HO-1 induction lowers blood pressure and superoxide production in the renal medulla of angiotensin II hypertensive mice

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Heme oxygenase-1 (HO-1) induction lowers blood pressure and superoxide production in the renal medulla of angiotensin II hypertensive mice. Am J Physiol Regul Integr Comp Physiol 292: R1472–R1478, 2007. First published December 28, 2006; doi:10.1152/ajpregu.00601.2006.—Heme oxygenase-1 (HO-1) induction can attenuate the development of angiotensin II (ANG II)-dependent hypertension. However, the mechanism by which HO-1 lowers blood pressure in this model is not clear. The goal of this study was to test the hypothesis that induction of HO-1 in the kidney can attenuate the increase in reactive oxygen species (ROS) generation in the kidney that occurs during ANG II-dependent hypertension. Mice were divided into four groups, control (Con), cobalt protoporphyrin (CoPP), ANG II, and ANG II + CoPP. CoPP treatment (50 mg/kg) was administered in a single subcutaneous injection 2 days prior to implantation of an osmotic minipump that infused ANG II at a rate of 1 μg·kg⁻¹·min⁻¹. At the end of this period, mean arterial blood pressure (MAP) averaged 93 ± 5, 90 ± 5, 146 ± 8, and 105 ± 6 mmHg in Con, CoPP-, ANG II-, and ANG II + CoPP-treated mice. To determine whether HO-1 induction resulted in a decrease in ANG II-stimulated ROS generation in the renal medulla, superoxide production was measured. Medullary superoxide production was increased by ANG II infusion and normalized in mice pretreated with CoPP. The reduction in ANG II-mediated superoxide production in the medulla with CoPP was associated with a decrease in extracellular superoxide dismutase protein but an increase in catalase protein and activity. These results suggest that reduction in superoxide and possibly hydrogen peroxide production in the renal medulla may be a potential mechanism by which induction of HO-1 with CoPP lowers blood pressure in ANG II-dependent hypertension.

Induction of HO-1 has been demonstrated to lower blood pressure in several models including: the spontaneously hypertensive rat (SHR), experimental renovascular hypertension, as well as in angiotensin-II (ANG II)-dependent hypertension (5, 26, 42). However, the mechanism by which HO-1 induction lowers blood pressure in these models has yet to be determined. There are several potential mechanisms for the blood pressure lowering effects of HO-1 induction in these models. One potential mechanism is decreased vascular resistance due to HO-1 induction in the vasculature. Several studies have demonstrated that HO-derived CO is an important regulator of both renal cortical and medullary blood flow (3, 43). HO-1 induction in the kidney could increase renal medullary blood flow shifting the pressure-natriuretic response towards the left and lowering blood pressure in these models.

HO-1 and its metabolites can also affect renal tubule cell function which may contribute to the blood pressure lowering effects of HO-1 induction. The thick ascending loop of Henle (TALH) exhibits a high level of NADPH-mediated superoxide generation (12). Superoxide generated in this segment of the nephron is an important regulator of sodium reabsorption, and excess superoxide production in the renal medulla has been linked to the development of hypertension (16, 22). The potential antioxidant role of HO-1 induction has been suggested in recent studies in which targeted overexpression of HO-1 in TALH cells is able to significantly attenuate oxidative damage induced by ANG II (24). However, the role of HO-1 to specifically reduce ANG-II-mediated superoxide production in the kidney has yet to be determined. The goal of the current study was to determine whether HO-1 induction with cobalt protoporphyrin (CoPP) could prevent the development of ANG II-dependent hypertension in mice and to determine whether induction of HO-1 with CoPP alters superoxide production in the renal medulla.

METHODS

Animals. Studies were performed on 16- to 24-wk-old male C57BL/6J mice purchased from Jackson Labs (Bar Harbor, ME). All mice were housed under standard conditions and allowed full access to food and water. HO-1 was induced by treatment with CoPP (50 mg/kg body wt sc; Frontier Scientific, Logan, UT) as previously described (1). In the first set of experiments, the mice were pretreated with CoPP or vehicle (0.1 M NaOH, pH 8.3) 2 days prior to ANG II administration. ANG II (1 μg·kg⁻¹·min⁻¹ sc) was delivered by using an osmotic minipump that was subcutaneously implanted. In the second set of experiments, mice were treated with CoPP or vehicle 5 days after implantation of the ANG II minipump. All procedures were in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center.

**Blood pressure.** Blood pressure was directly measured via microne- nthane catheters implanted into the carotid artery using aseptic surgical technique as previously described (27). Surgeries were performed 5 days after implantation of the minipumps, and the mice were allowed 2 days to recover from surgery. Mean arterial blood pressure (MAP) was recorded from conscious, freely-moving mice for 3 h per day for 5 consecutive days, and the data was averaged.

**Measurement of renal superoxide production.** Superoxide production in the renal medulla was measured by using the lucigenin technique as previously described (41). Briefly, kidneys were removed, and the medulla was homogenized (1:8 wt/vol) in RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail; Sigma, St. Louis, MO). The samples were centrifuged at 12,000 g for 20 min at 4°C. The supernatant was incubated with lucigenin at a final concentration of 5 μM and NADPH at a final concentration of 100 μM. The samples were allowed to equilibrate for 3 min in the dark, and luminescence was measured every second for 5–15 min with a luminometer (Berthold, Oak Ridge, TN). Luminescence was recorded as relative light units (RLU) per min. An assay blank with no homogenate but containing lucigenin was subtracted from the reading before transformation of the data. The amount of heme in each sample was then normalized to the total protein and expressed as nanomoles heme per mg protein.

**HO assay.** HO assay was performed on lysates prepared from the renal medulla as described above. The protein concentration was measured using a Bio-Rad protein assay with BSA standards. Reactions were carried out in a 1.2 ml containing: 2 mM glucose-6-phosphate, 0.2 units glucose-6-phosphate dehydrogenase, 0.8 mM NADP, 20 μM hemin, and 0.5 mg of lyses, as previously described (2). The reactions were incubated for 1 h at 37°C in the dark. The formed bilirubin was extracted with chloroform, and the change in optical density at 464–530 nm was measured by using an extinction coefficient of 40 mM/cm for bilirubin. HO activity was expressed as picomoles of bilirubin formed per hour per milligram of microsomal protein.

**Western blot analysis.** Western blot analysis for HO-1 protein was performed on lysates prepared from the medulla as described above. Samples of 50 μg of protein were boiled in Laemmli sample buffer for 5 min at room temperature and the organic phase was removed. The protein containing membrane was then visualized using an Odyssey infrared imager (LI-COR) which allows for the simultaneous detection of two proteins. The membranes were then incubated with Alex Fluor 680 goat anti-IgG (Rockland, Gilbertsville, PA) for 1 h at room temperature, and then incubated with rabbit anti-HO-1 antibody (Zymed, 1:5,000) overnight at 4°C. The membranes were then blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE) for 20°C overnight. Samples were then thawed and centrifuged at 13,000 g for 5 min at room temperature and the organic phase was collected. The amount of heme in each sample was then normalized to the total protein and expressed as nanomoles heme per mg protein.

**Heme content.** Heme content in the medulla was measured spectrophotometrically as previously described (14). Medullary lysates (500 μg) were added to 50 mM glycine-HCl buffer, pH 2.0, with 5 μl of 4 M HCl, 50 μl of 5 M NaCl, and 100 μl of DMSO in a volume of 1 ml. Samples were then extracted with 400 μl of chloroform and stored at −20°C overnight. Samples were then thawed and centrifuged at 13,000 g for 5 min at room temperature and the organic phase was collected. Heme content of each sample was then determined by the absorbance (A) of each sample at 388, 450, and 330 nm using the correction formula: $A_h = 2 A_{388} - (A_{450} + A_{330})$ as previously described (14). The amount of heme in each sample was then normalized to the total protein and expressed as nanomoles heme per mg protein.
Densitometry analysis was performed using Odyssey software (LI-COR). Levels of HO-1 protein are expressed as the ratio of HO-1 to \( \beta \)-actin for each sample.

To measure the protein levels of each of the three isoforms of superoxide dismutase (SOD), extracellular (EC), copper-zinc (CuZn), and manganese (Mn), (1:1,000; StressGen, Vancouver, Canada) polyclonal antibodies were used in conjunction with mouse anti-\( \beta \)-actin antibody, and the levels of each isoform were measured as described above. Catalase protein was detected by using a monoclonal antibody (1:10,000, Sigma) in conjunction with a rabbit anti-\( \beta \)-actin antibody, and the protein levels also measured as described above.

**Catalase activity.** Catalase activity was measured by using a commercially available colorimetric assay kit (Catalase Assay Kit; Sigma) according to the manufacturer’s guidelines. Catalase activity was measured in 3 g of medullary homogenate incubated in the absence of SOD for 5 min at room temperature and the reaction was stopped. Then 10 µl of the reaction was added to 1 ml of the colorimetric dye and incubated for 15 min at room temperature. The absorbance of each sample was then measured in triplicate at 520, and activity was expressed as micromoles per minute per milligram protein.

**Fig. 3.** A: representative Western blot of heme oxygenase-1 (HO-1) from renal medulla of Control, CoPP-, ANG II-, and ANG II + CoPP-pretreated mice. B: amount of HO-1 protein in the cortex and medulla normalized to \( \beta \)-actin (n = 6 each group). * Significant difference from control (\( P < 0.05 \)).

**Fig. 4.** A: HO activity in the medulla of control, ANG II-, and ANG II + CoPP-pretreated mice. * Significant difference from control (\( P < 0.05 \)). B: heme content in the medulla of control, ANG II, and ANG II + CoPP pretreated mice. * Significant difference from control (\( P < 0.05 \); † significant difference (\( P < 0.05 \)) from ANG II alone.

**Fig. 5.** Renal medullary superoxide production in control, CoPP-, ANG II-, and ANG II + CoPP-pretreated mice. Superoxide production was measured by using the lucigenin technique as outlined in METHODS. RLU, relative light unit. * Significant difference from control (\( P < 0.05 \)); † Significant difference (\( P < 0.05 \)) from ANG II alone.

**Fig. 6.** Renal medullary superoxide production in control, CoPP-, ANG II-, and ANG II + CoPP-pretreated mice. Superoxide production was measured using 2-dihydroxyethidium fluorescence as outlined in the METHODS. * Significant difference from control (\( P < 0.05 \)); † Significant difference (\( P < 0.05 \)) from ANG II alone.
**Statistics.** Values are means ± SE. Significant differences between mean values were determined using an unpaired t-test or with the use of an ANOVA followed by a post hoc test (Dunnett’s). A \( P < 0.05 \) was considered to be significant.

**RESULTS**

Pretreatment with CoPP prevents ANG II-dependent hypertension. In this set of experiments, mice were treated with one dose of CoPP (50 mg/kg body wt sc) 2 days prior to the implantation of an osmotic minipump that delivered ANG II at a rate of 1 \( \mu \text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \). MAP measured on days 7–12 postimplantation of the ANG II minipump. MAP was not different between control and CoPP-treated mice and averaged 93 ± 5 vs. 90 ± 5 mmHg in each group, respectively. MAP increased significantly after ANG II administration to 146 ± 8 mmHg and this increase in MAP was significantly attenuated by CoPP pretreatment with MAP averaging 105 ± 6 mmHg in ANG II + CoPP-treated mice (Fig. 1A). Heart rate was similar in all of the groups (Fig. 1B).

CoPP lowers blood pressure in established ANG II-dependent hypertension. In this set of experiments, mice were treated with one dose of CoPP (50 mg/kg body wt sc) 5 days after the implantation of an osmotic minipump that delivered ANG II at a rate of 1 \( \mu \text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \). Blood pressures were measured...
between days 7 and 12 postimplantation of the osmotic minipump, CoPP treatment significantly lowered blood pressure in ANG II-dependent hypertensive mice from 158 ± 4 to 114 ± 8 mmHg when measured at days 7–12 post-ANG II infusion (Fig. 2A). There was no significant difference in heart rate between the two groups (Fig. 2B).

CoPP induces HO protein, activity and decreases heme content in the medulla of ANG II hypertensive mice. The level of HO-1 protein was similar in the medulla of control and ANG II-infused mice and was significantly increased by CoPP treatment (Fig. 3). HO activity was measured in the medulla of control, ANG II, and ANG II mice pretreated with CoPP. In the medulla, HO activity was not significantly increased by ANG II infusion alone but was significantly increased with CoPP pretreatment averaging 43 ± 7 vs. 61 ± 6, vs. 141 ± 3 pmol bilirubin·mg⁻¹·h⁻¹ in control, ANG II, and ANG II + CoPP-treated mice (Fig. 4A). Heme content in the medulla was significantly increased in ANG II-infused mice compared with control and CoPP-treated mice 4.69 ± 0.41 vs. 2.62 ± 0.44 and 2.26 ± 0.79 nmolles/mg protein. Treatment with CoPP significantly reduced heme levels in the medulla of ANG II-infused mice to 2.35 ± 0.40 nmolles/mg protein (Fig. 4B).

CoPP decreases ANG II-mediated superoxide production in the medulla. Superoxide production in the medulla was measured by two independent methods: lucigenin chemiluminescence and 2-dihydroxyethidium fluorescence. CoPP treatment alone had no effect on superoxide production in the medulla. A significant increase in superoxide production in the medulla was observed by using each of these methods in ANG II-infused mice (Figs. 5 and 6). The increase in superoxide production in the medulla of ANG II-infused mice was normalized by CoPP treatment.

CoPP and antioxidant proteins in the medulla. To determine whether induction of HO-1 with CoPP was associated with any alteration in antioxidant proteins in the medulla, we measured the levels of the three isoforms of SOD and catalase in control, CoPP-, ANG II-, and ANG II + CoPP-pretreated mice. The protein levels of the mitochondrial isoform MnSOD and the intracellular isoform CuZnSOD were not different between the treatment groups (Fig. 7, A and B). EC-SOD protein was significantly increased in the medulla of ANG II-treated mice; however, pretreatment with CoPP prevented the induction of EC-SOD in ANG II + CoPP-treated mice (Fig. 7C). The levels of catalase in the medulla were lower in mice treated with ANG II and brought back to control levels in mice pretreated with CoPP (Fig. 8). Catalase activity was increased from 0.29 ± 0.05 to 0.43 ± 0.02 μmol·min⁻¹·μg protein⁻¹ in the medulla of ANG II + CoPP mice compared with mice infused with ANG II alone (Fig. 9).

DISCUSSION

Our results demonstrate the antihypertensive effects of HO-1 induction with CoPP in ANG II-dependent hypertension in the mouse. These results are in agreement with previous studies in the rat in which induction of HO-1 was able to lower blood pressure in several models, including ANG II-dependent hypertension (5, 26, 42). However, we report for the first time the lowering of blood pressure in established ANG II hypertension by HO-1 induction. Treatment with CoPP 5 days after the establishment of ANG II-dependent hypertension resulted in a ~40 mmHg decrease in MAP, which was similar to the blood pressure effect of induction of HO-1 prior to the onset of ANG II-dependent hypertension. These results are similar to those observed in a recent study in which induction of HO-1 with hemin was able to chronically lower blood pressure in SHR with established hypertension (38). These results, taken together with the results of the present study, suggest that induction of HO-1 to lower blood pressure is an area worthy of further investigation.

While the results from the current study indicate that the antihypertensive effect of HO-1 induction is reproducible in rodent models, the mechanism by which induction of HO-1 lowers blood pressure is not currently known. There are several potential mechanisms for the antihypertensive effects of HO-1 induction, which could include 1) improvement in systemic vascular relaxation via increased CO production (36); 2) in-

![Fig. 8. Representative Western blots of catalase in the medulla of control, CoPP-, ANG II-, and ANG II + CoPP-pretreated mice. *Significant difference from control (P < 0.05); †significant difference (P < 0.05) from ANG II alone.](image)

![Fig. 9. Catalase activity in the medulla of control, CoPP-, ANG II-, and ANG II + CoPP-pretreated mice.](image)
duction in the brain, specifically in the nucleus tractus solitari, which has been previously linked to changes in blood pressure (8, 13); and 3) renal vascular and/or tubular mechanisms. We decided to determine whether the antihypertensive effect of HO-1 induction in ANG II-dependent hypertension was associated with a decrease in the levels of reactive oxygen species (ROS) in the renal medulla. Recent evidence suggests that increased ROS production in the renal medulla is a key component of ANG II-dependent hypertension (25). The levels of superoxide production in the TALH are the highest in the kidney; and ANG II can increase superoxide production in the TALH by increasing the levels of NADPH oxidase (12, 18). Recent studies have also demonstrated that increased superoxide and hydrogen peroxide production, specifically in the renal medulla, can produce hypertension (15, 16). In the present study, ANG II not only increased superoxide production but also led to increases in EC-SOD and decreases in catalase, both of which are likely to result in an increase in hydrogen peroxide production in the medulla. Treatment of ANG II-infused mice with CoPP decreased superoxide generation and also normalized both the levels of EC-SOD and catalase as well as increasing catalase activity in the medulla. Thus, it is possible that blood pressure-lowering effects of CoPP could be attributed to both the reduction in superoxide anion and hydrogen peroxide mediated through changes in EC-SOD and catalase in the medulla.

The effects of CoPP on SOD and catalase protein levels in the mouse kidney are in contrast with studies conducted in diabetic rats where CoPP treatment led to an increase in the vascular levels of EC-SOD but not catalase (34). However, the levels of catalase in the kidney of the CoPP-treated diabetic rats were not measured. Previous studies have demonstrated that ANG II can increase the levels of EC-SOD in the vasculature presumably as a compensatory response to increases in superoxide production (31). While CoPP alone did not have any effect on EC-SOD levels in the medulla in the current study, it is possible that CoPP pretreatment in ANG II-infused mice prevented the compensatory increase in the levels of EC-SOD by reducing superoxide production. We did not observe any differences in the levels of MnSOD protein in the medulla between the different groups in the present study. MnSOD has previously been demonstrated to be inactivated by protein tyrosine nitration in the kidney of ANG II-treated rats due to increases in peroxynitrite (6). This mechanism of MnSOD inactivation was not investigated in the present study but could be an additional mechanism by which induction of HO-1 can reduce superoxide production in the medulla of ANG II-treated mice.

One of the major mechanisms by which induction of HO-1 may reduce superoxide production is through the generation of bilirubin, a potent antioxidant (30). Studies utilizing the hyperbilirubinemic Gunn rats, which have increased levels of unconjugated bilirubin due to the absence of UDP glucuanyl transferase, have shown that increased levels of bilirubin can prevent the pressor actions of ANG II through the scavenging of superoxide anion in the vasculature (23). Bilirubin has also been demonstrated to directly inhibit NADPH oxidase (11). Similar actions of bilirubin may be important in lowering superoxide production in CoPP-treated ANG II-hypertensive mice. Another potential mechanism by which superoxide production can be lowered in response to HO-1 induction is through the generation of CO. While CO is not traditionally thought of as an antioxidant, it can bind to the heme group on NADPH oxidase, as well as mitochondrial respiratory chain enzymes, to decrease superoxide production (32). Since equal amounts of bilirubin and CO are produced upon induction of HO-1, it is difficult to separate the effects of each metabolite individually in vivo. Specific analysis of the effect of each of these metabolites on superoxide production in vivo could be determined in future studies in which either bilirubin or a CO donor compound is infused specifically into the renal medulla of ANG II-dependent hypertensive animals.

HO-1 induction may also lower superoxide production through its effects on cellular heme levels. In the present study, ANG II infusion resulted in an increase in heme content in the medulla, which was normalized by CoPP treatment. Heme, which is a prooxidant capable of producing reactive oxygen species, could be a source of peroxynitrite and contribute to increases in protein nitrosylation (4). The increase in heme levels in the medulla may also be reflective of increases in heme-containing proteins, such as cyclooxygenase-2, which have been demonstrated to mediate ANG II-mediated superoxide production and hypertension (21, 40).

In summary, the data in the present study indicate that induction of HO-1 with CoPP either prior to or during established ANG II-dependent hypertension can lower blood pressure. This reduction in blood pressure is associated with a decrease in ANG II-stimulated ROS, superoxide, or perhaps hydrogen peroxide, production in the renal medulla. The reduction of ANG II-stimulated ROS production in the renal medulla may be due to direct effects of the metabolites of HO-1, CO, and bilirubin, or may be mediated through alterations in other important antioxidant proteins, such as superoxide dismutase and catalase. Alterations of medullary superoxide production in the renal medulla may be a novel mechanism for the antihypertensive properties of HO-1 induction by CoPP.

GRANTS

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