Expression and Actions of Heme Oxygenase in the Renal Medulla of Rats

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Abstract— Recent studies have shown that the heme oxygenase (HO) product, carbon monoxide (CO), induces vasodilation and that inhibition of HO produces a sustained hypertension in rats. Given the importance of renal medullary blood flow (MBF) in the long-term control of arterial blood pressure, we hypothesized that the HO/CO system may play an important role in maintaining the constancy of blood flow to the renal medulla, which in turn contributes to the antihypertensive effects of the renal medulla. To test this hypothesis, we first determined the expression of 2 isoforms of HO (HO-1 and HO-2) in the different kidney regions. By Northern blot analyses, the abundance of both isozyme mRNAs was found highest in the renal inner medulla and lowest in the renal cortex. The transcripts for HO-1 in the renal outer medulla and inner medulla were 2.5 and 3.7 times that expressed in the renal cortex and those for HO-2 in the outer medulla and inner medulla were 1.3 and 1.6 times that expressed in the renal cortex, respectively. Western blot analyses of both enzymes showed the same expression pattern in these kidney regions as the mRNAs. To determine the role that HO plays in the control of renal MBF, we examined the effect of the HO inhibitor zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG) on cortical blood flow and MBF in anesthetized rats. ZnDPBG was given by renal medullary interstitial infusion, and cortical blood flow and MBF were measured by laser Doppler flowmetry. Renal medullary interstitial infusion of ZnDPBG at a dose of 60 nmol/kg per minute produced a 31% decrease in MBF over a period of 60 minutes as measured by laser Doppler flow signal (0.62±0.02 vs 0.43±0.04 V in control vs ZnDPBG). With the use of an in vivo microdialysis technique, ZnDPBG was found to significantly reduce renal medullary cGMP concentrations when infused into the renal medullary interstitial space. These results suggest that both HO-1 and HO-2 are highly expressed in the renal medulla, that HO and its products play an important role in maintaining the constancy of blood flow to the renal medulla, and that cGMP may mediate the vasodilator effect of HO products in the renal medullary circulation. (Hypertension. 2000;35[part 2]:342-347.)

Key Words: oxygen ■ blood pressure ■ kidney ■ hemodynamics

Heme oxygenase (HO) is a rate-limiting enzyme involved in the cellular degradation of heme. In the presence of oxygen and nicotinamide adenine dinucleotide phosphate (NADPH), HO converts heme into biliverdin and carbon monoxide (CO), simultaneously releasing iron. Recent studies have indicated that HO is a ubiquitous protein and primarily exists in 2 different isoforms that are the products of different genes. HO-1 is inducible and well known as heat shock protein 32, which is sensitive to a variety of stimuli and agents that cause oxidative stress and pathological conditions such as heat shock, ischemia, radiation, hypoxia, and cellular transformation. HO-2 is constitutively expressed and is not inducible by the factors that induce HO-1, except adrenal glucocorticoids. Although another gene similar to HO-2 has been cloned and named as HO-3, the product of this gene displayed very little heme-degrading activity. The transcripts and proteins of HO, especially HO-1 and HO-2, have been detected in a variety of mammalian tissues. There is general agreement that the catalytic activity of HO in cellular heme homeostasis and its products possess beneficial or protective effects on cell injury in response to various stress or stimuli. All products of heme by HO have been reported to be biologically active. CO is an activator of soluble guanylate cyclase and relaxes vascular smooth muscle by means of a cGMP-dependent or cGMP-independent mechanism. Iron released by HO activity regulates gene expression, and bilirubin formed by conversion of biliverdin by a reductase is a potent antioxidant. Recent studies have demonstrated that the HO/CO system participates in the control of arterial blood pressure. Systemic inhibition of HO elicited peripheral vasoconstriction and produced a sustained hypertension in rats. It has been proposed that the hypertensive effects of HO inhibition are associated with either blunting of vasodilation and/or a reduction of the depressor effect of the central nervous system mediated by endogenous CO. There is
substantial evidence that endogenous CO produced by HO activity plays an important role in the control of vascular tone.1,6–8 HO-2 has been found to be expressed constitutively in arterial smooth muscle and endothelial cells, and HO-1 is induced at high levels in the heart and blood vessels.12–14 HO displays great activity in blood vessels because the substrate heme is readily available.15,16 Inhibition of HO increases vascular tone and blocks vasodilation induced by heme.8 All these data support the view that the vascular HO/CO system may contribute to the regulation of peripheral vascular resistance and control of arterial blood pressure.

Given the central role of the kidney and in particular the renal medullary blood flow (MBF) in the long-term control of arterial blood pressure,17,18 the present studies aimed to determine whether the HO/CO system is involved in the regulation of renal MBF and contributes to a long-term antihypertensive effect of the renal medulla. Previous studies demonstrated that induction of renal HO by different pharmacological interventions significantly blocked the development of hypertension in spontaneously hypertensive rats. This antihypertensive effect of HO induction is attributed to a decrease in P450 metabolites of arachidonic acid, 19- or 20-HETE.19,20 We have shown that 20-HETE produced renal vasconstriction and blockade of 20-HETE production increased renal MBF.21 Therefore it appears that the antihypertensive effect of HO inhibition may be due to blunting the vasoconstrictor action of 20-HETE in the renal medullary circulation. Taken together, the data suggest that the HO/CO system may mediate a mechanism important for maintaining the constancy of renal MBF and is consequently of importance in the long-term control of arterial blood pressure. To test this hypothesis, the present study was designed first, to characterize the expression of the HO catalytic isoforms HO-1 and HO-2 in the renal medulla with the use of Northern blot and Western blot analyses, and second, to examine the direct effect of HO inhibition on renal MBF by renal medullary interstitial infusion of zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG) and laser Doppler flowmetry. Third, because CO has been reported to stimulate guanylate cyclase activity, the effect of HO inhibition on renal medullary cGMP concentrations was also determined with the use of microdialysis and enzyme immunoassay techniques.

Methods

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction

To extract total RNA, male Sprague-Dawley rats (weight 250 to 280 g, Harlan Sprague-Dawley Inc) were anesthetized with sodium pentobarbital, and the kidneys were removed. The renal cortex, outer medulla, and papilla were dissected at 4°C. The dissected renal cortex, outer medulla, or inner medulla (100 mg) was transferred into individual tubes containing 1 mL TRIzol reagent (Life Technologies), then incubated at room temperature for 5 minutes. Total RNA was extracted, precipitated, and washed according to the protocol described by the manufacturer. The resultant RNA was resuspended in 50 μL of RNase-free water. The HO-1 and HO-2 cDNAs from rat kidney were cloned by reverse transcription polymerase chain reaction (RT-PCR) with primer pairs designed and synthesized based on the sequences of rat HO-1 and HO-2 cDNAs in GeneBank (accession number M12129 for HO-1 and J05405 for HO-2).2,3 The First-Stand cDNA Synthesis Kit (Amersham Pharmacia) was used to generate single-strand cDNA by RT, which was then used as a template for PCR with the primers for HO-1: 5'-GCTTATGCCCGCTTACCTC-3' (sense), position 4458 to 4478 and 5'-GTCTTAGCTCTTCTGACC-3' (antisense), position 4835 to 4855; for HO-2: 5'-GAATTCCGGAAAGGACG-ACAT-3' (sense), position 294 to 311 and 5'-CTTAGACTAT-GTAATACGCTCAAGAA-3' (antisense), position 1105 to 1122. The PCR products were fractionated in 1.5% agarose gels, excised, and extracted with the use of the Qiagen Gel Extraction Kit. The resulting cDNA (396 bp for HO-1 and 828 bp for HO-2) was cloned into pCR2.1-TOPO vector with the use of the TOPO TA cloning system (Invitrogen) and sequenced to confirm the identity of cDNA with an autossequencer by McConnell Inc. The plasmid DNA was digested with Eco I, purified, and used as a cDNA probe for Northern blot analysis.

Northern Blot Analysis

Northern blot analysis of HO mRNA was performed as described previously.22 Total RNA of 15 μg was fractionated on a 1.0% formaldehyde-agarose gel, stained with ethidium bromide (0.5 g/mL), washed, photographed, and transferred onto a Qiagen nylon membrane. Hybridization was carried out using the gene Rapidhyb (Amersham Pharmacia) with [32P]dCTP-labeled probes, namely plasmid DNA fragment containing HO-1 and HO-2 insert. The membrane was then exposed to Fuji x-ray film (Fisher) at ~80°C for 1 to 4 days. Molecular size of the HO-1 and HO-2 transcripts was determined by comparison to 18 S and 28 S rRNA.

Preparation of the Homogenate From Renal Tissues

The dissected cortical, outer medullary, and papillary tissues were homogenized with a glass homogenator in ice-cold HEPES buffer containing (in mmol/L): Na-HEPES, 25; EDTA, 1; and phenylmethylsulfonyl fluoride, 0.1. After centrifugation of the homogenate at 6000 g for 5 minutes at 4°C, the supernatant containing membrane and cytosolic components, termed homogenate, was aliquoted, frozen in liquid nitrogen, and stored at −80°C until used.

Western Blotting

Western blotting was performed as we described previously.23 Forty micrograms of protein of the homogenate was subjected to SDS-PAGE and transferred onto nitrocellulose membrane. Then, the membrane was washed and probed with 1:1000 specific polyclonal anti-HO-1 or anti-HO-2 antibodies (StressGen) and subsequently with 1:10000 horseradish peroxidase–labeled donkey anti-rabbit IgG. Finally, 10 mL of enhanced chemiluminescence detection solution (Amersham Pharmacia) was added, and the membrane was wrapped and exposed to Kodak Omat film. Each membrane was stripped of bound antibodies and reprobed with an anti-β-actin antibody to check the amount of loaded protein on each lane. Protein concentration of the tissue homogenate was measured with the use of a Bio-Rad protein assay kit according to the procedures described by the manufacturer.

Animal Preparation for Renal Medullary Flowmetry

Male Sprague-Dawley rats weighing between 250 and 300 g were fasted overnight but allowed free access to water. They were anesthetized with ketamine (30 mg/kg body wt IM) and inactin (50 mg/kg body wt IP) and placed on a thermostatically controlled warming table to maintain body temperature at 37°C. After tracheotomy, canulas were placed in the right femoral vein and artery for intravenous infusions and measurements of arterial pressure. An abdominal incision was made, and the left kidney was placed in a stainless steel cup to stabilize the organ for implantation of optical fibers to measure cortical and medullary blood flows or for implantation of microdialysis probes to dialyze cGMP from the renal interstitium as previously described.24–26 For renal medullary interstitial infusion of drugs, a 3-channel dialysis probe that contained an
infusion inlet was implanted into the renal medulla. This dialysis probe (Bioanalytical Systems) was constructed with an inlet and outlet channel for perfusion of the microdialysis fluid as described below. After implantations, 50 mmol/L of PBS was infused continuously at a rate of 0.5 mL/h to maintain the patency of interstitial infusion. The animals received an intravenous infusion of 2% BSA in a 0.9% sodium chloride solution, at a rate of 1 mL/100 g per hour throughout the experiment to replace fluid losses and maintain a stable hematocrit of \( \sim 43 \pm 3\% \).

### Laser Doppler Flowmetry of Cortical and Medullary Blood Flows

Experiments were performed in 7 rats to evaluate the effects of renal medullary interstitial infusion of the HO inhibitor ZnDPBG on renal cortical blood flow (CBF) and MBF. The rats were anesthetized and surgically prepared as described above. Laser Doppler flowmeters (model P3, PERIMED) were used to simultaneously determine the changes in CBF and MBF. For measurement of changes in regional blood flows, we constructed 1 optical fiber (diameter 500 \( \mu \)m) that was implanted in the renal cortex (1.5-mm depth) and another that was in the inner medulla (5-mm depth) in the left kidney, as described previously.\(^ {24,25} \)

After surgery and a 60-minute equilibration period, continuous measurements of mean arterial pressure, CBF, and MBF were obtained throughout the experiment with the use of a digital on-line monitoring system. Saline was infused into the renal medullary interstitium for two 20-minute control periods. At the end of the second control period, ZnDPBG at a dose of 60 nmol/kg per minute was infused into the renal medullary interstitial space for 60 minutes, and arterial pressure and blood flows were recorded continuously. The dose chosen for the present study was based on our preliminary experiments in which ZnDPBG, when infused into the renal medullary interstitium, did not produce acute systemic hemodynamic changes, thereby minimizing the influence of systemic hemodynamic change on MBF. ZnDPBG was first dissolved in 50 mmol/L of \( \text{Na}_2 \text{CO}_3 \) solution and then diluted into 50 mmol/L of PBS (pH 7.4). In preliminary experiments, we also confirmed that intravenous infusion of this dose of ZnDPBG had no effect on systemic hemodynamic changes, thereby minimizing the influence of systemic hemodynamic changes in CBF and MBF. For measurement of changes in regional blood flows, we constructed 1 optical fiber (diameter 500 \( \mu \)m) that was implanted in the renal cortex (1.5-mm depth) and another that was in the inner medulla (5-mm depth) in the left kidney, as described previously.\(^ {24,25} \)

### In vivo Microdialysis and Enzymatic Immunoassay of cGMP

In vivo microdialysis studies of cGMP in the renal medulla were performed as described previously.\(^ {24–28} \) Briefly, the rats were anesthetized and surgically prepared as described above. The microdialysis probes had a 0.5-mm tip diameter and a 20-kDa transmembrane diffusion cutoff (Bioanalytical Systems). A probe was inserted into the renal medulla (5.5 mm in depth) and perfused at a rate of 2 \( \mu \)L/min with 50 mmol/L PBS containing 205 mmol/L of NaCl, with an osmolarity of 500 mOsm/L. After a 1.5-hour equilibration period, dialysate fluid was collected at 20-minute intervals for a 40-minute control measurement period with the medullary interstitial infusion of PBS. In one group of rats \(( n = 7)\), ZnDPBG was then infused at a dose of 60 nmol/kg per minute. After 30 minutes, two 20-minute dialysate samples were collected. In another group of rats, PBS instead of ZnDPBG was infused into the renal medulla for 1 hour, and two 20-minute dialysate samples were collected. The quantitative determination of cGMP concentrations in the dialysate was performed with a cGMP assay kit (R&D Systems), according to the manufacturer’s protocol. All protocols listed above were approved by the Animal Care Committee of Medical College of Wisconsin.

### Statistical Analysis

Data are presented as mean±SEM. Significance of difference in mean values within and between multiple groups was examined with an ANOVA for repeated measures followed by a Duncan’s post hoc test (SigmaStat, SPSS Inc). A value of \( P<0.05 \) was considered statistically significant.

### Results

#### HO-1 mRNA and Protein Levels in the Renal Cortex and Medulla

The results of Northern blot and Western blot analyses of HO-1 are presented in Figure 1. Panel A shows a typical autoradiographic document of the membrane carrying renal cortical and medullary RNAs probed with the HO-1 cDNA probe. The abundance of a 1.8-kb HO-1 transcript was detected highest in the renal inner medulla and lowest in the renal cortex. The same amount of loaded RNA on each lane was documented by staining 28S rRNA in the gel with ethidium bromide before transferring RNA onto a nylon membrane. Panel B presents a typical gel document of the immunoreactive blots on the nitrocellulose membrane carrying renal cortical and medullary protein probed with a specific antibody against HO-1. A 32-kDa protein was detected by the Western blotting. Panel C summarizes the results of these HO-1 mRNA and protein analyses. The blot intensity of HO-1 mRNA and protein was significantly higher in the renal outer medulla and inner medulla compared with that in the renal cortex. The HO-1 transcripts in the outer medulla and inner medulla \(( n = 8 \) rats) were 2.5 and 3.7 times
that expressed in the renal cortex, respectively. The HO-1 protein levels in the outer medulla and inner medulla (n = 11 rats) were 1.7 and 2 times that detected in the renal cortex.

**HO-2 mRNA and Protein Levels in the Renal Cortex and Medulla**

The results of Northern blot and Western blot analyses of HO-2 are presented in Figure 2. Panel A shows a typical autoradiographic document of the membrane probed with the HO-2 cDNA probe. The abundance of a 1.3-kb HO-2 transcript was detected. In some gels, a light band that represented 1.9 kb HO-2 transcripts could also be seen. Panel B presents a typical gel document of the immunoreactive blots on the nitrocellulose membrane probed with a specific antibody against HO-2. A 36-kDa protein was detected and represented HO-2. Similar to the expression pattern of HO-1, the renal inner medulla exhibited the greatest expression of both HO-2 mRNA and proteins. Panel C summarizes the results of these experiments. The blot intensities showing the mRNA and protein levels of HO-2 were significantly higher in the outer medulla and inner medulla than in the renal cortex. The HO-2 transcripts in the outer medulla and inner medulla (n = 7 rats) were 1.3 and 1.6 times that expressed in the renal cortex, respectively. The HO-2 protein levels in the outer medulla and inner medulla (n = 8 rats) were 2.3 and 3.4 times that detected in the renal cortex.

**Effect of the HO inhibitor ZnDPBG RI on CBF and MBF**

The results of these experiments are presented in Figure 3. Renal medullary infusion (RI) of the HO inhibitor ZnDPBG (60 nmol/kg per minute) produced a time-dependent decrease in renal MBF, but it had no effect on CBF (n = 7 rats). The renal medullary laser Doppler flow signal was decreased to 0.42 ± 0.04 during 60 minutes of infusion from 0.61 ± 0.04 of control, a 31% reduction. During the 1-hour postcontrol period, MBF slowly returned but remained significantly lower than control value at the end of 60 minutes. Arterial blood pressure was not altered throughout the ZnDPBG infusion period (112 ± 4 mm Hg in control vs 114 ± 3 mm Hg during ZnDPBG infusion).

**Effect of HO inhibitor ZnDPBG RI on cGMP Concentrations in Renal Medullary Dialysate**

Figure 4 presents cGMP concentrations in renal medullary dialysate during vehicle and ZnDPBG infusion. Basal cGMP concentrations in renal medullary dialysate were 1.99 ± 0.02 pmol/μL (n = 7 rats). Vehicle RI (2% 50 mmol/L Na2CO3 in PBS, pH 7.4 to 7.5) had no effect on cGMP concentrations in renal medullary dialysate (n = 5 rats). ZnDPBG RI significantly decreased cGMP concentrations in renal medullary dialysate by 50%

**Discussion**

In the present study, the expression of HO-1 and HO-2 mRNAs and proteins were detected in the renal cortex and outer and inner medulla. Previous studies by RNAase protection assay from whole kidney total RNA have shown that 2 isoforms of HO were expressed in the kidney, but these studies did not determine the HO expression in different kidney regions. The present study demonstrated that the expression of both HO-1 and HO-2 exhibited a corticomedullary gradient, with the highest levels in the renal papilla. The greatest expression of HO in the renal medulla may be of functional significance.
particular importance because this kidney region is relatively underperfused and has the lowest PO₂ levels compared with other kidney regions. High levels of HO expression and activity may protect renal medullary cells or tissues from ischemic injury or oxidant stress through CO-induced vasodilation and a bilirubin-mediated antioxidant effect.

The mechanism of increased HO expression in the renal medulla was not explored in the present study. Previous studies have demonstrated that HO expression and activity in renal tissue were significantly upregulated in acute ischemic renal failure, which suggests that hypoxia may be one of the important stimulations of HO expression or activity in the kidney. In vitro tissue or cell systems exposed to hypoxia have been shown to upregulate HO-1. Given a low PO₂ milieu in the renal medulla, increased HO expression may be associated with hypoxia-induced stimulation in this kidney region.

Recent work in our laboratory has demonstrated that a hypoxia-sensitive transcription factor, hypoxia-inducible factor-1α (HIF-1α), is highly expressed in the renal medulla. HIF-1α may mediate increased expression of renal medullary HO mRNA and consequently protein expression for HO-1, since HO-1 was found to be controlled by HIF-1α-mediated transcription. It is possible, therefore, that this hypoxia-induced transcription may upregulate HO expression and activity in the renal medulla. It remains to be determined whether hypoxia upregulates HO-2, but this possibility cannot be excluded in the renal medulla because it is subjected to a long period of low PO₂.

The most important finding of the present study is that inhibition of medullary HO by ZnDPBG significantly reduced renal MBF. These results are in agreement with recent studies in other vascular beds indicating that the vascular HO/CO system plays a role in the regulation of basal tone in the resistance vessels. Because the constancy of renal MBF plays a critical role in the long-term control of arterial blood pressure by regulating sodium excretion, the tonic vasodilator effect of the renal medullary HO/CO system may represent an important intrarenal antihypertensive mechanism. However, this conclusion cannot be drawn until the effects of chronic alterations of renal medullary HO activity and expression on arterial blood pressure have been directly elucidated. In addition, despite the relatively low abundance of HO in total tissue of the renal cortex, we cannot exclude the possibility that this enzyme system is highly expressed in different individual structures, such as the renal vasculature. Considering the large portion of the kidney that makes up the renal cortex, the total capacity to produce HO reaction products may be substantial in this kidney region. Therefore the role of the HO/CO system in the integrated control of total renal function remains to be further determined. In the present study, renal medullary interstitial infusion of ZnDPBG had no effect on CBF. The lack of measurable change in CBF indicates that there was no significant physiologic effect directly resulting from the movement of the ZnDPBG compound from the renal medulla to the cortex. This does not mean that the effect would not be observed if ZnDPBG was infused directly into the renal artery or into the cortical interstitial space.

The present study did not attempt to define whether the vascular or tubular HO/CO system participates in the control of renal MBF. Previous studies have demonstrated that both renal vessels and tubules expressed 2 isoforms of HO. It is possible that the vascular HO/CO system is importantly involved in the regulation of vascular tone in the renal medulla, as shown in other vascular beds. However, the effect of the tubular HO/CO system cannot be excluded. More recently, Liu et al. have shown that inhibition of HO by chromium mesoporphyrin reduced the apical potassium channel activity in thick ascending limbs, which suggests that CO endogenously produced from heme through an HO-dependent metabolic pathway stimulates potassium channels in this tubular segment. CO derived from these tubules also may serve as a paracrine factor to induce vasodilation of vasa recta. Furthermore, high levels of HO-1 in medullary interstitial cells may be important in the control of renal MBF.

There is a large body of evidence indicating that the HO/CO system acts through activation of soluble guanylate cyclase. To explore the role of this enzyme in mediating the action of the renal medullary HO/CO pathway, we determined changes in cGMP concentrations of medullary dialysate when the HO inhibitor ZnDPBG was infused into the renal medullary interstitial space. The results showed that renal medullary infusion of ZnDPBG significantly decreased cGMP concentrations, which suggests that the effect of HO inhibitor on MBF is probably associated with blockade of CO-induced activation of guanylate cyclase. However, since high levels of nitric oxide (NO) concentrations and synthesis by nitric oxide synthase (NOS) have been found in the renal medulla and NO also stimulates guanylate cyclase, it is also possible that the HO/CO system alters cGMP levels by interacting with NO. In this regard, there is substantial evidence suggesting that the HO/CO system possesses inhibitory effects on NOS activity or NO production by CO binding to NOS with consequent reduction in heme availability for NOS and NOS heme degradation. According to this view, HO inhibition should increase rather than decrease cGMP concentrations. Taken together, the evidence presently is consistent with the idea that the renal medullary HO/CO system activity directly stimulates guanylate cyclase, resulting in cGMP production and vasodilation of vasa recta.

Other mechanisms mediating the vasodilator effects of the HO/CO system in the renal medulla cannot be excluded. Recent studies by Kozma et al. have indicated that CO-induced vasodilation in gracilis arterioles is independent of activation of guanylate cyclase. In the presence of NADPH, heme-L-lysinate as HO substrate has been reported to increase potassium channel activity of the thick ascending limb cells in the inside-out membrane patch, suggesting the possible direct effect of HO/CO on ion channels. Moreover, inhibition by CO of production of different endogenous vasoconstrictors such as 20-HETE, endothelin, and thromboxane A₂ may also contribute to the action of the HO/CO system activity on renal MBF.

In summary, the present study demonstrated a high level of HO expression in the renal medulla. HO and its products participate in the control of basal MBF. Activation of guanylate cyclase appears to be one mechanism responsible for
the HO-mediated vasodilation in the renal medulla. Most importantly, we conclude that the HO/CO system plays an important role in the control of the renal medullary circulation.

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References

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