Heme Oxygenase-2 Deficiency Contributes to Diabetes-Mediated Increase in Superoxide Anion and Renal Dysfunction

Alvin I. Goodman,* Praveen N. Chander,† Rita Rezzani,‡ Michal L. Schwartzman,§ Raymond F. Regan,¶ Luigi Rodella,† Saadet Turkseven,§ Elias A. Lianos,¶ Phyllis A. Dennery,** and Nader G. Abraham§

Departments of *Medicine, †Pathology, and §Pharmacology, New York Medical College, Valhalla, New York; ‡Department of Biomedical Science, Division of Anatomy, University of Brescia, Brescia, Italy; ¶Department of Emergency Medicine, Thomas Jefferson University Hospital, Philadelphia, Pennsylvania; ‡Department of Medicine, Division of Nephrology Robert Wood Johnson Medical School, New Brunswick, New Jersey; and **Department of Pediatrics, University of Pennsylvania School of Medicine, Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania

Heme oxygenase-1 (HO-1) and -2 play an important role in cytoprotection and are physiologic regulators of heme-dependent protein synthesis in renal tissues. The impact of HO-2 deletion comparing hyperglycemic HO-2 (+/+) mice and HO-2 knockout (−/−) mice was examined. Hyperglycemia was induced by streptozotocin (STZ) injection, and its effect on renal HO-1/HO-2 protein, HO activity, and creatinine levels were assessed. The effect of HO induction using systemic administration of the HO inducers heme or cobalt protoporphyrin and the effect of HO inhibition using systemic administration of the HO inhibitor tin mesoporphyrin also were assessed in STZ-treated mice. In STZ-treated HO-2 (−/−) mice, there was marked renal functional impairment as reflected by an increase in plasma creatinine, associated with acute tubular damage and microvascular pathology as compared with HO-2 (+/+). In these animals, HO activity was decreased with a concomitant increase in superoxide anion. Upregulation of HO-1 in HO-2 (−/−) mice by weekly administration of cobalt protoporphyrin prevented the increase in plasma creatinine levels and tubulointerstitial and microvascular pathology. Inhibition of HO activity by administration of tin mesoporphyrin accentuated superoxide production and increased creatinine levels in hyperglycemic HO-2 (−/−) mice. In conclusion, HO-2 deficiency enhanced STZ-induced renal dysfunction and morphologic injury and HO-1 upregulation in HO-2 (−/−) mouse rescue and prevented the morphologic damage. These observations indicate that HO activity is essential in preserving renal function and morphology in STZ-induced diabetic mice probably via mitigation of concomitant oxidative stress.

Received December 14, 2004. Accepted January 25, 2006.

Published online ahead of print. Publication date available at www.jasn.org.

Address correspondence to: Dr. Nader G. Abraham, New York Medical College, Department of Pharmacology, Grassland Road, Valhalla, NY 10595. Phone: 914-594-4132; Fax: 914-594-4119; E-mail: nader_abraham@nymc.edu

Copyright © 2006 by the American Society of Nephrology

ISSN: 1046-6673/1704-1073

Heme oxygenase-1 (HO-1) and -2 gene expression exert antioxidant effects (1). These are attributed to the capacity of HO to degrade heme that is released from oxidant destabilized heme proteins (2) and to the production of bilirubin, which has potent antioxidant effects (3), as well as the generation of the vasodilator gas carbon monoxide (CO) (1). HO-1 and HO-2, the inducible and constitutive HO forms, respectively, play an important role in maintaining renal blood flow (4). HO-2 expression along the nephron follows regional distributions, the highest levels being detected in the medullary thick ascending limb and proximal tubules (5–7). It plays a regulatory role in renal cytochrome P450 expression and 20-hydroxyeicosatetraenoic acid synthesis; the latter regulates renal function and vascular response (8–12). HO-2–derived CO from renal small arteries regulates 105 psK channels and modulates vascular reactivity in response to vasoconstrictors (13,14). Bilirubin has been shown to inhibit NADPH oxidase (15) and protein kinase C activities (16,17).

The mechanism of hyperglycemia-induced tissue injury, as seen in type 1 diabetes, is under intense investigation. Streptozotocin (STZ), which commonly is used to induce diabetes, frequently results in acute tubular damage in mice (18). In vitro (19–21) and whole-animal studies have implicated superoxide anion (O2−) (22,23), which promotes formation of cytotoxic lipid peroxides (24) by a variety of mechanisms (22). O2− is vasoconstrictive through the removal of vasodilators and the stimulation of vasoconstrictor (25,26). O2− can convert nitric oxide (NO) to peroxynitrite, thereby consuming the endogenous vasodilator in the vasculature (27) and inducing vasoconstrictors, such as endothelin, PDGF, TxA2, and isoprostanes (27,28).

The objectives of this study were to determine the impact of
HO-2 deficiency on O$_2$~ levels and renal function and morphology under conditions of hyperglycemia. We examined whether upregulation of HO-1 can reduce oxidant production and preserve normal renal function and prevent tissue damage. Our results demonstrate that HO-2 deficiency, with reduced total renal HO activity, causes renal dysfunction and renal tubular and microvascular damage in diabetic mice. Induction of HO-1 in HO-2--deficient mice maintains normal renal function, attenuates the effects of the hyperglycemia-mediated increase in superoxide formation, and prevents renal tissue injury.

Materials and Methods

Animal Treatment

Three-month-old homozygote HO-2 (--/--) null (courtesy of Dr. Denry and Dr. Tonegawa) B6/129 mice and BALB/c genetic background HO-2 (+/+ ) mice were used for the studies. We used five mice per group with a total of 25 mice in five groups. The experiments were repeated three times for a total of 75 mice [total of 150 mice for both HO-2 (--/--) and HO-2 (+/+ )]. Diabetes was induced by a single injection, via the tail vein, of STZ (Aldrich/Sigma, St. Louis, MO), dissolved in 0.05 mol/L citrate buffer (pH 4.5) and given at 50 mg/kg intravenously once a day for 4d as has been described previously (29). Control mice received an injection of an equal volume (0.1 ml intravenously) of vehicle solution (sodium citrate buffer [pH 4.5]). Blood glucose levels after STZ were 420 ± 35 ng/dL. Glucose levels were maintained between 200 and 340 ng/dL in all STZ-treated mice for the 6-wk duration of the study by administration of insulin (neutral protamine hagedorn 40 to 60 U/d per kg). Insulin was essential for ensuring that ketosis and weight loss were NS. Glucose monitoring was performed using an automated analyzer (Lifespan Inc., Miligitas, CA). All animals were maintained on a standard laboratory diet and water, and every effort was made to minimize animal suffering according to the National Institutes of Health Institutional Animal Care and Use Committee guidelines.

HO-2 (--/--) and (+/+) mice were divided into groups as follows: Group A were diabetic controls that received STZ and group B received saline solution (STZ was injected intravenously, and all other drugs in the following groups were injected intraperitoneally); group C received STZ and cobalt protoporphyrin (CoPP), 0.5 mg/100 g weekly; group D received STZ and stannous mesoporphyrin (SnMP), 20/100 g body wt weekly (30); group E received heme. Heme, CoPP, and SnMP were dissolved in Tris base as described previously (30). The last injection of CoPP or SnMP was 3 d before the mice were killed. Mice were followed for 6 wk (duration of the experiments), at which time they were killed with CO$_2$ gas. Few HO-2 (--/--) mice that received heme and STZ survived; heme was also injected into nondiabetic mice at a dose of 1.0 mg/100 g intraperitoneally weekly.

Blood was centrifuged at 2500 x g for 10 min at 4°C and stored at −20°C. Plasma creatinine levels were measured using a commercial kit (Aldrich/Sigma), according to the manufacturer’s recommendation. In renal tissue, superoxide measurements were performed using a cytochrome C assay that has been described previously (31).

Morphology and Protein Analysis

Kidneys from HO-2 (+/+) and HO-2 (--/--) control, STZ-treated diabetic, STZ-CoPP--treated, and heme injected mice were harvested and cross-sectioned. A portion was fixed in formalin, paraffin embedded, and processed for histology with the standard techniques. Tissue sections were cut at 3- to 4-μ thickness and stained with hematoxylin and eosin and periodic acid Schiff. Sections from mice that received injections also were stained for iron with Prussian blue reaction. A portion of kidney was snap-frozen and kept at −8°C for further protein determinations and Western blot analysis.

Measurement of HO Activity.

Renal tissues were homogenized (4 ml/g wet wt) in 10 mM Tris-buffered solution (pH 7.5) that contained 0.25 M sucrose. The homogenates were centrifuged at 27,000 x g for 20 min at 4°C. The supernatant then was centrifuged at 105,000 x g for 1 h at 4°C, and the resulting microsomal pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.6). Microsomal HO activity was assayed by the method of Abraham et al. (32) in which bilirubin, the end product of heme degradation, was extracted with chloroform, and its concentration was determined spectrophotometrically (Dual UV/VIS Beam Spectrophotometer Lambda 25, Perkin-Elmer, Norwalk, CT) using the difference in absorbance at wavelength from 4460 to 4530 nm with an absorption coefficient of 40 mM$^{-1}$·cm$^{-1}$.

Superoxide Anion (O$_2$~) Production.

O$_2$~ production was assayed by the spectrophotometric measurement of ferricytochrome c reduction. Tissue homogenates from diabetic and nondiabetic rats were frozen at −80°C until use. Tissue homogenates were incubated with 0.5 ml of reaction mixture that consisted of Krebs Ringer phosphate buffer that contained 80 μM Cytochrome c and 2 mM Na$_2$EDTA. After 1 h of incubation at 37°C, the supernatants were collected and used to assay the amount of reduced Cytochrome c by the difference in absorbance at 550 to 468 nm using the extinction coefficient μM/L as described previously (32).

Western Blot Analysis of HO-1 and HO-2.

Homogenates were used as a source for the measurement of HO-1 and HO-2 proteins as described previously (31). Tissues were lysed in a buffer (lysis buffer B) that consisted of 0.5% Nonidet P-40, 0.5% deoxycholate, and 10 mM EDTA in Tris-buffered saline (20 mM Tris and 150 mM NaCl [pH 7.4]) that contained 10 μg/ml each of leupeptin, antipain, and pepstatin and 1 mM PMSF. Unbroken cells or cell debris was removed by centrifugation at 300 x g for 10 min. Protein from the supernatants was precipitated by adding 5 volumes of methanol, and the resulting pellet then was dissolved in sample buffer and separated on 10% SDS–polyacrylamide gel. For immunoblotting, the separated proteins (20 μg of total protein) were electrophoretically transferred to membrane after 2 h with 100 V. The membranes were blocked with 10% milk in 10 mM Tris·HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20 (TBST) buffer at 4°C overnight. After washing with TBST, the membranes were incubated with a 1:1000 dilution of anti-HO-1 or anti-HO-2 antibodies (Stressgen Biotechnologies, Victoria, British Columbia, Canada) for 1 h at room temperature with constant shaking. The filters then were washed and subsequently probed with horseradish peroxidase–conjugated donkey anti-rabbit (Amersham, Piscataway, NJ) at a dilution of 1:2000. Chemiluminescence detection was performed with the Amersham ECL detection kit according to the manufacturer’s instructions.

Statistical Analyses

The data are presented as mean ± SEM for the number of experiments. Statistical significance (P ≤ 0.05) was determined by the Fisher method of multiple comparisons. For comparison between treatment groups, the null hypothesis was tested by a single factor ANOVA for multiple groups or unpaired t test for two groups.

Results

As seen in Figure 1, glucose levels were maintained at similar levels and resulted in prevention of weight loss between the groups. Insulin dose was individualized; therefore, dosages were different within the same group and nonsignificantly
different between the groups. Under these conditions, body weight changed significantly (Figure 1B).

**Effect of HO-2 Deficiency on Renal Morphology**

As expected, renal histology was unremarkable in the control HO-2 (+/+) mice. As compared with the latter, the HO-2 (−/−) control mice showed occasional arteriolar hyalinosis, scattered hyaline casts in the medullary distal tubules, and one to two small foci of tubulointerstitial scarring with mild lymphoplasmacytic infiltrate close to the corticomedullary junction. These findings were very minor and not considered significant (Figure 2), although there was a slight increase in plasma creatinine levels in the control HO-2 (−/−) mice compared with HO-2 (+/+) mice.

Similarly, no significant abnormalities, in particular tubular damage, was apparent on histology in STZ-treated HO-2 (+/+) mice despite a slight increase in plasma creatinine levels compared with the control HO-2 (+/+) mice (Figure 3, A, D, and G). In contrast, STZ-treated HO-2 (−/−) mice exhibited widespread acute damage in cortical and outer and inner medullary tubules. This was characterized by frequent tubular dilation and by matting, attenuation, and focal loss of brush borders in the cortical as well as in the outer medullary proximal tubules (some denoted with *; E) and microvascular thickening and hyalinosis (B, arrows). Tubular and microvascular (C, arrows) pathology is entirely abrogated in STZ+cobalt protoporphyrin (CoPP)-treated HO-2 (−/−) mice. Magnification, ×50 (PAS stain).

**Figure 1.** (A) Serum glucose in control and streptozotocin (STZ)-treated heme oxygenase-2–deficient [HO-2 (−/−)] and HO-2 (+/+) mice. Five mice of each group received 50 mg/kg STZ for 4 consecutive days. The mean fasting glucose ± SEM for each group of mice at each treatment are shown. *P ≤ 0.05 is significant compared with the corresponding control (n = 5). (B) Total body weight gain or loss for control HO-2(−/−) and HO-2 (+/+) mice or in STZ-induced diabetes; *P < 0.05.

**Figure 2.** Representative images of renal cortex showing no significant morphologic abnormalities in control HO-2(+/+) (A) and HO-2 (−/−) (B) mice, despite slightly elevated plasma creatinine in the latter. Magnification, ×50 (periodic acid-Schiff [PAS] stain).

**Figure 3.** Representative images of renal tissue in STZ-treated mice. HO-2 (+/+) mice (A, D, and G) show no significant morphologic abnormalities; HO-2 (−/−) mice display widespread tubular damage (B, E, and H), including loss of brush borders in outer medullary proximal tubules (some denoted with *; E) and microvascular thickening and hyalinosis (B, arrows). Tubular and microvascular (C, arrows) pathology is entirely abrogated in STZ+cobalt protoporphyrin (CoPP)-treated HO-2 (−/−) mice. Magnification, ×50 (PAS stain).
with acute tubular necrosis of the toxic type. In addition, small foci of tubulointerstitial scarring were accompanied by foci with small aggregates of lymphoplasmacytic infiltrates affecting approximately 5% of the cortical interstitium predominantly in the juxtamedullary location. Approximately 5% of the tubules and Bowman’s capsules displayed segmental thickening commensurate with early interstitial scarring (Figure 4B). STZ-treated HO-2 (−/−) mice also revealed microvascular pathology. Approximately 40% of the sampled arterioles were markedly thickened with focally nodular hyaline-type deposits associated with myocytic degeneration and focal loss (Figure 4E). A few smaller arteries revealed mild acellular intimal thickening. Glomerular capillary tufts, however, showed no significant abnormalities (Figure 3B). CoPP treatment entirely abrogated the STZ-induced tubulointerstitial and microvascular pathology in HO-2 (−/−) mice (Figures 3, C, F, and I, and 4, C and F).

Effect of Heme on the Morphologic Changes
HO-2 (+/+) mice that received injections of heme showed no significant abnormalities except that approximately 50% of the cortical proximal tubules contained scattered, granular intracytoplasmic deposits of iron-positive material, consistent with heme. There was no demonstrable associated tubulointerstitial scarring (Figure 5, A and C). HO-2 (−/−) mice that received injections of heme showed early mild tubular atrophy and focal coarse vacuolation in the proximal tubular cells (Figure 5B). The iron staining of the cortical proximal tubules was more intense and was seen in approximately 65% of the tubules (Figure 5D) as compared with HO-2 (+/+) mice that received injections of heme. Medullary tubules contained occasional small hyaline casts, and small foci of tubulointerstitial scarring were noted close to the corticomedullary junction.

Effect of Hyperglycemia on Tissue Levels HO-1 and HO-2 Proteins
We assessed the basal levels of HO-1 and HO-2 proteins in whole-kidney homogenates from nondiabetic and diabetic HO-2 (+/+) and HO-2 (−/−) mice that were treated weekly with CoPP, heme, or SnMP. HO-1 and HO-2 proteins were measured 3 d after the last injection of CoPP, SnMP, or heme. Figure 6 shows representative Western blots depicting HO-1 and HO-2 expression in control and diabetic mice. In STZ-treated HO-2 (−/−) mice, levels of HO-1 protein were undetectable (Figure 6A, top). STZ treatment had no effect on HO-2 protein levels in HO-2 (+/+) (Figure 6B, bottom). In both HO-2 (+/+) and HO-2 (−/−) mice that were treated with STZ, both inducers and inhibitors of HO had an activating effect on HO-2

Figure 4. Representative images of renal tissue in STZ-treated mice. HO-2 (+/+) mice (A and D) show no significant abnormalities; HO-2 (−/−) mice display focally prominent tubular basement membrane thickening and interstitial fibroblastic proliferation (B, bottom left), and microvascular (∗) nodular hyalinosis (arrows) and focal myocytic degeneration (arrowhead). Tubulointerstitial (I) and microvascular (∗) pathology is entirely abrogated in STZ + CoPP-treated HO-2 (−/−) mice. Magnification, ×100 (PAS stain).

Figure 5. Representative images of renal cortex in mice that received heme injections. HO-2 (+/+) mice show no significant abnormalities on histology (A) and mild intracytoplasmic accumulation of iron pigment in the proximal tubules (C); HO-2 (−/−) mice display focal coarse vacuolation (B, middle top), and excessive iron deposits in the tubules. Magnification, ×100 (PAS stain in A and B, Prussian blue stain in C and D).

Figure 6. Western blot analysis of HO-1 and HO-2 proteins in kidney homogenates of HO-2 (+/+) (A) and HO-2 (−/−) mice (B). Control or diabetic mice received injections of heme (1 mg/100 g), or CoPP (0.5 mg/100 g), or stannous mesoporphyrin (SnMP; 2 mg/100 g) once a week for 4 weeks. Immunoblots were performed using antibodies against mice and HO-1 and HO-2 proteins. Blots are representative of five separate experiments.
gene expression (Figure 6). In STZ-HO-2 (+/+) mice, CoPP, heme, and SnMP caused a similar pattern of increase in HO-1, markedly inducing the levels of HO-1 protein, suggesting that HO-1 gene response to the inducers is not impaired in HO-2 (−/−) mice. During the course of the study, approximately 30% of HO-2 (−/−) mice died after heme administration, but no deaths occurred in the HO-2 (+/+) mice, suggesting that heme was a potent cytotoxic agent in HO-2–deficient mice.

Assessment of Renal HO Activity and Superoxide Production

To define further the effect of hyperglycemia on renal HO-1, we measured HO activity in control and hyperglycemic mice after administration of the HO inducers CoPP and heme or the HO inhibitor SnMP. As seen in Figure 7A, HO activity was 0.467 ± 0.17 nmol bilirubin/mg per 30 min in control mice compared with 0.24 ± 0.09 nmol bilirubin/mg per 30 min in STZ-treated mice (n = 5; P < 0.05). Treatment with heme or CoPP significantly increased HO activity in STZ-treated HO-2 (+/+) mice. Heme was a weaker inducer of HO activity compared with CoPP (Figure 7A). CoPP treatment increased HO activity in both HO-2 (+/+) and HO-2 (−/−) hyperglycemic mice by approximately four- to five-fold compared with hyperglycemic controls that were not treated with CoPP or heme (Figure 7A). Weekly administration of SnMP for 6 wk inhibited HO activity by 50 to 60% (Figure 7A), despite that it increased HO-1 protein levels (Figures 6A and 4B, bottom). This is due to the effect of SnMP on activation of the HO-1 gene, owing to structural similarities with heme and binding at the same binding site (1,30).

Because HO-2 deficiency resulted in decreased HO activity, we evaluated the possible role of O$_2^-$ in mediating this effect. Figure 7 demonstrates that STZ treatment increased O$_2^-$ production in both HO-2 (−/−) and HO-2 (+/+) mice; however, hyperglycemia led to a greater increase in the level of O$_2^-$ formation in HO-2 (−/−) mice compared with hyperglycemic HO-2 (+/+) mice. In contrast, the increase in HO-1 proteins and HO activity, as a result of CoPP administration, was closely associated with a decrease in O$_2^-$ production in both groups of mice. However, increase in HO-1 proteins but decrease in HO-1 activity as seen in rats that were treated with SnMP magnified O$_2^-$ production in both groups (Figure 7).

Effect of HO-2 Deficiency on Renal Function in STZ-Treated Mice

As seen in Figure 8, there was a significant increase in plasma creatinine in control HO-2 (−/−) mice compared with control HO-2 (+/+) mice before the administration of STZ (P < 0.05). Plasma creatinine in HO-2 (+/+) mice was 0.26 ± 0.06 versus 0.43 ± 0.057 in HO-2 (−/−) mice. After STZ treatment, plasma creatinine levels were significantly increased in both HO-2 (+/+) and HO-2 (−/−) mice compared with control, the increment being most pronounced in HO-2 (−/−) mice. CoPP administration prevented an increase in plasma creatinine both
in HO-2 (+/+) and in HO-2 (−/−) STZ-treated mice (P < 0.05). In contrast, inhibition of HO activity using SnMP increased plasma creatinine levels in both groups.

Discussion
In this study, we have demonstrated that HO-2 is essential in preserving normal renal function and morphology. Its deficiency causes major renal morphologic injury and impairs renal function in diabetic mice. Several important findings support this conclusion.

The first was that kidneys that were obtained from HO-2 (−/−) mice showed a significant increase in plasma creatinine as compared with HO-2 (+/+) mice. STZ-treated diabetic HO-2 (−/−) mice not only revealed a further rise in plasma creatinine but also displayed marked damage on renal morphology that was not evident in STZ-induced diabetes in HO-2 (+/+) mice. The latter, however, displayed a mild, albeit significant, rise in plasma creatinine levels. In mouse models of diabetes, no specific histopathology criteria associated with diabetic nephropathy have been formulated. For example, thickening of the glomerular basement membrane has been reported in several but not all mouse models (33). Furthermore, nodular glomerular sclerosis generally is absent in mice as is arteriolar hyalinosis, which are two features that classically are seen only in humans with advanced diabetic nephropathy (34). Moreover, Breyer et al. (35) reported that administration of STZ to mice caused glomerular hypertrophy. However, in humans, the best histopathologic correlate of renal function in patients with diabetic nephropathy is the fractional volume of the mesangium, which is an index of mesangial expansion (34,36). It therefore is not surprising that these human-type morphologic changes were not seen in glomeruli of mice with STZ-induced diabetes of short duration. However, there were marked changes in tubules in the diabetic mice that were deficient in the HO-2 gene, similar to those reported when the HO-1 gene was deficient in human studies (37,38). Therefore, we postulate that the HO-2 deletion is responsible for the impairment in renal functional and histologic alterations that were observed and amplified as a result of STZ-induced diabetes in HO-2 (−/−) mice. Recently, Tay et al. (18) also reported STZ-induced renal toxicity with acute tubular necrosis in the murine model of diabetic nephropathy. Consonant with our findings in the wild type, renal functional impairment without evidence of histologic damage was noted even at very low doses of STZ (75 mg/kg) by these authors. Our findings suggest that the diabetes-induced renal impairment is markedly accentuated even at a lower dosage in HO-2 (−/−) mice and became morphologically manifested as acute tubular necrosis.

An additional crucial finding is that HO activity is low in renal tissues of HO-2 (−/−) mice, and this may have contributed to the abnormal renal function without STZ treatment, albeit without any significant morphologic abnormalities. In agreement with this result, Chen and Regan (39) showed that HO-1 levels were normal in astrocytes of HO-2 knockout mice and reported that supplementation with heme potentiated cell injury in these HO-2 (−/−) mice. Our results also indicate that renal injury can be exaggerated by heme administration in HO-2 (−/−) mice. It is known that with the increase in HO activity, there is a parallel increase in ferritin that sequesters the released iron (40). Heme administration in both HO-2 (−/−) and HO-2 (+/+) leads to accumulation of intracytoplasmic iron in proximal cortical tubules, but the accumulations are more abundant in HO-2 (−/−) mice and are associated with mild early tubulointerstitial scarring and associated inflammation. These findings indicate that HO-2 may be involved in eliminating heme from the renal tubules and thus abrogating iron-induced tubular damage. This effect is in agreement with results that have been described by others. Nath et al. (24,41,42) showed that a decrease in HO activity predisposed rats to marked tubular interstitial inflammation and mortality after repetitive dosing with hemoglobin. It is also in agreement with those who have studied HO-2 deficiency in the heart and lung (43). A lowered HO activity in HO-2 (−/−) compared with HO-2 (+/+) mice therefore would diminish ferritin synthesis and increase tissue iron deposits. Increased iron deposits in the proximal tubules with slightly morphologic tubular injury was seen in our own heme-injected HO-2 (−/−) compared with HO-2 (+/+) mice. This could be corrected with iron-dependent oxidative stress, which has been shown to be a major instigator of renal damage (41,44). Excessive heme-iron accumulated in the tissues may contribute toward increased death of STZ-treated HO-2 (−/−) mice. No tissue evaluation of iron deposit accumulation was done in the STZ-treated animals as 30% of the STZ-treated HO-2 (−/−) mice that received injections of heme died, compared with HO-2 (+/+) mice, further supporting the cytoprotective importance of the constitutive HO-2 and the rescue by inducing HO-1.

Our study, in addition, suggests that HO-2 deficiency sensitizes renal tissues to STZ-mediated injury. Elevation in plasma creatinine levels was more pronounced in diabetic HO-2 (−/−) compared with wild type HO-2 (+/+) mice. HO-2 (−/−) mice showed reduced HO activity, whereas inducing HO-1 with CoPP prevented renal tissue damage and further rise in plasma creatinine. In contrast, inhibition of HO activity by SnMP magnified the diabetes-induced plasma creatinine elevations. On the basis of our data, it is clear that HO-2 deficiency per se contributes to renal dysfunction. It is known that STZ alone may be an instigator of the renal tissue injury manifested by an increase in plasma creatinine and tubular injury and expression of Ig antigen (18,45).

Despite a slight increase in plasma creatinine, HO-2 (+/+) mice that were treated with STZ showed no significant glomerular, vascular, or tubular abnormalities. In contrast, HO-2 (−/−) mice revealed changes of widespread toxic tubular damage with the same dosage of STZ, suggesting that HO-2 deficiency renders tubules more vulnerable to toxic insult. Small foci of interstitial inflammation and tubulointerstitial scarring, which possibly are reactive and secondary to toxic tubular damage, were noted in HO-2 (−/−) mice. Furthermore, our morphologic findings indicate that myocytes in microvessels are also more prone to STZ-induced injury in mice with HO-2 deficiency, leading to focally prominent arteriolar hyalinosis. Under the conditions of these experiments, no classic diabetes-specific glomerular changes were noted in wild-type or HO-2 (−/−) mice.
Another important finding is that preconditioning or inducing HO-1 expression, via a weekly injection of CoPP, prevents severe diabetes-mediated damage to the kidney in HO-2 (−/−) mice. The vulnerability of HO-2 (−/−) mice may be due to the sensitivity of the renal tubules and microvessels to the various inflammatory molecules that are associated with the increase in renal heme, and the latter may be related to lack of HO-1 induction in HO-2 (−/−) mice. Upregulation of HO-1 has been shown to diminish formation of vascular heme and O$_2^−$, increase extracellular superoxide dismutase in diabetic rats (30,46), and increase reduced glutathione (47). Acute and chronic nephropathies also are related to the elevation of cellular heme protein, such as that seen in sickle cell anemia and hemolysis (44,48). Furthermore, an increase in cellular heme results in elevation of gp91 (NOX4)-mediated superoxide formation (49).

There are several mechanisms by which upregulation of HO-1 compensates for HO-2 deficiency. Upregulation of HO-1 inhibits the inflammatory response, presumably by a decrease in cellular heme-mediated oxidative stress (50) and vasoconstrictors (46,51–55). Consistent with these observations, mice and humans who are deficient in HO-1 experience progressive chronic inflammation and are sensitive to stressful injury, presumably as a result of elevated cellular heme and iron (56,57). Mice that are deficient in HO-2 show impairment in oxygen sensing in the lung (58) as well as increased oxidative stress. HO may preserve renal function and tissue integrity through increased HO activity, leading to higher levels of bilirubin, an antioxidant, and CO, an antiapoptotic agent.

The benefits of increased HO activity by CoPP with respect to renal function also may be related to the production of both bilirubin and CO. HO-1 induction by CoPP decreases superoxide, plasma creatinine levels, and renal tubular and microvascular damage in HO-2 (−/−) mice. The increase in HO-1 protein but inhibition of HO activity as a result of SnMP administration leads to increased susceptibility to diabetes-mediated renal injury and supports the concept that HO-2 gene expression and its products, CO and bilirubin, are essential in preserving renal structural and functional integrity.

Acknowledgments

This work was supported by National Institutes of Health grants DK56601 and HL34300 (N.G.A.); Westchester Kidney Foundation, Inc., and Philip Morris USA, Inc.; and Philip Morris International.

We thank Jennifer Brown for excellent secretarial assistance and Suseela Gogeneni for technical assistance with histology.

References

12. Zou AP, Ma YH, Sun ZH, Ortiz-de-Montellano PR, Clark JE, Masters BS, Roman RJ: Effects of 17-octadecenoic acid,
a suicide-substrate inhibitor of cytochrome P450 fatty acid omega-hydroxylase, on renal function in rats. J Pharmacol Exp Ther 268: 474–481, 1994


Access to UpToDate on-line is available for additional clinical information at http://www.jasn.org/