Heme Oxygenase-1 Protects Rat Kidney from Ureteral Obstruction via an Antiapoptotic Pathway

Jin Hyun Kim,*Jung Il Yang,†Myeong Hee Jung,*Jeong Seok Hwa,‡Kee-Ryeong Kang,* Dong Jun Park,†Gu Seob Roh,*Gyeong Jae Cho,*Wan Sung Choi,* and Se-Ho Chang*†‡§

Departments of *Anatomy and Neurobiology, †Internal Medicine, ‡Urology, and §Biochemistry, College of Medicine, Institute of Health Sciences, Gyeongsang National University, Gyeongnam, Republic of Korea

This study examined the functional significance of heme oxygenase-1 (HO-1) expression on renal injury induced by ureteral obstruction in the rat kidney. Male Sprague-Dawley rats were divided into three groups, after which unilateral ureteral obstruction (UUO) was performed: untreated (group 1), treated with 30 mg/kg body wt hemin (group 2), and treated with 50 μg/kg body wt zinc (α) protoporphyrin η (ZnPP) and 30 mg/kg hemin (group 3). After 7 and 14 d, histologic changes and the expression of HO-1, Bcl-2, Bad, TGF-β, and cleaved caspase-3 were examined. Tubular lumens were dilated and epithelial cells were flattened on day 7 after UUO. Interstitial fibrosis and separation of the tubules were markedly increased on day 14. In contrast, the kidneys that were treated with hemin exhibited minimal interstitial fibrosis and flattening of epithelial cells on day 7 and fewer changes on day 14 than in the controls. However, treatment with ZnPP, an inhibitor of HO enzyme activity, eliminated the beneficial effect of hemin on interstitial fibrosis and tubular dilation. Increased HO-1 expression was associated with increased Bcl-2. In the ZnPP-treated rats, Bcl-2 signals were decreased compared with the hemin group. The level of proapoptotic Bad was not changed in any group. The positive cells for cleaved caspase-3 were significantly increased in renal tubular epithelial cells and tubulointerstitial cells in the obstructed rats, and hemin treatment decreased the caspase-3 activation. This study demonstrates that upregulation of HO-1 provides protection against renal injury that follows UUO. This effect is dependent on modulation of the antiapoptotic pathway by HO-1 expression.


Ubilointerstitial disease is one of the common histopathologic features of progressive renal disease of diverse cause (1–3) and strongly correlates with deterioration of renal function. Inflammation of the tubulointerstitial compartment leads to fibrosis (4,5) via excessive matrix protein production, fibroblast activation or proliferation, monocyte and macrophage infiltration, and tubular atrophy (6,7).

Kidneys with ureteral obstruction develop progressive tubulointerstitial damage. Unilateral ureteral obstruction (UUO) is a representative model of tubulointerstitial renal fibrosis that has many readily quantifiable cellular and molecular events, such as inflammation and apoptosis (8). UUO in rats and mice produces tubulointerstitial inflammation and fibrosis, which seems to mirror the human condition that is produced by obstructive nephropathy (9). In progressive obstructive nephropathy, the renal tubules dilate and tubular epithelial cells undergo apoptosis, leading to tubular atrophy. These changes may be mediated, in part, by intracellular pathways that are initiated by oxidative stress (10–12).

Oxidative stress is difficult to measure directly in vivo, and the alternative of monitoring stress-inducible genes such heme oxygenase-1 (HO-1) has found acceptance as a surrogate marker (13,14). Induction of HO-1 is an adaptive and protective response in several models, including renal ischemia-reperfusion injury (15), nephrotoxin-induced renal injury (16,17), organ transplantation (18), acute glomerulonephritis (19), rhabdomyolysis (20,21), and nonrenal settings of tissue injury (22). The evidence to support increased oxidative stress in obstructed kidneys is based on recent findings of increased expression of HO-1 mRNA in the UUO model (14). The physiologic importance of HO-1 as a beneficial response to oxidative stress has been confirmed by the phenotypic consequences of HO-1-deficient mice (23) and a patient with HO-1 deficiency (24). The mechanisms by which HO-1 confers protection against oxidative stress have not been defined exactly yet. It is believed that the byproducts that are derived from the HO reaction, biliverdin, bilirubin, iron, and carbon monoxide (CO), may mediate the beneficial effects of HO-1 (23,25–27). The increase in the levels of bilirubin and CO, which exhibit antioxidant and anti-apoptotic properties, may contribute to the cytoprotective action of HO-1 induction against oxidant injury that is brought about by obstructive nephropathy.

Although considerable advances have been made in understanding the pathogenesis of renal fibrosis as a result of UUO, few published data, either experimental or clinical, describe the early transduction events of fibrogenic signals. These pathways need further definition. Because HO-1 may play a cytoprotec-
tive role through an antiapoptotic pathway in vitro (26,28), several studies have suggested that one possible pathway by which HO-1 confers protection against oxidant injury is via its ability to impart antiapoptotic activity. This study was designed to examine whether the mitigation of renal injury in obstructive nephropathy by previous treatment with hemin, a chemical inducer of HO-1, may indeed involve an apoptotic pathway.

Materials and Methods
Reagents
Hemin chloride (H-5533) was purchased from Sigma-Aldrich Co. (St. Louis, MO), and zinc (α) protoporphyrin η (ZnP; 194988) was purchased from ICN Inc. (Costa Mesa, CA). Hemin (30 mg/kg body wt) and ZnP (25 mg/ml) were dissolved in 0.1 M NaOH, titrated to pH 7.4 with 0.1 M HCl, and diluted 1:10 with phosphate buffer (pH 7.4). ZnP was prepared in darkness and protected from light. Anti–HO-1 (SPA-895) used in immunohistochemistry and Western blot were from Stressgen Co. (Victoria, BC, Canada). Cleaved caspase-3 antibody was from Cell signalling (Beverly, MA). Antibodies against TGF-β (sc-146), Bcl-2 (sc-492), and Bad (sc-942) for immunohistochemistry were from Santa Cruz Inc. (Santa Cruz, CA).

Animals and Treatments
Adult male Sprague-Dawley rats (150 to 200 g; Samtako, Animal Breeding Center, O San, Korea) were housed in temperature-controlled conditions under a light/dark photocycle with food and water supplied ad libitum. Animal studies were conducted according to the National Institutes of Health Guide for Care and Use of Laboratory Animals. Hemin (30 mg/kg body wt) and ZnP (50 µg/kg body weight) were administered intraperitoneally with a single dose, and the same volume of saline was administered for the controls. After 48 h, UUO was performed. Animals were killed on days 7 and 14 after obstruction injury.

Surgery
Anesthetized rats were subjected to left flank incisions, and the UUO was performed by complete ligation of the left ureter at the ureteropelvic junction using double silk sutures. The right kidney was exposed, but the ureter was not ligated. For sham group, rats were subjected to left flank incisions, and the left kidney was exposed, but the ureter was not ligated. Animals were placed on regular diets, allowed free access to tap water, and killed at days 7 and 14. Five rats were used for each time point. These time points were chosen because they were shown to span the quantitative spectrum of tubular and interstitial cell apoptosis by previous studies (29).

Protein Preparation and Western Blot
For Western blot, kidneys were removed and the extracted tissues were homogenized in lysis buffer (1× PBS [pH 7.4] with 1% Triton X-100 and 1 mM EDTA) that contained 10 µM leupeptin and 200 µM PMSF. Protein concentrations were determined by Protein Assay kit (Bio-Rad, Hercules, CA) using BSA as standard. Thirty micrograms of total protein was applied to a 10 to 12% SDS–polyacrylamide gel. After electrophoresis, proteins in the gel were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), and the membrane was incubated with each antibody, incubated further with secondary antibodies, and visualized with ECL kit. Actin antibody (Sigma) was used as a loading control.

Determination of Renal HO Activity
HO activity was determined in rat kidney microsomes by bilirubin generation (30). Microsomes that were prepared by ultracentrifugation (105,000 × g, 60 min) were resuspended in 0.5 ml of 100 mM potassium phosphate buffer (pH 7.4) that contained 2 mM MgCl₂, frozen to −70°C, and thawed three times and sonicated on ice. The resulting supernatant (400 µl) was added to the reaction mixture (200 µl), which contained 2 mg of rat liver cytosol, 10 µM hemin, 0.2 mM glucose-6-phosphate, 0.2 units of glucose-6-phosphate dehydrogenase, and 0.8 mM NADPH, for 1 h at 37°C in the dark. The formed bilirubin was extracted with same volume of chloroform and was calculated by the difference in absorbance between 464 and 530 nm (extinction coefficient 40 mM/cm for bilirubin). HO activity was expressed as picomoles of bilirubin formed per milligram of protein per hour. The protein content of the microsomal fraction was determined by the method of Bradford (31).

Renal Pathology
Kidneys were routinely fixed in 4% phosphate-buffered paraformaldehyde and paraform embedded. Tissue sections at 5 µm were obtained. Paraffin wax was removed with xylene, and sections were rehydrated with ethanol. After washing, the sections were stained with hematoxylin and eosin. For analyzing the degree of tubulointerstitial collagen deposition, sections were stained with Masson trichrome. Twenty cortical tubulointerstitial fields that were randomly selected at ×400 magnification were assessed in each rat, and the density of trichrome-positive signals was analyzed by Image-Pro PLUS (Meyer Instruments, Houston, TX).

Immunohistochemical Detection
All immunohistochemical studies using the Avidin-Biotinylated-HRP complex (ABC; Vector Laboratories, Burlingame, CA) kits were performed on 5-µm-thick sections of paraformaldehyde-fixed and paraform-embedded kidney tissue. The sections were incubated with 1% normal serum and then treated successively with HO-1, Bcl-2, Bad, cleaved caspase-3, and TGF-β at 4°C for 16 h. After the sections were washed in PBS (pH 7.4), they were incubated for 90 min with secondary antibody. Finally, the sections were incubated with ABC for 60 min at room temperature, rinsed in PBS, and developed by 0.027% 3,3-diaminobenzidine tetrahydrochloride (Sigma) with 0.003% H₂O₂. The sections were counterstained with hematoxylin to visualize cell nuclei.

Statistical Analyses
Data were evaluated using one-way ANOVA and t test. P < 0.05 was considered to indicate statistical significance.

Results
Renal HO Activity and HO-1 Expression Increased by Hemin
To verify hemin-mediated induction of HO-1, we determined HO enzyme activity in rat kidney microsomes by bilirubin generation at 24 h after exposure (Figure 1A) and examined expression of HO-1 by Western blot (Figure 1B). As shown in Figure 1, pretreatment with hemin significantly increased not only HO enzyme activity but also HO-1 protein level. Surprisingly, the level of HO-1 protein also was increased with hemin and ZnP treatment (Figure 1B), whereas hemin/ZnP inhibited HO activity in kidneys (Figure 1A). It has been reported that HO-1 protein and mRNA expression are increased by ZnP treatment, although it inhibits HO enzyme activity (32-
To confirm this result, we also examined the effects of ZnPP on renal HO activity and protein level in vivo at various doses of ZnPP. The HO activity was not changed at any dose of ZnPP alone, but high dose of ZnPP blocked the hemin-induced HO activity, markedly (data not shown). This result suggests that the metalloporphyrin ZnPP used in this study is valuable for specific inhibition of HO induction.

**Hemin-Mediated HO-1 Induction Decreases Renal Injury and Interstitial Fibrosis in UUO**

To test whether induction of HO-1 could protect the cells from renal injury that was induced by UUO, we treated animals with hemin or hemin/ZnPP for 48 h and then operated on them. These time points were chosen because a pilot study showed that animals progress through the entire quantitative spectrum of tubular and interstitial cell death during this time (36). Histologic changes were determined by hematoxylin and eosin staining. The addition of HO inhibitor and hemin did not affect any apparent changes in morphology compared with control (Figure 2, A, D, and G). No changes were detected in the sham group (data not shown). In the saline-treated group, tubular lumens were significantly dilated and the epithelial cells were flattened 7 d and 14 d after UUO (Figure 2, B and C). In contrast, the kidney from animals that were treated with hemin exhibited very mild epithelial cell flattening on day 7 (Figure 2E). Induction of HO-1 considerably decreased structural renal injury as a result of obstruction (Figure 2, E and F). The interstitium was not apparent, and the tubules were similar to the saline-treated kidney on day 14 (Figure 2F).

**Figure 1.** Activity of heme oxygenase-1 (HO-1; A) and Western blot demonstrating expression of HO-1 (B) in kidneys that were treated with saline, hemin, and hemin with zinc (α) protoporphyrin η (ZnPP) in response to each treatment for 24 h. HO-1 activity was measured in kidney microsomes as described in Materials and Methods, determined as picomoles of bilirubin formed per milligram of protein per h and determined in duplicate for each rat. Values are means ± SD; n = 3 in each group. P < 0.0001.

**Figure 2.** Representative pictures of hematoxylin and eosin staining from kidneys of animals that received no treatment (A through C), HO-1 induction group with hemin (D through F), and HO-1 induction with hemin and blockade with ZnPP (G through I) at day 7 (B, E, and H in obstruction) and day 14 (C, F, and I in obstruction). (A, D, and G) Nonobstructed kidneys. HO-1 induction with hemin ameliorates (E and F) whereas blockade of the HO-1 worsens (H and I) tubulointerstitial damage after unilateral ureteral obstruction (UUO). Magnification, ×40, hematoxylin and eosin.
Hemin/ZnPP abolished the reduction of tubular dilation that was observed in the hemin-treated animals (Figure 2, H and I).

The degree of renal fibrosis was determined by Masson trichrome staining. Masson trichrome is most useful to differentiate collagen from other fibers. There was significant interstitial fibrosis that resulted in separation of the tubules at 14 d.

Masson trichrome staining of fibrosis in kidneys. Groups are animals that received no treatment (A-a, b, c), HO-1 induction group with hemin (A-d, e, f), and with hemin and blockade with ZnPP (A-g, h, i), after 7 d (A-b, e, h) and 14 d (A-c, f, i). In most of them, collagenous components are stained as blue color and cytoplasm is varying shades of red. Collagen deposits (blue) are evident within the fibrotic interstitial lesions between tubular cells (c and i) (A). Quantitative analysis of Masson trichrome- positive cells in the cortex of 7- and 14-d obstructed kidneys (B). Results are mean ± SEM. *P < 0.05, **P < 0.01. Magnification, ×200.

Figure 3. Masson trichrome staining of fibrosis in kidneys. Groups are animals that received no treatment (A-a, b, c), HO-1 induction group with hemin (A-d, e, f), and with hemin and blockade with ZnPP (A-g, h, i), after 7 d (A-b, e, h) and 14 d (A-c, f, i). In most of them, collagenous components are stained as blue color and cytoplasm is varying shades of red. Collagen deposits (blue) are evident within the fibrotic interstitial lesions between tubular cells (c and i) (A). Quantitative analysis of Masson trichrome- positive cells in the cortex of 7- and 14-d obstructed kidneys (B). Results are mean ± SEM. *P < 0.05, **P < 0.01. Magnification, ×200.

Figure 4. Immunohistochemistry for HO-1 in the renal cortex. Groups are animals that received no treatment (A, B, and C), HO-1 induction group with hemin (D, E, and F), and with hemin and blockade with ZnPP (G, H, and I), after 7 d (B, E, and H) and 14 d (C, F, and I). HO-1 immunoreactivity is increased in hemin-treated groups (E and F), and this is decreased in hemin/ZnPP groups (H and I). Arrows indicate HO-1-positive cells in tubular epithelial cells. Magnification, ×40.

Hemin/ZnPP abolished the reduction of tubular dilation that was observed in the hemin-treated animals (Figure 2, H and I).
Induction of HO-1 decreased interstitial collagen deposition on days 7 and 14 (Figure 3, A-e and A-f, and B) in comparison with saline-treated obstructed kidneys (Figure 3, A-b and A-c, and B). The suppression of tubulointerstitial fibrosis by hemin was partially blocked by the addition of ZnPP (Figure 3, A-h and A-i).

Hemin-induced HO-1 protein level in UUO and HO-1–positive cells were detected mainly in tubular epithelial cells (Figure 4, D through F, arrows). This upregulation of HO-1 was paralleled with suppression of tubulointerstitial fibrosis (Figure 3, A-e and A-f). Although positive signals for HO-1 protein still were detected in hemin/ZnPP-treated kidneys (Figure 4G), but this was less than compared with hemin alone (Figure 4D).

**HO-1 Induction Modulates Bcl-2 Expression in UUO**

Bcl-2 is known to play an antiapoptotic role in tubule damage (37). However, preliminary data regarding Bcl-2 in UUO have been contradictory (38). We sought to determine whether Bcl-2 was implicated in apoptosis in this model. To evaluate whether hemin affected the intrarenal apoptotic networks, we assessed by immunohistochemistry the expression of antiapoptotic Bcl-2 and proapoptotic Bad protein levels in rats from each group. The saline-treated group did not show positive signals (Figure 5, A through C). In contrast, in the rats that were treated with hemin alone, the positive staining for Bcl-2 was modestly increased on day 7 and markedly increased on day 14 (Figure 5, E and F). In the combined hemin/ZnPP-treated rats, they were decreased compared with the hemin group (Figure 5, H and I). Also, to examine whether increased Bcl-2 levels as a result of HO-1 induction can be detected early, we performed Western blot from obstructed kidneys. Levels of Bcl-2 protein were increased beginning at 6 h after obstruction, the same as HO-1 expression, and changes in the levels were comparable to other groups (Figure 6). This result suggests that HO-1 activation before obstruction can protect from renal damage by UUO by the induction of antiapoptotic function and occurs during early stages. In contrast, levels of proapoptotic Bad were unchanged in the hemin-pretreated group, as compared with saline controls and other groups (Figure 6).

**Increased HO-1 Level Reduces TGF-β Expression in UUO**

Although many factors are involved in the pathophysiology of UUO (8), it is well accepted that TGF-β has been implicated in the development of tubulointerstitial fibrosis in this model (8,38,39). There was prominent interstitial fibrosis as well as focal tubular atrophy in UUO in our study (Figure 3). The administration of hemin strongly decreased renal levels of TGF-β in the obstructed kidney (Figure 7, E and F), whereas combined treatment with hemin and ZnPP increased renal expression of TGF-β (Figure 7, H and I). The pattern of TGF-β

![Figure 5](image-url)
expression is consistent with the results of interstitial fibrosis shown in Figure 3. Therefore, these results provide evidence that renal fibrosis that is induced by UUO can be prevented by hemin-mediated HO-1 induction.

**HO-1 Induction Decreased Cleaved Caspase-3 Activation in UUO**

Very little tissue staining was observed in control kidneys (data not shown) by immunohistochemistry using an antibody against activated caspase-3. The number of caspase-3–positive cells significantly increased in renal tubular epithelial cells and tubulointerstitial cells from the untreated animals (Figure 8, A-a and A-b). Hemin was able to decrease the caspase-3 activation that was induced by UUO (Figure 8, A-c and A-d). As shown in Figure 8A-e and f, caspase-3 activation was increased in kidneys with combined hemin/ZnPP treatment. Positive cells for activated caspase-3 in the hemin-treated group were significantly decreased compared with saline group (Figure 8B). These results suggest that HO-1 is involved in apoptosis of renal tubular cells and tubulointerstitial cells in kidneys that undergo obstruction.

**Discussion**

The results of this study provide evidence to suggest that tubulointerstitial fibrosis and changes in renal tubular epithelial cells that are induced by UUO might be protected at least in part by HO-1 induction and that inhibition of HO enzyme activity with ZnPP reverses this protection. Administration of hemin decreased tissue TGF-β level and interstitial fibrosis and cleaved caspase-3 expression significantly. Decreased expression of activated caspase-3 was accompanied by increased Bcl-2 but not Bad expression. Furthermore, these beneficial effects in the obstructed kidney were abolished by ZnPP. These results suggest the possibility that HO-1 induction can preserve tubulointerstitial damage from the obstructed kidney.

Although the pathogenesis of tubular damage including tu-

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**Figure 6.** Western blot analysis of HO-1, Bcl-2, and Bad protein at 6, 12, 24, and 48 h after UUO. Thirty micrograms of protein was immunoblotted with each antibody. Actin was used to confirm the amount of proteins loaded in each lane.

**Figure 7.** Immunohistochemical changes of TGF-β. Groups are animals that received no treatment (A, B, and C), HO-1 induction group with hemin (D, E, and F), and with hemin and blockade with ZnPP (G, H, and I), after 7 d (B, E, and H) and 14 d (C, F, and I). Magnification, ×40.
bulointerstitial fibrosis undoubtedly is multifactorial, one of the early modulators is oxidative stress (13,14). Localized oxidative stress may be cytotoxic to renal tubular epithelial cells (40,41), resulting in increased apoptosis (42). Reduction of oxidative stress by statins has been shown to mitigate renal injury in UUO (14). Also, there are some indications that oxidative stress plays a role in TGF-β expression and in production of extracellular matrix in tubulointerstitial fibrosis (43).

Upregulation of HO-1 inhibits apoptosis both in vitro (26,28) and in vivo (17,44,45), consistent with our result that showed decreased frequency of active caspase-3–positive cells in hemin-pretreated animals as compared with controls. The cellular and physiologic mechanisms by which HO-1 exerts cytoprotective functions in UUO injury may involve expression of antiapoptotic proteins. Indeed, ZnPP treatment, an HO activity inhibitor, prevented the expression of Bcl-2 in this study. However, metalloporphyrin inhibitors have been shown to affect guanylate cyclases, nitric oxide synthase (46), and caspase-3 activity (47). Although these nonspecific effects for using this drug cannot be ruled out, various antiapoptotic proteins are induced after HO-1 gene transfer and play important roles in cell function and survival (50). That expression of HO-1 is tightly regulated in vivo and is specific to cell types strongly indicates that the enzyme might exert adverse effects on cell survival and that it has a relatively short half-life after induction.

Conclusion
Our study demonstrates that the upregulation of HO-1 in the rat provides protection against UUO-mediated renal injury. This beneficial effect depends, at least in part, on HO-1 modulation of the antiapoptotic pathway. This study documents the potential utility of HO-1–inducing agents in preventing renal dysfunction in obstructive nephropathy.

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Correction


The authors of this article wish to rectify a typographical error in the name of one of the authors of this paper. The fifth author’s name is correctly spelled as Kee Ryeon Kang. The authors regret this error.