

## Role of Renal Medullary Heme Oxygenase in the Regulation of Pressure Natriuresis and Arterial Blood Pressure

Ningjun Li, Fan Yi, Elisabete A. dos Santos, Dustin K. Donley, Pin-Lan Li

**Abstract**—Recent studies have demonstrated that inhibition of renal medullary heme oxygenase (HO) activity and carbon monoxide (CO) significantly decreases renal medullary blood flow and sodium excretion. Given the crucial role of renal medullary blood flow in the control of pressure natriuresis, the present study was designed to determine whether renal medullary HO activity and resulting CO production participate in the regulation of pressure natriuresis and thereby the long-term control of arterial blood pressure. In anesthetized Sprague–Dawley rats, increases in renal perfusion pressure induced significant elevations of CO concentrations in the renal medulla. Renal medullary infusion of chromium mesoporphyrin (CrMP), an inhibitor of HO activity, remarkably inhibited HO activity and the renal perfusion pressure-dependent increases in CO levels in the renal medulla and significantly blunted pressure natriuresis. In conscious Sprague–Dawley rats, continuous infusion of CrMP into the renal medulla significantly increased mean arterial pressure ( $129 \pm 2.5$  mm Hg in CrMP group versus  $118 \pm 1.6$  mm Hg in vehicle group) when animals were fed a normal salt diet (1% NaCl). After rats were switched to a high-salt diet (8% NaCl) for 10 days, CrMP-treated animals exhibited further increases in mean arterial pressure compared with CrMP-treated animals that were kept on normal salt diet ( $152 \pm 4.1$  versus  $130 \pm 4.2$  mm Hg). These results suggest that renal medullary HO activity plays a crucial role in the control of pressure natriuresis and arterial blood pressure and that impairment of this HO/CO-mediated antihypertensive mechanism in the renal medulla may result in the development of hypertension. (*Hypertension*. 2007; 49:148-154.)

**Key Words:** carbon monoxide ■ sodium excretion ■ bilirubin ■ chromium mesoporphyrin  
■ nitric oxide ■ high salt

The heme oxygenase (HO) catalyzes the rate-limiting step in heme degradation, producing equimolar quantities of biliverdin (BV), iron, and carbon monoxide (CO). BV is subsequently converted to bilirubin (BR) by BV reductase.<sup>1-3</sup> It has been shown that the products of HO are involved in the regulation of cardiovascular–renal function<sup>3</sup> and that induction of HO-1 by pharmacological/genetic interventions improves vascular function and ameliorates both genetic and experimental forms of hypertension.<sup>3-5</sup> However, most studies showing the contribution of HO to the regulation of blood pressure are systemic interventions and focused on the vasculature. Given the crucial role of the kidneys in the long-term control of arterial blood pressure, it is imperative to determine whether local HOs in the kidneys participate in the regulation of the renal function and arterial blood pressure and how HOs and their products work to alter the blood pressure.

The 2 major functional isoforms of HO are the inducible (HO-1) and the constitutive (HO-2) forms. Numerous tissues,<sup>1</sup> including the kidneys,<sup>6</sup> express HOs. Previous studies have reported that both HO-1 and HO-2 are more abundantly expressed in the renal medulla than in the renal cortex.<sup>6,7</sup> It

has been demonstrated that HO participates in the regulation of renal circulation and sodium excretion.<sup>6,8-10</sup> Renal medullary infusion of HO inhibitor decreased medullary blood flow (MBF) without changing cortical blood flow.<sup>6</sup> Because the renal medullary circulation plays a key role in the regulation of sodium excretion and arterial blood pressure,<sup>11,12</sup> we hypothesized that renal medullary HO activity, through its actions of increasing MBF and sodium excretion, thereby produces an antihypertensive effect. To test this hypothesis, we first determined the effects of HO inhibition on pressure natriuresis in anesthetized Sprague–Dawley rats. Correspondingly, we analyzed HO activity using high-performance liquid chromatography (HPLC) analysis and CO production in response to elevations of renal perfusion pressure (RPP) using a microdialysis-oxyhemoglobin CO trapping technique. Then, we addressed the role of renal medullary HO activity in the long-term control of arterial blood pressure by chronic medullary infusion of an HO inhibitor. Our data suggest that the HO/CO system in the renal medulla plays an important antihypertensive role by maintenance of pressure natriuresis and reduction of salt sensitivity of arterial blood pressure.

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## Methods

### Animals

Experiments were performed on male Sprague–Dawley rats weighing between 300 and 350 g purchased from Harlan. All of the protocols were approved by the institutional animal care and use committee of Virginia Commonwealth University and Medical College of Wisconsin.

### HPLC Analysis of HO Activity

Renal tissue HO activities were determined using HPLC analysis of the conversion of heme to bilirubin by renal cortical, outer, and inner medullary tissues as described previously.<sup>13,14</sup> An expanded Methods section is available online at <http://hyper.ahajournals.org>.

### Effects of Renal Medullary Infusion of HO Inhibitor on Pressure—Natriuresis

Rats were prepared for the study of pressure natriuresis as described previously.<sup>15</sup> The left kidney was stabilized, and an interstitial catheter was placed into the renal medulla (5 mm in depth) for continuous renal medullary infusion of CrMP (10 nmol/kg per minute) or vehicle at a rate of 10  $\mu$ L/min throughout the experiment.<sup>6</sup> Renal blood flow (RBF) was measured with a transonic flow probe around the left renal artery (Transonic) as described previously.<sup>16</sup> Glomerular filtration rate was measured by a fluorescein isothiocyanate–inulin (Sigma–Aldrich) clearance rate as described previously by us and others.<sup>16,17</sup> Mean arterial pressure (MAP) and RBF were recorded, and urine samples were collected for 30 minutes at each RPP level after a 10-minute equilibration period. GFR, urine flow, and urinary Na<sup>+</sup> excretion were factored per gram of kidney weight (kw). For the details, see the expanded Methods section online.

### Assay of Renal Medullary CO Concentrations

An *in vivo* microdialysis–oxyhemoglobin CO trapping assay was established to determine renal medullary CO concentrations. CO reacts with ferrous oxyhemoglobin (OxyHb) to form carboxyhemoglobin (CO–Hb), which can be spectrophotometrically detected to measure the endogenous CO formation.<sup>9,18–21</sup> *In vivo* microdialysis experiments were performed as described in our previous studies.<sup>22,23</sup> The animals were prepared as described above for the studies of pressure natriuresis. At each RPP level, a 30-minute dialysate sample was collected for measurement of CO–Hb and calculation of CO concentration using the formulas reported previously.<sup>18,21,24</sup> For the details, see the expanded Methods section online.

### Assay of Renal Medullary NO Concentrations

An *in vivo* microdialysis–OxyHb NO trapping assay was used to determine renal medullary NO concentrations as described previously.<sup>23</sup>

### Effects of Chronic Renal Medullary Infusion of HO Inhibitor on Arterial Blood Pressure

The rats were surgically prepared for chronic renal medullary infusion and arterial blood pressure monitoring with a telemetry system. There were 3 groups including vehicle+high salt, CrMP+high salt, and CrMP+normal salt. The animals were fed with a normal salt diet for 3 days and then a high-salt diet for 10 days. For the details, see the expanded Methods section.

### Effects of High Salt Intake and Chronic Renal Medullary Infusion of HO Inhibitor on the mRNA and Protein Expressions of HO-1 and HO-2 in the Renal Medulla

After the chronic CrMP infusion described above, the expression of HO-1 and HO-2 mRNA and protein in the renal medulla were detected using real-time RT-PCR (TaqMan Gene Expression Assays kits, Applied Biosystems) and Western blot analyses using antibodies against rat HO-1 and HO-2 (Santa Cruz Biotechnology).

## Statistical Analysis

Data are presented as mean  $\pm$  1 SE. The significance of differences in mean values within and between multiple groups was evaluated using an ANOVA followed by a Duncan's multiple range test. Student *t* test was used to evaluate the statistical significance of differences between 2 groups. *P* < 0.05 was considered statistically significant.

## Results

### Effects of Renal Medullary Infusion of CrMP on HO Activity in the Kidneys

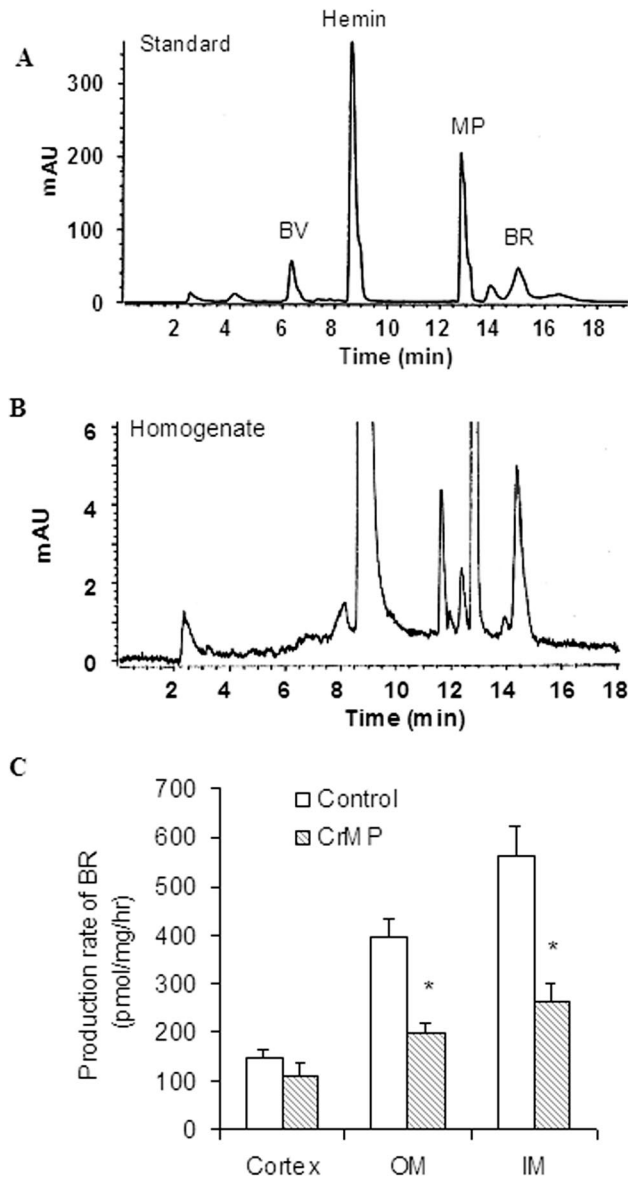
Hemin, the substrate of HO, and its products, BV and BR, and internal standard (mesoporphyrin) were clearly separated by an HPLC chromatogram when mixed in standard solution (Figure 1A). After incubation of renal tissue homogenate with hemin, the production of BR was detected (Figure 1B). HO activities, as presented by the production rates of BR, were significantly decreased by 50% in the outer medulla and inner medulla by renal medullary infusion of CrMP, whereas no significant decrease in HO activity was observed in cortical tissue homogenates (Figure 1C).

### Effects of Renal Medullary Infusion of CrMP on Pressure—Natriuresis

Acute renal medullary infusion of CrMP did not change the baseline MAP. Elevation of RPP in control rats significantly increased sodium excretion from  $2.26 \pm 0.28$  to  $13.71 \pm 1.87$   $\mu$ mol/min per gram of kw. In CrMP-treated animals, the diuretic and natriuretic responses to the elevations of RPP were blunted by 52% (from  $1.54 \pm 0.32$  to  $6.64 \pm 1.57$   $\mu$ mol/min per gram of kw; Figure 2). There was no significant difference in RBF and GFR between control and CrMP-treated rats (data not shown).

### Effects of RPP on the CO Concentrations in the Renal Medulla

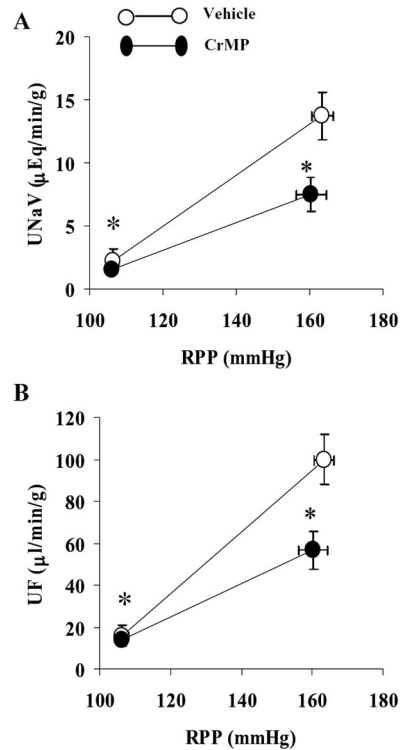
Figure 3A shows the linear correlation between the measured CO concentrations and the standard concentrations of CO-saturated solution (*r* = 0.999). The minimal detectable CO concentration was 10 nmol/L. The CO concentrations > 40  $\mu$ mol/L saturated the OxyHb at the OxyHb concentration used in the present experiment. Dialysis efficiency of CO through microdialysis probe was determined from *in vitro* dialysis experiments in which concentrations of CO in the dialysates were compared with CO concentrations in the serial dilutions of CO-saturated solution. An 86.9% recovery was observed in the effluent dialysate solution. Figure 3B presents CO concentrations in renal medullary dialysates at different RPP levels with and without renal medullary infusion of CrMP. Basal CO concentrations in the renal medulla averaged  $334 \pm 58$  nmol/L, which were significantly increased to  $873 \pm 184$  nmol/L after elevation of RPP. In the animals treated with interstitial infusion of CrMP (10 nmol/kg per minute), basal CO concentrations in renal medullary dialysates were significantly decreased by 63% ( $129 \pm 27$  nmol/L), and the increases of renal medullary CO concentration in response to the elevations of RPP were also significantly attenuated by 54% ( $399 \pm 52$  nmol/L) compared with the vehicle-treated animals.



**Figure 1.** Effects of renal medullary infusion of CrMP on HO activities in renal medullary tissue homogenates. A, Representative HPLC chromatogram of hemin, BR, BV, and mesoporphyrin ([MP] internal standard). B, Representative HPLC chromatogram of the metabolites produced after incubation of renal tissue homogenate with hemin. C, Production rate of BR by renal tissue homogenates to represent HO activity. \* $P < 0.05$  vs control (n=6).

**Effects of RPP on the NO Concentrations in the Renal Medulla**

The NO concentrations in the renal medulla were significantly increased after elevation of RPP ( $95 \pm 4.6$  to  $196 \pm 17.5$  nmol/L). In the animals treated with interstitial infusion of CrMP, basal NO concentrations in renal medullary dialysates were slightly, but significantly, decreased by 22% ( $69 \pm 6.4$  nmol/L), and the increases of renal medullary NO concentration in response to the elevations of RPP were also significantly attenuated by 31% ( $131 \pm 15.2$  nmol/L) compared with the vehicle-treated animals (Figure 4).



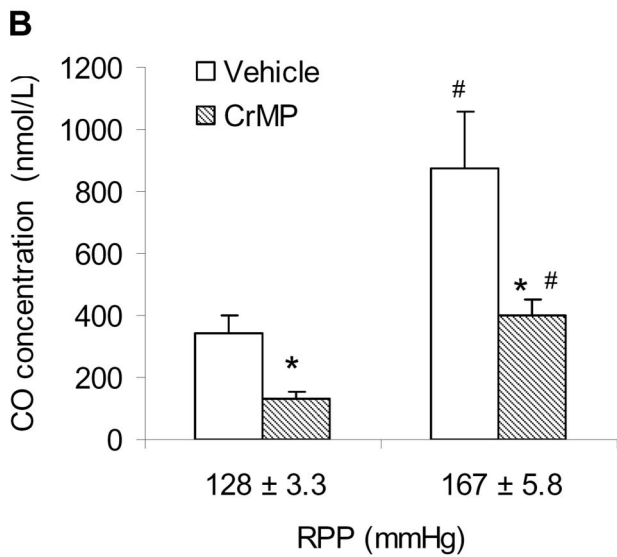
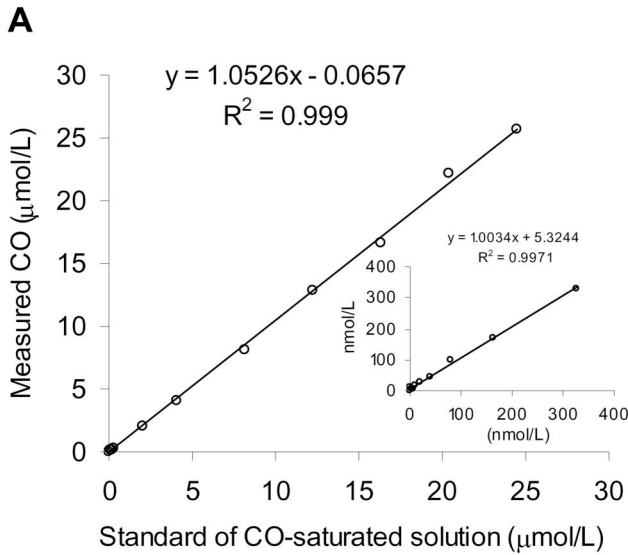
**Figure 2.** Effects of renal medullary infusion of CrMP on  $\text{Na}^+$  excretion (A) and urine flow (B) in response to elevation of RPP. \* $P < 0.05$  vs vehicle (n=8).

**Effects of High Salt Intake and Chronic Renal Medullary Infusion of HO Inhibitor on the Protein and mRNA Expressions of HO-1 and HO-2 in the Renal Medulla**

Both protein and mRNA expressions of HO-1 in the renal medulla were remarkably increased, whereas the expressions of HO-2 were not significantly changed in high-salt group compared with the normal salt group. In CrMP-treated rats, both protein and mRNA expressions of HO-1 were also significantly increased. In contrast, HO-2 expressions were considerably inhibited in CrMP-treated rats (Figure 5).

**Effects of Chronic Renal Medullary Infusion of CrMP on the Arterial Blood Pressure**

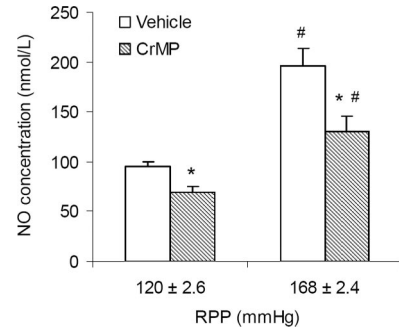
Figure 6A shows the effects of chronic medullary infusion of CrMP on MAP. There was no significant difference in baseline MAP between the vehicle and CrMP groups at the beginning of this chronic infusion protocol. Continuous infusion of CrMP into the renal medulla induced a significant increase in MAP ( $129 \pm 2.5$  mm Hg in the CrMP group versus  $118 \pm 1.6$  mm Hg in vehicle) when animals were fed a normal salt diet (1% NaCl). The MAP was further increased in CrMP-infused animals after the animals were challenged with a high-salt diet for 10 days compared with vehicle-infused animals ( $152 \pm 4.1$  versus  $130 \pm 4.2$  mm Hg). The increased MAP in the CrMP+normal salt group returned to  $124 \pm 2.9$  mm Hg 7 days later. HO activities in renal medullary tissue were significantly lower in CrMP-infused rats ( $302 \pm 42$  pmol/mg protein per hour in the high-salt group and  $258 \pm 33$  in the normal salt group) than that in vehicle-infused rats ( $590 \pm 52$  pmol/mg protein per hour; Figure 6B).



**Figure 3.** Effects of elevation of RPP and renal medullary infusion of CrMP on CO concentrations in the renal medulla. A, Standard curve of CO-saturated solution measured by spectrophotometric assay of CO-Hb. Insert, Low range of CO-saturated solution. B, Renal medullary interstitial CO concentrations in response to elevation of RPP with and without interstitial infusion of CrMP. \**P*<0.05 vs vehicle; #*P*<0.05 vs values at lower RPP (n=6).

**Discussion**

In the present study, increases in RPPs elevated CO concentrations in the renal medulla in anesthetized Sprague–Dawley rats, whereas renal medullary infusion of CrMP blocked HO activities and RPP-dependent increases in CO levels in the renal medulla, which was accompanied by a significant blunting of diuretic and natriuretic response to the elevation of RPP. The present study also demonstrated that high salt intake upregulated HO-1 expression, and chronic inhibition of renal medullary HO activity increased arterial blood pressure and salt sensitivity of MAP. These data suggest that HO in the renal medulla plays an important role in the

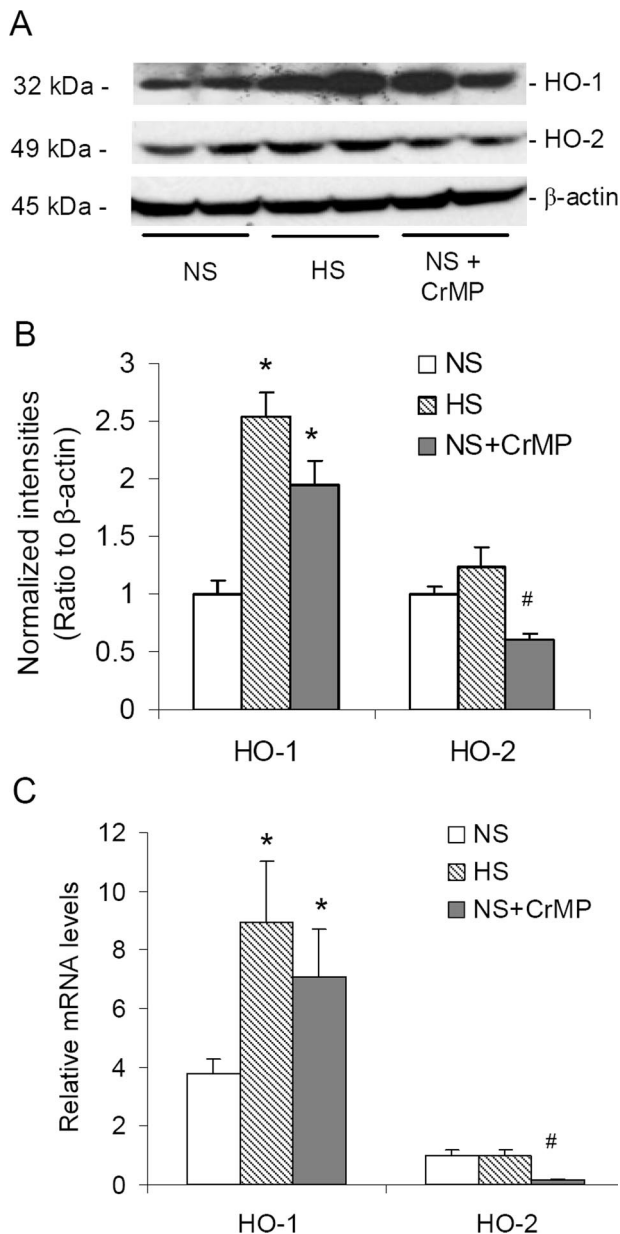


**Figure 4.** Effects of elevation of RPP and renal medullary infusion of CrMP on NO concentrations in the renal medulla. Renal medullary interstitial NO concentrations in response to elevation of RPP with and without interstitial infusion of CrMP. \**P*<0.05 vs vehicle; #*P*<0.05 vs values at lower RPP (n=6).

regulation of renal function and long-term control of blood pressure.

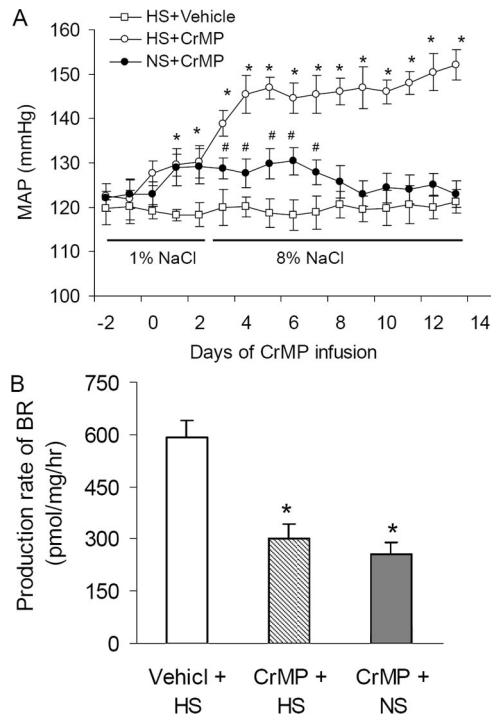
Inhibition of renal medullary HO activity has been shown to significantly reduce renal MBF.<sup>6</sup> Given the important role of MBF in the control of sodium excretion and arterial blood pressure,<sup>11,12,25</sup> the renal medullary HO/CO system, by altering MBF, may be an important determinant of pressure natriuresis and thereby the long-term control of arterial blood pressure. In support of this notion, our data demonstrated that inhibition of renal medullary HO activity remarkably blunted the pressure-natriuretic response to elevations of RPP. This alteration of pressure-natriuretic response by HO inhibition may be associated with the vasodilator actions of CO in the renal medulla and consequent increases in MBF, as shown in a previous study.<sup>6</sup> However, because the HO/CO system has also been shown to directly inhibit tubular reabsorption<sup>10</sup> and to participate in the regulation of tubular function in the loop of Henle,<sup>26</sup> we cannot rule out a possible direct action of CO on tubular sodium reabsorption. In addition, the products of HO, including CO, BV, and BR, have been indicated to affect the activities of NO synthase (NOS), cyclooxygenase, and cytochrome P450 (CYP450)<sup>27,28</sup> and minimize oxidative stress,<sup>3,29</sup> which can also mediate the indirect tubular actions of HO in the renal medulla. As shown for many other regulators of pressure natriuresis, it is believed that the HO/CO system may participate in the regulation of pressure-natriuretic responses through both medullary hemodynamic and tubular actions.

To further clarify the role of the renal medullary HO/CO system in the regulation of pressure natriuresis, we measured the CO levels in the renal medulla at different levels of RPP by spectrophotometric analyses of the formation of CO-Hb in the renal medullary microdialysates. In previous studies, this combination of in vivo microdialysis and hemoglobin-trapping techniques has been successfully used to examine NO levels in the kidneys and brain.<sup>23,30,31</sup> The adequate sensitivity of CO-Hb assay in determining CO concentrations and the high dialysis efficiency of CO as shown in the present study make this method a useful tool in monitoring the CO levels in the renal medulla. We found that the elevation of RPP significantly increased the CO concentration, and inhibition of HO activity blunted the RPP-dependent increases of CO concentration in the renal medulla, indicating that there



**Figure 5.** Effects of high salt intake and renal medullary infusion of CrMP on protein and mRNA levels of HO-1 and HO-2 in the renal medulla. A, Representative ECL gel documents of Western blot analyses depicting the protein levels of HO-1 and HO-2 in the renal medulla. B, Summarized intensities of these HO-1 and HO-2 blots normalized to  $\beta$ -actin in the renal medulla. C, Summarized relative mRNA levels of HO-1 and HO-2 in the renal medulla. The Ct values were first normalized with respect to 18S rRNA levels to obtain  $\Delta$ Ct values. The  $\Delta$ Ct value of HO-2 from the normal salt group was used as a reference to calculate  $\Delta\Delta$ Ct values for all other samples. Relative mRNA levels were expressed by the values of  $2^{-\Delta\Delta Ct}$ . HS indicates high-salt diet; NS, normal salt diet. \* $P < 0.05$  vs NS; # $P < 0.05$  vs NS and HS (n=7).

was an activation of HO activity during the elevation of RPP. These results provide direct evidence that the HO/CO system may be one of the important mechanisms that mediate or modulate pressure natriuresis. However, the present study did not attempt to explore the mechanism by which elevation of RPP activates HO activity. In this regard, it has been reported that a variety of stimuli, including shear stress and stretch,



**Figure 6.** Effects of chronic renal medullary infusion of CrMP on MAP. A, MAP in vehicle- and CrMP-treated rats. HS indicates high-salt diet; NS, normal salt diet. B, HO activities represented by the production rate of BR in renal medullary tissue homogenates. \* $P < 0.05$  vs vehicle; # $P < 0.05$  vs vehicle and HS+CrMP (n=7).

can induce HO-1 and result in elevated CO production.<sup>32,33</sup> Therefore, it is possible that an increase in RPP stimulates HO activity and consequent CO production through shear stress, stretch, pressure itself, and/or other unknown pathways. Detailed mechanisms remain to be defined in future studies.

It should be noted that the basal levels of CO in the renal medulla detected in the present study were much lower than that in a recent report, which showed that the tissue CO concentration in the kidneys was  $5 \pm 2$  pmol/mg of fresh tissue weight (corresponding to  $5 \mu\text{mol/L}$ ).<sup>34</sup> The reason for this discrepancy is probably because of the difference of measurements used in the studies. The present study measured free CO trapped by Hb, but the tissue CO measured in the other report was the total amount of CO including CO as a free form in the interstitial fluid and that bound to hemoglobin, myoglobin, cytochrome C, and other proteins. Because CO level was shown to be much higher in the blood ( $47 \pm 10$  pmol/mg),<sup>34</sup> contamination of red blood cells might also contribute to the high tissue CO concentrations. The present study measured interstitial CO in the in vivo animal preparations, which may be an optimal method to continuously monitor CO production in different experiment conditions.

The most important bioactive factor produced by HO is CO. In addition to the direct vasodilator<sup>2,3</sup> and tubular<sup>10,26</sup> effects of CO, however, CO has also been demonstrated to interact with other vascular and tubular regulators, such as the metabolites of NOS,<sup>35-37</sup> CYP450,<sup>27</sup> and cyclooxygenase<sup>28,38</sup>

to exert related vascular and tubular regulatory action. In this regard, the evidence for the interaction of HO/CO and NO is conflicting. It has been reported that low level of CO induces NO release, whereas a high level of CO suppresses NOS activity and decreases NO production.<sup>36</sup> To address whether inhibition or activation of HO in the present study would increase or decrease the NO production, we also measured the NO concentrations in the renal medulla during activation of HO by elevated RPP and the inhibition of HO by CrMP. Our results demonstrated that elevation in RPP increased NO production in the renal medulla, which is consistent with previous studies.<sup>39</sup> Inhibition of HO caused small but significant decreases in both basal and RPP-induced production of NO in the renal medulla. Because concentration ranges of CO reported to stimulate NO release are at nanomoles per liter and to inhibit NO production at micromoles per liter, the nanomoles per liter range of CO detected in the renal medulla in the present study falls into the "low" level of CO, which acts to stimulate NO release. Therefore, inhibition of HO blocked the stimulation of NO production by CO and decreased the NO concentration in the renal medulla. These data further support the view that there is an interaction between HO/CO and NO in the renal medulla and that both of them are involved in the pressure natriuresis process.

There is a concern regarding the role of CO-mediated regulation of CYP450 in the pressure natriuresis. CO inhibits the activity of CYP450 and thereby blocks the generation of vasoconstrictive substances, such as 20-HETE.<sup>3,27,40</sup> It has been shown that 20-HETE has potent natriuretic effects, and decreased 20-HETE levels are responsible for the blunted pressure natriuresis and salt-sensitive hypertension in the Dahl salt-sensitive rat.<sup>15,41</sup> Therefore, inhibition of HO in the present study could be speculated to increase the production of 20-HETE, which could result in natriuretic effects. However, 20-HETE possesses both natriuretic tubular actions and antinatriuretic vascular actions.<sup>40,41</sup> Its natriuretic tubular actions mainly target proximal tubules,<sup>15,41</sup> and inhibition of 20-HETE has been shown to increase renal MBF.<sup>40</sup> With respect to the possible increase of 20-HETE by CrMP in the present study, blunt of pressure natriuresis by renal medullary inhibition of HO could be partially attributed to the vasoconstrictor effect of 20-HETE in the renal medulla, because CrMP was administered locally.

Because of a major role of pressure natriuresis in the long-term control of arterial blood pressure and sodium balance,<sup>42</sup> the regulation of pressure natriuresis by the renal medullary HO/CO system may represent an important intrarenal antihypertensive mechanism. It was assumed that increased production of CO in response to elevations of RPP importantly contributes to the long-term control of sodium balance and arterial blood pressure. To test this hypothesis, we further examined the effects of chronic inhibition of renal medullary HO activity on MAP and the salt sensitivity of MAP. Our results showed that high salt intake significantly upregulated HO-1 expression, which may be mediated by high-salt-induced activation of hypoxia-inducible factor 1 $\alpha$  in the renal medulla.<sup>43–45</sup> It was also found that chronically continuous infusion of CrMP into the renal medulla inhibited HO activity and resulted in hypertension and increased salt

sensitivity of MAP. Interestingly, in the animals kept on normal salt diet, CrMP initially induced an increase of MAP, but the MAP returned to normal 7 days later. This is probably because of other adaptive mechanisms to compensate for the inhibitory effect of CrMP on sodium excretion. However, the compensative mechanisms could not overcome the antinatriuretic effect induced by CrMP under high-salt diet, and the MAP continuously increased, indicating that the HO/CO system is an important pathway in renal adaptation to high-salt intake. These results provide direct evidence that renal medullary HO activity possesses important antihypertensive action and thereby contributes to the long-term control of arterial blood pressure and the determination of salt sensitivity of arterial blood pressure. As discussed above, this renal medullary antihypertensive action of the HO/CO system may be attributed to its action on both vascular bed and tubules in the renal medulla.

### Perspectives

The present study showed that elevation in RPP activated HO activity in the renal medulla and that locally inhibition of HO activity in this kidney region significantly blunted natriuretic response to the elevation of RPP, and chronic inhibition of renal medullary HO activity induced hypertension and increased salt sensitivity of arterial blood pressure. These results suggest a contributing role of renal medullary HO activity to the renal adaptation to high-salt intake and the long-term control of arterial blood pressure. Therefore, impairment in the HO/CO system in the renal medulla with consequent blunting of pressure natriuresis may be one of the important renal mechanisms leading to the development of hypertension.

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### Disclosures

None.

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