activity reversed the cytoprotective effect. Overexpression of HO-1 resulted in a significant reduction in cisplatin-induced cytotoxicity. These studies provide a basis for future studies using targeted gene expression of HO-1 as a therapeutic and preventive modality in high-risk settings of acute renal failure.

acute renal failure; oxidative stress; cytoprotection; carbon monoxide; cancer chemotherapy

REACTIVE OXYGEN SPECIES PLAY an important role in the pathogenesis of a variety of renal diseases, including acute renal failure (3, 28). The kidney is one of the prominent sites for intense oxidative processes in the body (28) and is, therefore, extremely vulnerable to free radical-mediated injury. Recent studies have reported that in states of heightened oxidant stress, heme oxygenase-1 (HO-1) is induced as a beneficial response in cells exposed to a diverse array of toxic insults (22, 30, 37). HO-1 is a ~32-kDa microsomal enzyme that catalyzes the initial and rate-limiting reaction in heme catabolism. It cleaves heme molecules to yield equimolar quantities of biliverdin, carbon monoxide, and iron. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. Three isoforms of heme oxygenase, HO-1, HO-2, and HO-3, have been described (22, 24). HO-1 is inducible, whereas HO-2 is constitutively expressed in the brain, testes, endothelium, and the medullary thick ascending limb (mTAL) segment of the rat kidney (21, 22). A third isoform, HO-3, with properties similar to HO-2, has recently been described (24). HO-1 is induced by heme products and a variety of nonheme stimuli, including heavy metals, hydrogen peroxide, nitric oxide, endotoxin, cytokines, ultraviolet A irradiation, shear stress, hyperoxia, and oxidized low-density lipoproteins (LDL). Although the cellular processes underlying HO-1 induction are complex and tightly regulated, one denominator common to most of these stimuli is a significant shift in cellular redox (30).

With regard to acute renal injury, previous studies have shown that expression of HO-1 in renal tubules is cytoprotective in both heme- and nonheme-mediated models of renal failure. For example, in heme-mediated renal injury in a rat model of acute renal failure associated with rhabdomyolysis, administration of a specific inhibitor of HO-1, tin protoporphyrin, worsens renal damage. Prior induction of HO-1 with hemoglobin leads to a considerable decrease in mortality (29). Vogt et al. (39) demonstrated a novel phenomenon of acquired resistance to renal tubular injury in acute glomerular inflammation that was dependent on the induction of HO-1 in renal tubules. Induction of HO-1 occurs in immune-mediated renal injury, as demonstrated by the expression of this protein in infiltrating macrophages in acute renal transplant rejection (2). Expression of HO-1 has been linked to prolonged xenograft survival by investigators, showing shortened survival in hearts transplanted from HO-1-knockout mice compared with wild-type or heterozygote mice (36). Previous studies have shown that in cisplatin-induced toxic nephropathy, a model of acute oxidative stress not directly dependent on the delivery of heme proteins to the kidney, HO-1 is induced in renal tubules as early as 6 h after cis-diamminedichloroplatinum(II) (cisplatin) administration (1). Inhibition of HO-1 by tin protoporphyrin significantly worsens both struc-
tural and functional parameters of renal injury, providing indirect evidence for a role of HO-1 in CP nephrotoxicity.

The chemotherapeutic agent CP is widely used for the treatment of several human malignancies, including ovarian, testicular, bladder, head and neck, esophageal, and small-cell lung cancers (17). The beneficial antineoplastic effects of this drug are often mitigated by significant side effects, including nephrotoxicity, bone marrow suppression, peripheral neuropathy, ototoxicity, nausea, vomiting, and anaphylaxis. CP accumulates in the kidney to a greater degree than in other organs and preferentially injures the S3 segment of the proximal tubule and the mTAL, both situated in the outer medullary region of the kidney (33, 34). After a single injection of CP (50–100 mg/m²) for chemotherapy, 28–36% of patients develop dose-dependent nephrotoxicity (17, 33). Urinary levels of CP approach 50–250 mg/ml (~100–500 µM) within 2–6 h of CP infusion (20) and progressively decrease over a few days. Early reductions in glomerular filtration rate are associated with decreases in renal plasma flow and increased renal vascular resistance, making CP nephrotoxicity an ideal model to study the early pathophysiological and biochemical determinants of acute renal failure (34). The pathogenesis of CP-induced acute renal failure is characterized by both necrosis and apoptosis of renal tubular cells (19, 20). High concentrations of CP cause necrosis, whereas lower concentrations induce apoptosis (19). In fact, apoptosis has been recognized as the main mechanism responsible for the antineoplastic action of CP (13). It is, therefore, not surprising that CP-induced apoptosis is important in nephrotoxicity, the major adverse effect of this agent.

The purpose of the present study was to evaluate the specific role of HO-1 in CP-induced renal injury in transgenic mice with targeted deletion of the HO-1 gene. In vitro studies were performed to assess the effects of chemical inducers and inhibitors of HO-1 on CP-mediated necrosis and apoptosis in human renal proximal tubule cells. To further corroborate our findings with chemical inducers and inhibitors of HO-1, we also performed studies in human renal epithelial cells to manipulate the HO-1 gene at the molecular level by selective overexpression.

METHODS

Reagents

CP, hemin, fetal bovine serum, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and blood urea nitrogen assay kit were obtained from Sigma Chemical (St. Louis, MO). Zinc protoporphyrin (ZnPP) was obtained from Porphyрин Products (Logan, UT). The cytotoxicity detection kit for lactate dehydrogenase (LDH) assay was obtained from Boehringer Mannheim (Indianapolis, IN), anti-HO-1 antibody (SPA-895) from StressGen Biotechnologies (Victoria, BC), lipofectamine from Life Technologies (Grand Island, NY), and the mammalian expression vector pcDNA3.1/Zeo and Zeein from Invitrogen (Carlsbad, CA).

Studies In Vivo

Study protocol. Homozygous mice, HO-1 (−/−), carrying a targeted deletion of a large portion of the HO-1 gene, were selected from the offsprings of heterozygous/homozygous mating by Southern blotting of tail DNA (31). Wild-type (+/+) and heterozygous (+/−) littermates were used as controls. Mice from 12–16 wk of age were studied. CP, 20 mg/kg (1.0 mg/ml solution in sterile normal saline) or vehicle (normal saline) was administered by a single intraperitoneal injection. Mice were maintained on standard chow and tap water ad libitum. The induction of acute renal failure was confirmed by increases in blood urea nitrogen (BUN) and histological parameters of acute renal injury (tubular necrosis, casts, loss of brush border, red blood cell extravasation). The study protocol was approved by the Institutional Animal Care and Use Committee at the University of Florida, Gainesville.

In preliminary experiments to determine the course of renal function in these mice, we measured BUN at days 3 and 5 after CP (20 mg/kg ip). These results showed significantly higher BUN values at day 5 in all animals with a significant increase in mortality, limiting the number of animals in each group available for experimental data. However, we observed that homozygote mice had a significant increase in BUN at day 3, whereas the wild-type and heterozygote mice showed only a trend toward increasing BUN values; hence we performed all experiments at this time point.

At day 3 after CP administration, mice were anesthetized with pentobarbital sodium and perfused through the heart with PBS (pH 7.4), followed by perfusion with paraformaldehyde-lysine-periodate fixative for 5 min. Kidneys were excised and placed in fresh fixative overnight at 4°C. The next day, kidneys were washed with PBS and sliced into multiple 1-mm sections. After dehydration through a graded ethanol series, slices were immersed in a 1:1 mixture of 100% ethanol and wax for 1 h at 37°C, followed by two 1-h incubations in pure wax at 37°C and then embedded in wax and sectioned. Tissue sections were stained with hematoxylin and eosin for assessment of acute tubular necrosis by light microscopy.

Apoptosis detection and quantification. Wax sections of 5–6 µm were placed on gelatin-coated slides and processed for the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay by a modification of previously described methods (12). Briefly, after dewaxing and inactivation of endogenous peroxidases with hydrogen peroxide, sections were incubated with TdT reaction as described, and labeling was detected by using the avidin-biotin method. Counterstaining with methyl green for 3–5 min facilitated visualization of the sections. To localize the site of apoptotic nuclei, double staining was performed by using an antibody to Na⁺-K⁺-ATPase alpha-1 subunit (Upstate Biotechnology, Waltham, MA). Analyses were performed in a blinded fashion. Random areas were viewed at a magnification of ×40 and scored for the number of apoptotic nuclei present in renal tubules in at least three to four slices of the kidney. An average of 266 ± 55 fields were examined, and the total number of apoptotic nuclei per square millimeter was calculated for each animal. For additional confirmation of apoptosis, transmission electron microscopy was performed from glutaraldehyde-fixed kidney tissue by previously described methods (15).
Plasmid construction for HO-1 overexpression. A plasmid, pcDNA3.1/HO-1, was constructed by using the mammalian expression vector pcDNA 3.1/Zeo and a 1.0-kb EcoRI/XbaI fragment containing the entire protein coding region of the HO-1 gene. The expression plasmid was designed to include the HO-1 DNA encoding an expressed transgene mRNA ~300 bp smaller than the endogenous message. The vector alone was used as control. Transfections were done by using lipofectamine. Transfected cells were selected with Zeocin (100 µg/ml) for a week and incubated for 3 days without Zeocin before experiments. Successful overexpression was confirmed by Northern and Western analyses.

Northern analysis. Total cellular RNA was extracted from transfected cells in 10-cm plates by using the method of Chomczynski and Sacchi (7). The RNA was electrophoresed on 1% agarose-formaldehyde gels, blotted onto nylon membranes, and hybridized to a 32P-labeled cDNA probe for HO-1 gene. The expression plasmid was designed to include the entire protein coding region of the HO-1 gene. The expression plasmid was designed to include the entire protein coding region of the HO-1 gene. The expression plasmid was designed to include the entire protein coding region of the HO-1 gene. The expression plasmid was designed to include the entire protein coding region of the HO-1 gene.

MTT stock solution were added, and cells were incubated for 4 h. One hundred microliters of acidic isopropanol (0.04 N hydrochloric acid in isopropanol) were added. The absorbance of samples at 550 and 690 nm was measured. Results are expressed as percent change in absorbance of CP-treated cells over control (untreated) cells in each group (vector alone and HO-1 transfected cells, respectively).

Quantification of apoptosis by using the annexin V binding assay. Binding of annexin V to the phosphatidylserine residues on the cell membrane in the presence of an intact cell membrane has been used as a marker for early apoptosis (9, 35). In these experiments, HPTC were pretreated with 5 µM hemin for 12 h, washed with Hank’s balanced salt solution, and exposed to CP (10 µM) in complete media for 5 days. To inhibit HO-1 activity, ZnPP (5 µM) was added, to cells pretreated with hemin, for 2 h before exposure to CP. Annexin V binding assay was performed by using an apoptosis-detection kit (R&D systems, Minneapolis, MN) for analysis of adherent cells as per the manufacturer’s recommendations. Cells were harvested by gentle trypsinization and stained in suspension for immunofluorescence studies by using annexin V-FITC. The concentration of trypsin-EDTA used (0.025%) does not interfere with the annexin V assay, as shown by negative staining of control cells. Apoptotic cells were identified as cells staining positive for annexin V and negative for propidium iodide. Necrotic cells stained positive for both annexin V and propidium iodide. The number of apoptotic cells is expressed as a percentage of the total number of cells counted. An average of 700–800 cells was counted from multiple fields under both phase-contrast and fluorescence microscopy in each experimental group from at least three independent experiments.

Statistical analysis. Data are represented as means ± SE. For comparisons involving two groups, the t-test was used. For comparisons involving more than two groups, ANOVA and the Student-Newman-Keuls test were applied. All results are considered significant at P < 0.05.

RESULTS
Assessment of Renal Function

All animals (+/+; +/− and −/−) that received CP showed a statistically significant (P < 0.01) decrease in body weight at day 3 compared with day 0 (Table 1). The decrease in weight was similar in wild-type, heterozygote, and homozygote mice treated with CP. No significant change in weight occurred in the mice that received normal saline. The results of renal function as

<table>
<thead>
<tr>
<th>Wild-type</th>
<th>Day 0</th>
<th>Day 3</th>
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<tbody>
<tr>
<td>Saline (n = 6)</td>
<td>26.5 ± 1.5</td>
<td>27.1 ± 1.5*</td>
</tr>
<tr>
<td>Cisplatin (n = 8)</td>
<td>25.0 ± 1.6</td>
<td>22.7 ± 1.5*</td>
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<tr>
<td>Heterozygote</td>
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<tr>
<td>Saline (n = 5)</td>
<td>26.1 ± 1.8</td>
<td>25.7 ± 1.4*</td>
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<tr>
<td>Cisplatin (n = 7)</td>
<td>29.7 ± 1.9</td>
<td>26.5 ± 1.8</td>
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<tr>
<td>Homozygote</td>
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<tr>
<td>Saline (n = 8)</td>
<td>30.0 ± 2.2</td>
<td>29.1 ± 2.0*</td>
</tr>
<tr>
<td>Cisplatin (n = 6)</td>
<td>26.2 ± 1.6</td>
<td>22.5 ± 1.5*</td>
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Values are means ± SE. n, No. of mice; cisplatin, cis-diammine-dichloroplatinum(II). *P = ns; †P < 0.01, day 3 vs. day 0.
measured by BUN are shown in Fig. 1. The BUN values in HO-1 (−/−) mice were significantly elevated at day 3 after the administration of CP compared with wild-type (+/+) and/or heterozygote (+/−) mice. These data demonstrate that CP causes significantly worse renal dysfunction in HO-1 (−/−) mice compared with HO-1 (+/+) or HO-1 (+/−) mice. Because no differences in renal function were observed between the HO-1 (+/+) mice and the HO-1 (+/+) mice, further histological comparisons were performed in the HO-1 (+/+) and the HO-1 (−/−) mice.

Morphological Evaluation and Quantification of Apoptosis In Vivo

Renal tissue sections in HO-1 (−/−) mice examined at day 3 after CP administration demonstrated increased evidence of acute renal injury, characterized by tubular necrosis, degeneration, casts, loss of brush border, and red blood cell extravasation, compared with HO-1 (+/+) mice, which showed only mild changes (Fig. 2, A–D). Apoptotic bodies were identifiable on hematoxylin- and eosin-stained kidney tissue sections in the HO-1 (−/−) mice (Fig. 2D). Electron microscopy was performed to further confirm the presence of apoptosis in the kidney. The characteristic features of chromatin condensation (Fig. 3, inset) and the presence of apoptotic bodies were easily identifiable in proximal and distal tubules in the HO-1 (−/−) mice (Fig. 3). To quantify the degree of apoptosis in the kidney, the TUNEL assay was performed at day 3 after administration of CP. As shown in Fig. 4A, significantly increased numbers of apoptotic nuclei were seen in the CP-treated HO-1 (−/−) mice. No staining was seen in saline-treated mice. Figure 4B shows the results of quantification of apoptotic nuclei at day 3 after saline/CP in HO-1 (−/−) and HO-1 (+/+) mice. Although CP-treated HO-1 (−/−) mice showed a 6.6-fold increase in apoptotic nuclei, the HO-1 (+/+) mice had a 3-fold increase over saline-treated animals, respectively. Significant differences were observed between CP-treated and saline-treated wild-type mice as well (P < 0.05, unpaired t-test). Apoptotic nuclei were identifiable in both proximal and distal tubules, as verified by dual staining using Na^+–K^+–ATPase, which gives much stronger staining of the distal tubule than the proximal tubule. Necrotic cells and necrotic debris did not stain positive with the TUNEL assay. These data demonstrate that, in this model of CP nephrotoxicity, the lack of HO-1 expression increased renal dysfunction and renal injury, with an accompanying increase in renal tubular apoptosis.

Induction of HO-1 by Hemin Attenuates CP-Mediated Cytotoxicity in HPTC In Vitro

To evaluate the cytoprotective role of HO-1 in cytotoxicity induced by CP, HPTC were pretreated with a low dose of hemin (5 µM), a potent inducer of HO-1, 12 h before exposure to CP (100 µM). The maximal time point of HO-1 induction by hemin was determined in HPTC. As shown in Fig. 5A, maximal induction (~6-fold) was observed at 12 h after hemin treatment. Cytotoxicity was determined by phase-contrast microscopy and the LDH assay in these experiments. Exposure of HPTC to CP resulted in morphological evidence of cytotoxicity, as demonstrated by cell rounding, swelling, and detachment (Fig. 5B). Prior induction of HO-1, by hemin pretreatment, resulted in resistance of HPTC to CP-induced cytotoxicity (Fig. 5B). These data were corroborated by measurement of LDH activity, which demonstrated that hemin pretreatment significantly attenuated CP-induced cytotoxicity by 60% (CP alone: 16.1 ± 2.7%; hemin + CP: 6.35 ± 1.6%; P < 0.05; n = 4) at 12 h (Fig. 5C). The specificity of the cytoprotective effect of HO-1 was confirmed by cotreatment of cells with ZnPP (5 µM), an inhibitor of HO-1. Inhibition of HO-1 with ZnPP resulted in reversal of the cytoprotective effect conferred by hemin pretreatment (hemin + CP + ZnPP: 15.4 ± 1.8%). Hemin and ZnPP alone demonstrated no significant cytotoxicity. The protective effect of hemin pretreatment was also seen after 24 h of exposure to CP (100 µM) (CP: 36.5%; hemin + CP: 17.6%; P < 0.05; n = 2).

Induction of HO-1 by Hemin Decreases CP-Mediated Apoptosis In Vitro

Previous studies by Lieberthal et al. (20) demonstrated that low concentrations of CP (~8–10 µM) cause apoptosis, whereas higher concentrations cause necrosis in mouse proximal tubule cells. In view of these observations, we explored the effects of HO-1 expression on CP-induced apoptosis in HPTC. Apoptosis was quantified by the annexin V binding assay. As shown in Fig. 6, exposure of HPTC to CP (10 µM) for 5 days induced apoptosis (C and D). Induction of HO-1 by hemin pretreatment significantly attenuated apoptosis (E and F). Inhibition of HO-1 by ZnPP, after hemin pretreatment, reversed the protective effect, resulting in more severe cellular injury with extensive necrosis (G and H). A quantitative representation of these results is summarized in Fig. 7. Incubation of cells with hemin or ZnPP (5 µM) alone caused no cell injury (apoptosis or necrosis).
Overexpression of HO-1 Decreases CP-Mediated Cell Injury

To further corroborate the results with chemical inducers and inhibitors, HO-1 was overexpressed in our cellular model of CP-induced cytotoxicity. HEK-293 cells were transfected with a vector containing the entire protein coding region of the human HO-1 gene (pDNA3.1/HHO-1) under the promoter/enhancer of cytomegalovirus (pcDNA3.1/HHO-1). The vector alone was used as a transfection control. Northern analysis confirmed the presence of successful overexpression (~40-fold) in the form of a ~1.5-kb message under basal, unstimulated conditions (Fig. 8A). Immunoblot analysis in cells transfected with pcDNA3.1/HHO-1 revealed an approximately eightfold increase in HO-1 protein over control cells transfected with vector alone (Fig. 8B).

Control and transfected cells were exposed to CP (100 µM) for 16 h, and cytotoxicity was assessed by phase-contrast microscopy, LDH, and MTT assays. Morphological indexes of injury, such as cell rounding, vacuolization, and detachment, were significantly reduced in cells with elevated levels of HO-1 compared with cells transfected with the vector alone. These changes were accompanied by a 68% decrease in CP-induced cytotoxicity as determined by the LDH release assay (vector alone: 20 ± 6.8%; HO-1 overexpression: 6.6 ± 2.82%; P < 0.02; n = 4) (Fig. 9A). Treatment of cells with a competitive inhibitor, ZnPP, reversed the cytoprotective effects of HO-1 (data not shown). Mitochondrial viability, assessed by the MTT assay, revealed significantly decreased CP-induced injury in cells transfected with HO-1. Cells transfected with HO-1 and treated with CP had more viable mitochondria, represented by a smaller change in absorbance, over control (untreated), HO-1 transfected cells (untreated, 100% vs. CP-treated, 94.7 ± 3.5%). Cells transfected with vector alone had significantly greater mitochondrial injury (untreated, 100% vs. CP-treated, 83.7 ± 4.9%). The percent change in absorbance in vector alone vs. HO-1-transfected cells is shown in Fig. 9B.

DISCUSSION

Acute renal failure due to ischemia and nephrotoxic drugs results in varying degrees of morphological damage, especially in the S3 segment of the proximal tubule (5). Nephrotoxicity due to the chemotherapeutic agent, CP, is characterized by similar morphological changes in this segment of the nephron. In addition to injury in...
More recently, studies have demonstrated a role for cytochrome P-450 and release of its catalytic iron as a potential mediator of CP nephrotoxicity (4). The propensity of iron to participate in the propagation of free radical generation via the Haber-Weiss reaction and the recent demonstration that HO-1 is required for the maintenance of iron homeostasis (31) implicate iron as a potential candidate molecule responsible for the injurious effects of CP in the HO-1 (−/−) mice.

Apoptosis of renal tubular cells plays an important pathogenic role in CP nephrotoxicity as well as in other models of acute renal failure (19, 20). Studies in mouse proximal tubular cells provided evidence that CP-induced apoptosis is mediated via the generation of oxygen free radicals, on the basis of the cytoprotective effect of antioxidants such as catalase, superoxide dismutase, deferoxamine, and probucol (20). In contrast, antioxidants did not protect against CP-induced necrosis. Our in vitro studies showed significant attenuation of CP-induced apoptosis as well as necrosis in human renal epithelial cells by chemical inducers and overexpression of HO-1. Increased apoptosis and necrosis were also observed in the HO-1 (−/−) mice. In this regard, the protection afforded by HO-1 expression is unique compared with other antioxidants.

In our model of CP nephrotoxicity, a higher degree of apoptosis was seen in both proximal and distal tubules of HO-1 (−/−) mice kidneys. These results are similar to observations in the p21-knockout mouse model of CP-induced renal injury (25). Mice deficient in p21 (WAF1, CIP-1, or Sid-1), a cell-cycle inhibitory protein, develop a rapid onset of renal failure and have a higher mortality after CP administration compared with wild-type littermates (25). The mice also have a higher degree of CP-induced apoptosis in proximal and distal tubules, implicating the importance of cell-cycle regulation in the pathogenesis of CP-induced renal failure.

Renal epithelial cells transfected with HO-1 have slower growth rates compared with cells transfected with vector alone (Shiraishi F and Agarwal A, unpublished observations), similar to findings in pulmonary epithelial cells after overexpression of HO-1 (18). The cellular mechanisms for this phenomenon are being evaluated in our laboratory. It is tempting to extrapolate the effects of nitric oxide-induced cell growth arrest mediated via activation of cGMP to carbon monoxide released from the HO-1-catalyzed reaction, because carbon monoxide has been shown to decrease proliferation of vascular smooth muscle cells (26).

Our findings of severe CP-induced renal dysfunction in the HO-1 (−/−) mice cannot be explained by increased renal tubular apoptosis alone. Most likely, the renal dysfunction involves a combination of increased apoptosis, necrosis, and possible vascular effects because of the absence of HO-1 expression (1, 11). Previous studies have demonstrated that inhibition of HO-1 exacerbates changes in renal hemodynamics in CP-induced nephrotoxicity (1). Foremost, inhibition of HO-1 with tin protoporphyrin increases renal vascular resistance and decreases renal blood flow in CP nephrotoxicity before detectable changes in renal function. These
effects could be attributable to decreased generation of carbon monoxide, a metabolite with vasodilatory effects mediated via cGMP (11). Second, increased intracellular heme associated with CP nephrotoxicity may quench nitric oxide, further exacerbating the vascular effects that occur during the early stages in this model. Enhanced HO-1 activity would reverse these deleterious vascular effects by generating carbon monoxide and degrading intracellular heme, thereby providing a cytoprotective effect.

CP-induced mitochondrial injury (6) was significantly reduced by overexpression of HO-1 in our study. Recent studies suggest that mitochondria are the principle sensors and, thus, critical in the initiation of the

Fig. 4. Apoptotic nuclei in kidneys of HO-1 (+/+) and (-/-) mice. A: apoptotic nuclei in kidneys of mice treated with saline (top row) or CP (20 mg/kg; bottom row) at day 3. Terminal deoxy nucleotide mediated dUTP nick end-labeling (TUNEL) staining in kidney nuclei is indicated by arrows (magnification: ×100). Inset: higher magnification (×400) showing apoptotic nucleus in renal tubule of a HO-1 (-/-) mouse kidney. B: quantitation of apoptotic nuclei in wild and homozygote mice at day 3 after saline or CP administration. Values are means ± SE of apoptotic nuclei observed per mm² section of kidney prepared from each animal. *P < 0.01 (ANOVA). #P < 0.05 vs. saline (unpaired t-test).
apoptotic cascade (16, 32, 38). Mitochondrial dysfunction with liberation of cytochrome C, a mitochondrial heme protein, occurs as an early event in CP-induced apoptosis and results in activation of further downstream pathways (10). The processes involved in the antiapoptotic effects of HO-1 expression are not entirely clear. Although tubular injury in CP nephrotoxicity is not directly dependent on the delivery of heme proteins to the kidney, increased amounts of endogenous heme are observed in the kidney, specifically in the cytosolic fraction. This occurs as early as 6 h after CP administration and contributes, at least in part, to the induction of HO-1 and the injury that ensues (1). We speculate that CP-induced oxidant stress destabilizes heme proteins, resulting in the liberation of heme...
from its binding protein. The heme moiety, either directly or via the effects of its released iron, can damage a number of cellular targets including the lipid bilayer, the cytoskeleton, mitochondria, and the nucleus. Elevated levels of HO-1 will degrade the potentially prooxidant heme groups, released as a consequence of CP-induced oxidant stress and would limit the increase in heme that would otherwise occur. In addition to the induction of HO-1, administration of CP also leads to a concomitant increase in ferritin in the kidney. The increase in ferritin is an additional protective response, because ferritin safely sequesters the iron released from the heme ring (30). Other mechanisms by which the induction of HO-1 may confer protection include increased generation of bilirubin, a metabolite with potent antioxidant activity (8), and improved renal hemodynamics via generation of carbon monoxide (1). Studies to explore the cellular basis of the cytoprotective effects of HO-1 overexpression are presently underway in our laboratory.

Higher concentrations of heme (>50 µM) are cytotoxic whereas lower concentrations (5 µM) are cytoprotective, possibly via induction of HO-1. We do not think that hemin or a heme metabolite is inactivating CP for several reasons. First, in all our experiments where we used hemin pretreatment, the cell monolayer was washed and fresh media were added before CP exposure. Second, we have shown reversal of the cytoprotective effect with a competitive inhibitor of HO-1. Third,
to obviate the concerns over chemical inducers and inhibitors, we have performed studies to selectively overexpress HO-1 in our cell culture model as well as used knockout mice to confirm our findings.

In summary, our study demonstrates that the expression of HO-1 protects renal epithelial cells against CP-induced cytotoxicity. CP nephrotoxicity, as assessed by structural and functional changes, was significantly greater in HO-1-knockout mice compared with wild-type mice. In contrast, induction of HO-1 by chemical inducers or by overexpression in renal epithelial cells in vitro protected cells against CP-induced cytotoxicity. Our data provide the impetus to design possible strategies for targeted gene expression of HO-1 as well as the development of novel, physiologically relevant inducers of the endogenous HO-1 gene as a therapeutic and preventive modality in high-risk settings of acute renal failure.

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Address for reprint requests and other correspondence: A. Agarwal, Div. of Nephrology, Hypertension and Transplantation, Box 100224 J HMMC, 1600 SW Archer Rd., Univ. of Florida, Gainesville FL 32610 (E-mail: agarwal@nerp.nerd.cfl.ufl.edu).

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