Decreased renal heme oxygenase-1 expression contributes to decreased renal function during cirrhosis

MOTOAKI MIYAZONO, CHRYSSELLE GARAT, KENNETH G. MORRIS, JR., AND ETHAN P. CARTER
Cardiovascular-Pulmonary Research Laboratory and Departments of Medicine and Physiology and Biophysics, University of Colorado Health Sciences Center, Denver, Colorado 80262

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Miyazono, Motoaki, Chrystelle Garat, Kenneth G. Morris, Jr., and Ethan P. Carter. Decreased renal heme oxygenase-1 expression contributes to decreased renal function during cirrhosis. Am J Physiol Renal Physiol 283: F1123–F1131, 2002. First published July 24, 2002; 10.1152/ajprenal.00363.2001.—Heme oxygenase (HO) is the rate-limiting enzyme in the degradation of heme, catalyzing the oxidative cleavage of heme molecules to biliverdin, carbon monoxide, and iron. The present study was designed to investigate the role of HO-1 in the pathogenesis of renal dysfunction during cirrhosis. Biliary cirrhosis was induced in rats by common bile duct ligation (CBDL). Animals were studied 2 and 5 wk after surgery. In kidney from CBDL rats, HO-1 protein expression increased slightly at 2 wk but was abolished at 5 wk. In addition, we confirmed histologically that HO-1 expression was suppressed in renal tubules and interlobular arterioles in 5-wk-old CBDL rats. Conversely, HO-1 expression in liver was strongly increased. Consistent with the development of cirrhosis and renal dysfunction means arterial pressure (MAP), glomerular filtration rate (GFR), and renal blood flow (RBF) were decreased in CBDL rats compared with sham-operated controls. In sham rats, treatment with the selective HO inhibitor zinc protoporphyrin markedly decreased GFR and RBF to values similar to those measured in CBDL rats without decreasing MAP. In conclusion, decreased renal HO-1 expression contributes to deteriorated renal function and hemodynamics during cirrhosis. This finding provides a novel mechanism for the pathophysiology of renal dysfunction during cirrhosis.

significant renal complications frequently occur during liver cirrhosis. These complications can include an entire spectrum, from water-balance abnormalities, sodium retention, and activation of intrarenal hormones (12) to the most extreme manifestation of functional renal failure, known as hepatorenal syndrome (6).

There are two animal models of cirrhosis that are commonly used to investigate the renal and vascular complications, carbon tetrachloride (CCl4) toxicity and common bile duct ligation (CBDL). The complications that arise from the CCl4-treated model of liver cirrhosis are thought to be the result of either 1) an early decrease in peripheral vascular resistance, mostly in the splanchnic area, as the primary event that produces the hyperdynamic circulation and sodium retention; or 2) activation of a hepatorenal reflex, which induces primary renal sodium retention, followed later by development of the hyperdynamic state. The complications that arise from CBDL are generally milder than that of CCl4 toxicity and typically result in increased cardiac output, water balance abnormalities, sodium retention, and activation of intrarenal hormones (12, 23). Renal failure is a common observation in CCl4-induced cirrhosis but is much less frequent during biliary cirrhosis induced by CBDL. While there is a significant body of research using the CCl4-model, there is less information on the CBDL model, particularly the temporal events leading to vascular and renal dysfunction (23), as well as the mediators of these complications.

Nitric oxide (NO) has been shown to be central to the development of the hyperdynamic syndrome during cirrhosis (22). Levels of NO and endothelial NO synthase (eNOS) protein expression are increased in aortic and pulmonary tissue, contributing to the vasodilation in those tissues (3, 5, 22). However, in the renal circulation, the site of vasoconstriiction and reduced renal blood flow (RBF) during CBDL-induced cirrhosis (11, 12, 13, 26), it is reasonable to hypothesize that eNOS protein expression and locally produced NO are decreased. In addition, recent studies have demonstrated that while NO is central to the vascular dysfunction during cirrhosis, there is a significant NO-independent component that contributes to the vascular dysfunction (2, 3). A gene with close regulatory and functional ties to NO that could be serving as an NO-independent modulator of vascular and renal function is heme oxygenase (HO). HO-1 is a stress-response gene that has recently emerged as a potential regulator of vascular tone by its generation of CO during its enzymatic conversion of heme to bilirubin (20). The HO-2 isofrom has similar enzymatic action but is constitutively expressed in a wide variety of tissues, including the kidney (4). CO acts as a vasodilator similarly to NO by
activating guanylate cyclase, which ultimately leads to the opening of vascular smooth muscle K⁺ channels (32). HO-1 expression is regulated under a variety of conditions, including regulation by NO (2, 7). Recently, it has been reported in an in vitro model that exposure of proximal tubules to NO-generating systems induced HO-1 expression (15).

An understanding of the factors that may regulate renal function during biliary cirrhosis is important because of the frequency and severity of the complications. While the temporal events that occur after CBDL are becoming clear (14, 23), the factors that regulate these events are unknown. Using the CBDL model of renal and vascular dysfunction, our first objective was to investigate whether eNOS, HO-1, and HO-2 are implicated in renal dysfunction during CBDL-induced cirrhosis. Because we found that HO-1, HO-2, and eNOS expression in the kidney after CBDL were drastically reduced, the second objective was to begin to evaluate the functional consequences of reduced renal expression of both HO isoforms and eNOS in CBDL-induced cirrhosis.

METHODS

Animal model of liver cirrhosis. Biliary cirrhosis was induced in rats by CBDL (2, 3). The surgical procedures and experimental protocols were approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center (Denver, CO). Male Sprague-Dawley rats (body wt 200–250 g) were allowed to acclimate to Denver’s altitude (1,500 m) for 1 wk before any experimental protocols were performed. Animals had continuous access to food and water. A laparotomy was performed in animals under anesthesia with intramuscular ketamine (100 mg/kg) and xylazine (4 mg/kg). The bile duct was isolated, doubly ligated with 3-0 silk, and resected between the two ligatures. The abdomen was closed with 4-0 silk sutures. Buprenorphine (0.25 mg/kg) powder was sprinkled over the closure, and the skin was sutured with 3-0 silk, and resected between the two ligatures. The abdomen was closed with 4-0 silk sutures. Buprenorphine (0.25 mg/kg) powder was sprinkled over the closure, and the skin was sutured.

Physiological studies. In anesthetized [intramuscular ketamine (100 mg/kg) and xylazine (4 mg/kg)] sham or CBDL rats, a tracheotomy was performed and a tracheal cannula was inserted to facilitate breathing. Polyethylene catheters (Intramedic, Clay Adams, Parsippany, NJ) were placed in the right carotid artery for measuring mean arterial pressure (MAP) and withdrawing blood and in the right jugular vein for administering insulin infusions. The right carotid artery catheter was connected to a pressure transducer (UFI model 1050 or TSD 104A, Biopac Systems) for continuous MAP monitoring, and data were recorded using a Biopac MP 100 system with AcqKnowledge software (Biopac Systems). Insulin was infused through the right jugular vein catheter at a rate adjusted to give a plasma level of 50–100 µg/100 ml. After a left-flank incision to expose the left kidney, a small catheter (~0.5 mm OD) pulled from heated polyethylene tubing (PE-240) was placed into the isolated left ureter. A small diameter probe (EP102.5, lumen diameter 2.5 mm; Carolina Medical Electronics, King, NC) was placed around the left renal artery and connected to a square-wave electromagnetic flowmeter (model 501D; Carolina Medical Electronics) for measurement of RBF. After insulin infusion was started, an equilibration period of at least 60 min was allowed. Then, urine was collected for 20 min and blood samples (~150 µl each) were slowly drawn at the midpoint of the 20-min clearance period. Packed red blood cells were resuspended in saline and reinjected into the rats to maintain hematocrit. Urine volume was measured gravimetrically. The concentration of insulin in the plasma and urine was determined according to the Anthrone reaction (33). To evaluate effects of HO inhibition, some sham rats were subjected to an intraperitoneal injection of HO-1 inhibitor zinc protoporphyrin (ZnP, 50 µmol/kg, Porphyrin Products, Logan, UT) at 60 min before the start of the experiments.

Metabolic studies. Sodium and water excretion and creatine clearance were measured in rats housed in metabolic cages. Sham (n = 4) and CBDL (n = 7) rats were housed in metabolic cages for a total of 48 h. The first 24-h period was allowed for animal acclimation. During the final 24-h period, water and food intake were monitored and urine was collected for volume and sodium determinations. Plasma and
urine sodium were measured in the clinical laboratory (Beckman CX3, Beckman Instruments, Fullerton, CA). Sodium excretion was calculated by multiplying urine sodium content by daily urine production; fecal sodium was not measured.

Creatinine clearance was measured as an index of GFR and renal function. Plasma creatinine was measured in blood samples drawn from tail veins and in the urine collected from the metabolic cages. Plasma and urine creatinine concentrations were determined using a Beckman creatinine analyzer 2 (Beckman Instruments).

Measurement of blood carboxyhemoglobin. Blood carboxyhemoglobin (COHb) was used as an index for the amount of CO in the blood. Arterial blood (30–50 μl) was collected in a heparinized syringe at the beginning of the studies. Samples were measured by using an OMS3 hemoxymeter (Radiometer, Copenhagen, Denmark).

Statistical analysis. All data are reported as means ± SE. Comparisons between two groups were made with Student's unpaired t-test. Comparisons between three or more groups were made with ANOVA with Tukey's post hoc analysis. In all cases, P < 0.05 was considered significant.

RESULTS

Body weight was monitored in all rats. Before CBDL or sham surgery, average body weight was 232 ± 12 g. At the 2-wk time point, sham rats weighed 261 ± 11 g and CBDL rats weighed 252 ± 9 g. By 5 wk, sham rats weighed 348 ± 9 g and CBDL rats weighed 310 ± 17 g. Liver function was assessed by the measurement of serum bilirubin. Sham values were 0.12 ± 0.02 gm/dl, and CBDL values were significantly elevated at 12.67 ± 1.98 gm/dl and remained elevated at 5 wk.

HO-1, HO-2, and eNOS protein expression in kidney are decreased during cirrhosis. We first sought to confirm our initial hypothesis that renal eNOS expression was reduced in CBDL rats. As shown in Fig. 1A, eNOS protein expression in a total kidney homogenate was decreased to 42% in CBDL rats beginning at 2 wk. This decrease was sustained at the 5-wk time point. A striking observation was that HO-1 protein expression in kidney, while slightly increased at 2 wk, was abolished in the 5-wk CBDL kidney (Fig. 1B). Renal HO-2 protein expression was also decreased in the 5-wk CBDL rats, but to a much lesser degree than the decrease in HO-1 (Fig. 1C).

Immunohistochemistry was used to investigate the location of the changes in HO-1 expression in the kidney. In kidney from 5-wk sham rats, HO-1 was present on the apical sides of distal renal tubules (Fig. 2A) and interlobular arteriolar endothelium (Fig. 2A, inset). Conversely, after CBDL there was a marked decrease in the number of HO-1-positive tubular and vascular regions compared with in sham rats (Fig. 2B). HO-1 staining was difficult to detect in either renal tubules (Fig. 2B) or interlobular arterioles (Fig. 2B, inset) in the kidney of CBDL rats.

Fig. 1. Immunoblot analysis of endothelial nitric oxide synthase (eNOS), heme oxygenase (HO)-1, and HO-2 protein expression in kidney. Western blot analysis of eNOS (A), HO-1 (B), and HO-2 protein expression (C) in kidney from rats 2 or 5 wk after sham or common bile duct ligation (CBDL) surgery is shown. Averaged protein expression was determined by densitometric analyses and is displayed in histograms for each protein. Values are means ± SE from 5–7 rats/group (nos. on bars). *P < 0.05 vs. sham.
eNOS and HO-1 protein expression in liver are increased in cirrhotic rats. To determine whether decreased HO-1 and eNOS were unique to the kidney, we also investigated eNOS and HO-1 protein expression in the liver from sham and CBDL rats at 2 and 5 wk. In the liver, eNOS protein expression was increased by 66% (P < 0.05) and 85% (P < 0.05) at 2 and 5 wk after CBDL, respectively (Fig. 3A). Similarly, hepatic HO-1 protein expression was increased by approximately threefold (P < 0.05) at 2 and 5 wk after CBDL, respectively (Fig. 3B). These findings are in stark contrast to the declines observed in the kidney.

In sham liver, HO-1 expression was primarily in hepatic stellate cells and Ito cells, with a small amount of staining detected in vascular smooth muscle cells (Fig. 2C). Five weeks after CBDL, there was a dramatic increase in the number of HO-1-positive cells as well as in the staining intensity per cell. As seen basally, there was no hepatocyte staining nor any staining in the proliferating cholangiocytes during CBDL (Fig. 2D).

Blood COHb levels are increased during cirrhosis. Arterial blood COHb reflects the amount of CO that is produced in tissues and circulating in systemic blood. By 2 wk postsurgery, the COHb level in arterial blood was significantly increased in CBDL rats compared with sham rats. COHb levels remained elevated at the 5-wk time point (Fig. 4A). In CBDL rats, HO inhibition with ZnPP reduced COHb levels to values intermediate from sham rats (Fig. 4A), whereas in sham rats ZnPP resulted in a slight but nonsignificant reduction in COHb levels (data not shown).

MAP, GFR, and RBF are decreased in CBDL rats. To verify the development of systemic and renal complications during liver cirrhosis, MAP was monitored and GFR and RBF were measured in sham and CBDL rats. At both 2- and 5-wk time points, MAP was significantly lower in the CBDL rats compared with sham (P < 0.05) (Fig. 4B). Renal function was significantly impaired after CBDL. GFR was decreased in the CBDL rats compared with sham rats (P < 0.05) (Fig. 4C). Consistent with this decrease, RBF was also reduced in the CBDL rats (Fig. 4D).

To begin to explore the hypothesis that HO-1 mediates the cirrhosis-induced decrease in renal function, sham rats were injected intraperitoneally with the selective HO inhibitor ZnPP. Although HO inhibition with ZnPP did not alter MAP (Fig. 4B), it did result in significantly reduced GFR and slightly reduced RBF to values similar to what was observed in CBDL rats (Fig. 4, C and D, respectively).

Metabolic studies. To assess the impact that CBDL had on water and sodium metabolism, rats were housed in metabolic cages for a total of 48 h. These studies corroborated earlier findings (23) and support the development of renal dysfunction after CBDL. CBDL was associated with a trend toward decreased
body weight (Fig. 5A) and significant increases in water excretion and plasma creatinine ($P < 0.05$) (Fig. 5, E and C, respectively). As with the GFR measured using inulin clearance, GFR estimated from creatinine clearance was significantly reduced in the CBDL rats ($P < 0.05$) (Fig. 5B). As recently reported (23), CBDL was associated with reduced sodium excretion ($P < 0.05$) (Fig. 5D).

**DISCUSSION**

Renal dysfunction is a common occurrence during liver cirrhosis. Although the severity of dysfunction can vary, renal failure during liver cirrhosis is a leading cause of morbidity and mortality in cirrhotic patients. Despite elevated levels of systemically circulating NO (3, 5), decreased production of NO locally in the kidney is thought to be central to the renal vasoconstriction and decreased renal function during cirrhosis. In addition to its vasodilatory action, a secondary role for NO as a regulator of gene expression in the kidney and other tissues has recently emerged. One gene whose expression is positively regulated by NO is HO-1 (7, 15). CO produced by HO-1 has biological actions quite similar to those of NO (28), and thus it is possible that the regulation of HO-1 by NO serves as a redundant stress-response mechanism. The purposes of our studies were, first, to determine how renal HO-1, HO-2, and eNOS expression were impacted by CBDL-induced cirrhosis. Having found that renal HO-1, HO-2, and eNOS expression were markedly decreased after CBDL despite increased expression in liver, our second objective was to determine the functional consequences of these changes.

The temporal changes to systemic hemodynamics and sodium balance during CBDL-induced cirrhosis have recently been documented (23). Prominent changes in increased MAP, increased heart rate, increased water excretion, and elevated serum creatinine were seen as early as 12 days after CBDL. Other changes such as a positive sodium balance and decreased sodium excretion became evident by 16 days after CBDL. These observations are in excellent agreement with our present findings showing significant cardiovascular and renal derangements beginning as early as 2 wk after CBDL. The bases for these changes are only beginning to be unraveled and are the focus of our studies.

HO is the rate-limiting enzyme in heme catabolism, catalyzing the oxidative cleavage of heme molecules to biliverdin, CO, and iron (20). HO exists in inducible (HO-1) and constitutive (HO-2) isoforms, which are distinct gene products. Both isoforms have wide tissue distribution, including multiple sites of expression within the rat kidney (4, 34). The role of HO in renal function has only recently begun to be investigated. Although the factors that induce HO-1 are well studied and appear to operate in all tissues studied including the kidney, the functional role of HO-2 is less clear, particularly in the kidney.

We evaluated the impact that CBDL has on HO-1 and HO-2 expression. We observed that although there was a slight increase in HO-1 expression in the kidney 2 wk after CBDL, there were profound decreases in renal expression of HO-1 by 5 wk. We were surprised to observe that constitutively expressed HO-2 was decreased in the kidney at both 2 and 5 wk. These decreases were in contrast to sharp increases in hepatic HO-1 expression at both time points. eNOS expression followed a similar, tissue-specific pattern. This differential, tissue-specific expression of HO-1 and eNOS presumably has significant physiological implications. In the kidney, the decreased renal function during CBDL that we report may be attributed, at least in part, to the elimination or reduction in the vasodilatory actions of NO and HO-derived CO. It is important to consider that the fall in renal function...
occurs in the setting of elevated systemic NO (3, 5) and CO, suggesting that locally produced NO and CO in the kidney are critical for normal renal vascular and tubular function.

A recent report investigating the relative roles of HO-1 and HO-2 in renal function found that under basal conditions, HO-2 is more abundant than HO-1 in the kidney and that HO-2 is responsible for basal HO activity in the kidney (4). Despite being characterized as a constitutively expressed protein, several factors (e.g., SnCl2) can regulate HO-2 expression in the kidney (4). HO-2 expression has not been investigated during cirrhosis. The immunolocalization of HO-2 in relatively high amounts in renal arterioles may have important functional consequences for the CO regulation of RBF. This combined with our observations of reduced renal HO-2 expression in CBDL rats, as well as reduced RBF and GFR after HO inhibition with ZnPP, suggest an important role for HO in the reduced renal function during CBDL cirrhosis that we observed. However, we acknowledge that additional studies using transgenic murine models, as well as further physiological studies using inhaled or infused CO, will provide more definitive answers in addressing the role of HO in both normal and pathophysiological renal function.

Our observation that renal HO-1 expression is slightly increased in the early period after CBDL has recently been observed by another group of investigators (14). Leung et al. (14) observed that renal HO-1 expression was increased 6 days after CBDL. They demonstrated that this rise in HO-1 was a critical protective mechanism against glycerol-induced acute renal failure in these 6-day CBDL rats. If HO-1 is critical in protecting renal function, then the dramatic reduction in renal HO-1 (and HO-2) expression in the later stages of CBDL that we report may be central to the deterioration in renal function that occurs concomitantly late in CBDL-induced cirrhosis.

To investigate our second objective of defining the role of HO and HO-produced CO in normal renal physiology, we used ZnPP, the selective inhibitor of HO. The porphyrin family of compounds inhibits HO-1 and HO-2 activity with a high degree of specificity. Although the exact half-life in the circulation of ZnPP and similar porphyrins is not known, it has been reported that ZnPP (40 μmol/kg body wt) effectively inhibits increased activity of HO when administered 12 h before and after treatment with phenylhydrazine, a potent inducer of HO-1 (21). We found that the acute administration of ZnPP via intraperitoneal injection resulted in decreases in both GFR and RBF in sham rats, as well as decreased COHb in CBDL rats. The magnitude of these declines in renal function is in good agreement with the decrements that we observed during cirrhosis. This provides evidence supporting a role for the HO-1-CO pathway in regulating renal function in both health and disease. It is not surprising that ZnPP did not completely reverse GFR and RBF to CBDL values. This is an indication that other redundant regulatory pathways (e.g., NO, thromboxane, prostaglandins) are likely operative.

The vascular, vasodilatory actions of NO and CO fall under the traditional paradigm, in which they relax vascular smooth muscle by increasing intracellular cGMP, leading to activation of K+ channels and hyperpolarization (31). However, CO can also cause

Fig. 4. Measurement of carboxyhemoglobin (COHb; A), mean arterial pressure (MAP; B), glomerular filtration rate (GFR; C), and renal blood flow (RBF; D) in sham and CBDL rats at 2 and 5 wk after either sham or CBDL surgery. In A, the zinc protoporphyrin (ZnPP) groups are 2- and 5-wk CBDL rats that received the selective HO inhibitor (50 μmol/kg body wt). In B, C, and D, measurements were repeated in a separate group of sham rats after ZnPP treatment. Values are means ± SE. *P < 0.05 vs. sham.
vasodilation by cGMP-independent mechanisms. The direct activation of big conductance calcium-dependent K⁺ channels by CO constitutes an important mechanism for cGMP-independent, CO-induced vascular smooth muscle relaxation (10). The present study and earlier work by our laboratory (3) and others (16, 18) demonstrates that the combined actions of NO and CO are central to the systemic and pulmonary vasodilation and reduced MAP during cirrhosis. Conversely, decreased eNOS and HO-1 expression in the kidney, presumably resulting in reduced amounts of NO and CO available locally in the kidney, could be a critical component in the renal vasoconstriction and reduced RBF during cirrhosis.

The reasons for the apparent contradictory observation of decreased renal HO-1, HO-2, and eNOS and renal function in the presence of elevated hepatic and pulmonary HO-1 (3) and eNOS (3, 5) and systemic NO and CO are unclear. Although we are not able to measure intrarenal levels of NO and CO, these observations suggest that NO and CO locally produced in the kidney are critical to normal renal function. Furthermore, the mechanisms behind the differential hepatic and pulmonary vs. renal HO-1 and eNOS expression are unclear. It is possible that during cirrhosis the upregulation of systemic CO resulting from increased HO-1 protein expression in the liver (29) and lung (2) may reduce HO-1 and HO-2 protein expression in the kidney due to a negative-feedback loop in an attempt to restore circulatory integrity. Clearly, more experiments are needed to definitively address this issue.

Besides the role of CO as a cGMP-dependent and -independent regulator of vasodilation, CO also activates K⁺ channels in renal tubular epithelial cells. In the thick ascending limb of Henle, HO-derived CO stimulates the apical 70-pS K channel (17), suggesting that CO is an endogenous modulator of ion transport and salt homeostasis in this segment. Although the role of CO in tubular ion transport has not been explored in the present study, it is clear that the complex relationship between not only CO and NO but also

Fig. 5. Results from metabolic cage studies. Sham (n = 4) and CBDL (n = 7) rats were housed in metabolic cages as described in METHODS. Body weight (A), creatinine clearance (B), plasma creatinine (C), sodium excretion (D), and urine production (E) were measured. Data were acquired over a 24-h period. *P < 0.05 vs. sham.
vasopressin and aquaporins in sodium retention and water reabsorption during liver cirrhosis needs further investigation.

HO-1 plays a protective role in mitigating tissue injury by virtue of its antioxidant actions. Oxidants are well known to cause localized vasoconstriction and to disrupt tubular function (1). Recent studies demonstrate that HO-1 is central to the prevention of renal failure after renal ischemia (30) or glycerol-induced (9, 14, 24) acute renal injury in rats. Taken together, these studies demonstrate that both the antioxidant actions of HO-1 and the production of CO act to preserve RBF. Cholestasis after CBDL causes an imbalance in the oxidant-antioxidant pathways in the hepatic and renal systems, leading to oxidative damage to tubular cells (25, 27) and increased lipid peroxidation in the kidney (19). These insults coincide with dramatic decreases in renal HO-1 and HO-2 expression. Therefore, despite elevated levels of circulating CO, the renal generation of oxidants during CBDL may not be adequately buffered locally. In our model of renal dysfunction during cirrhosis, decreased HO expression in the kidney of CBDL rats could result in the inability to buffer locally produced oxidants, leading to vasoconstriction and a deterioration of renal function. This CO-independent mechanism of vasoconstriction needs to be explored further.

In summary, we have shown that HO-1, HO-2, and eNOS protein expression decreased concomitantly with decreased renal function in CBDL rats. These results suggest that the decreased HO-1 and HO-2 expression in the kidney after CBDL may play an important role in the development of renal dysfunction during cirrhosis.

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