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Heme Oxygenase-1 Overexpression Fails to Attenuate Hypertension when the Nitric Oxide Synthase System Is Not Fully Operative

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Key Words

Antihypertensive · Aorta, blood pressure · Heme oxygenase · Heme oxygenase inhibitors · Hypertension · NG-nitro-L-arginine methyl ester · Nitric oxide synthase · Renal hypertension

Abstract

Heme oxygenase (HO) is an enzyme that is involved in numerous secondary actions. One of its products, CO, seems to have an important but unclear role in blood pressure regulation. CO exhibits a vasodilator action through the activation of soluble guanylate cyclase and the subsequent production of cyclic guanosine monophosphate (cGMP). The aim of the present study was to determine whether pathological and pharmacological HO-1 overexpression has any regulatory role on blood pressure in a renovascular model of hypertension. We examined the effect of zinc protoporyphyrin IX (ZnPP-IX) administration, an inhibitor of HO activity, on mean arterial pressure (MAP) and heart rate in sham-operated and aorta-coarcted (AC) rats and its interaction with the nitric oxide synthase (NOS) pathway. Inhibition of HO increased MAP in normotensive rats with and without hemin pretreatment but not in hypertensive rats. Pretreatment with NG-nitro-L-arginine methyl ester blocked the pressor response to ZnPP-IX, suggesting a key role of NOS in the cardiovascular action of HO inhibition. In the same way, AC rats, an experimental model of hypertension with impaired function and low expression of endothelial NOS (eNOS), did not show any cardiovascular response to inhibition or induction of HO. This finding suggests that eNOS was necessary for modulating the CO response in the hypertensive group. In conclusion, the present study suggests that HO regulates blood pressure through CO only when the NOS pathway is fully operative. In addition, chronic HO induction fails to attenuate the hypertensive stage induced by coarctation as a consequence of the impairment of the NOS pathway.

Introduction

Heme oxygenase (HO) is the rate-limiting enzyme in heme catabolism, which leads to the generation of CO, free iron and biliverdin [1]. Three mammalian HO isoforms have been identified [2], one of which, HO-1, is a stress-responsive protein that is induced by various oxidative agents and processes, including its own heme sub-

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strate [3], heavy metals [3, 4], glutathione depletion [5], UVA radiation [6], hypoxia [7], hyperoxia [7], ischemia-reperfusion [7] and hypertension [8, 9].

In blood vessels, CO produced by HO is reported to elicit relaxation of smooth muscle [10, 11] through an increase in cyclic guanosine monophosphate (cGMP) levels [12]. However, it is apparent that CO has a profound opposing effect in a dose-dependent manner. While a physiological dose of CO is a powerful vasodilator and attenuator of the phenylephrine-contracting effect [13–15], supraphysiological levels of CO-releasing molecules result in less relaxation or contraction [16]. In the same way, the moderate induction of HO-1 enhances cGMP cellular levels, but extremely high expression of HO-1 leads to a decrease in cGMP [16]. This decrease has been attributed to the stripping of heme from the soluble guanylate cyclase (sGC) protein. Moreover, although low levels of HO-1 inhibitors can reduce CO and bilirubin [16], they can also inhibit other heme proteins, including cytochrome P450 or cyclooxygenase, at high concentrations [17].

In experimental models of hypertension, such as spontaneously hypertensive rats, the administration of hemin, a pharmacological inducer of HO, decreases blood pressure by inducing and activating the HO/CO-sGC/cGMP system. These effects demonstrate the physiopathological importance of this system and open a new field for HO-1 as a possible therapeutic target to manage hypertension [18–20].

Currently, a large body of evidence supports a central role for oxidative stress in the pathogenesis of hypertension. We previously showed a redox imbalance in the aorta [9], heart [8] and kidney [21] and oxidative stress generation in aorta-coarcted (AC) hypertensive rats. As a consequence, HO-1 is overexpressed in these tissues exerting a protective response against reactive oxygen species [8, 9].

Considering these backgrounds, the aim of this work was to elucidate whether pathological or pharmacological HO-1 overexpression plays a protective role in a renovascular model of hypertension.

Methods

Reagents

Acetylcholine (Ach), NG-nitro-L-arginine methyl ester (L-NAME) and nitroglycerine were purchased from Sigma-Aldrich (St. Louis, Mo., USA), and all other chemicals were of analytical grade. Hemin and zinc protoporyphyrin IX (ZnPP-IX; Sigma-Aldrich) were dissolved in 0.1 mol/l NaOH, titrated to pH 7.4 with 0.1 mol/l HCl, and diluted 1:10 with phosphate buffer. ZnPP-IX was prepared in the dark and protected from light.

In vivo Experiments

Animals were treated in accordance with the Argentine National Institute for Health Guide for the Care and Use of Laboratory Animals. Three-month-old male Wistar rats (235–250 g) were anesthetized with ether and randomly assigned to groups that underwent either a sham operation or complete ligation of the abdominal aorta between the right and left renal arteries (AC rats), according to the method described by Rojo-Ortega and Genest [22]. Seven days after surgery, the rats were anesthetized with ether and the carotid artery was cannulated and connected to a Statham Gould P23ID pressure transducer coupled to a Grass 79D polygraph. The mean arterial pressure (MAP) was calculated according to the following formula: diastolic pressure + (systolic pressure – diastolic pressure)/3. The heart rate (HR) was estimated tachographically by counting the pulsatile waves of arterial pressure recordings.

After recovery and determination of basal MAP values for 30 min, ZnPP-IX was administered intraperitoneally (i.p.) (50 μmol/kg) in sham-operated (n = 7) or AC rats (n = 7); MAP and HR were recorded for 30 min thereafter.

To determine the role of nitric oxide synthase (NOS) in the response to HO inhibition, the blood pressure response to ZnPP-IX (50 μmol/kg, i.p.) administration was evaluated in rats pretreated for 45 min with L-NAME (75 mg/kg, i.v.) or vehicle (physiological solution, i.v.) in both experimental groups (n = 7 in each group). The indicated dose was chosen because it is specific only for the endothelial NOS (eNOS) isoform [23].

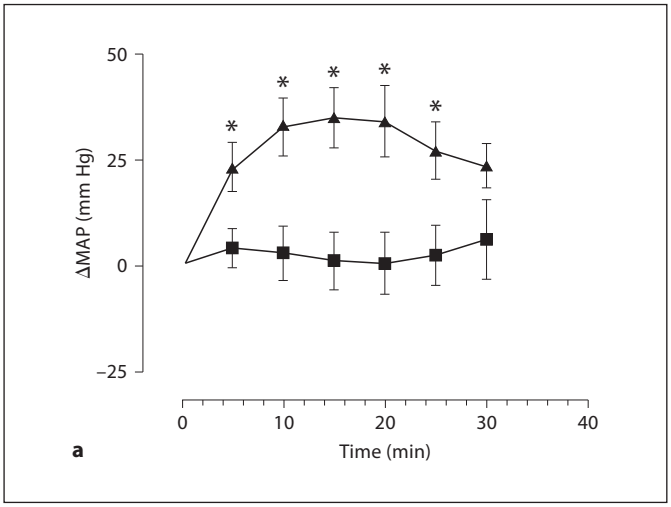
The possible role of HO-1 overexpression in blood pressure regulation was also evaluated in sham-operated and AC rats. As described in previous studies [24], hemin (ferriprotoporphyrin IX; 15 mg/kg per day) or vehicle (0.1 mol/l NaOH, titrated to pH 7.4 with 0.1 mol/l HCl and diluted 1:10 with phosphate buffer, pH 7.4) were administered to sham-operated (n = 7) and AC (n = 7) rats intraperitoneally on 4 consecutive days after aortic coarctation surgery (days 3, 4, 5 and 6). At day 7, rats were cannulated as described above, and basal MAP values were estimated after 30 min. Thereafter, ZnPP-IX was administered (50 μmol/kg, i.p.) to sham-operated or AC rats, and then the MAP and HR responses were monitored for 30 min.

In vitro Experiments

HO-1 and eNOS Expression by Western Blotting

The animals were sacrificed by cervical dislocation, and thoracic aortic segments were excised, washed with ice-cold saline solution (9 g/l NaCl) and weighed. Aortic homogenates were then prepared in a Potter-Elvehjem homogenizer using radioimmunoprecipitation assay buffer containing 50 mmol/l Tris, 150 mmol/l NaCl, 1 g/l sodium dodecyl sulfate (SDS), 5 g/l sodium deoxycholate, 10 g/l Triton X-100, 1 mmol/l phenylmethanesulfonylfluoride and Roche Complete Protease inhibitor tablets. The homogenates were then separated by centrifugation at 6,000 *g* for 20 min, and the supernatant was mixed with Laemmli 6× loading buffer and incubated at 92°C for 5 min.

Total tissue protein was separated by electrophoresis in 4–20% Tris-glycine SDS polyacrylamide gels (Mini Protean III System; BioRad Laboratories, Hercules, Calif., USA), transferred onto nitrocellulose membranes and blocked in 50 g/l dry milk in T-TBS



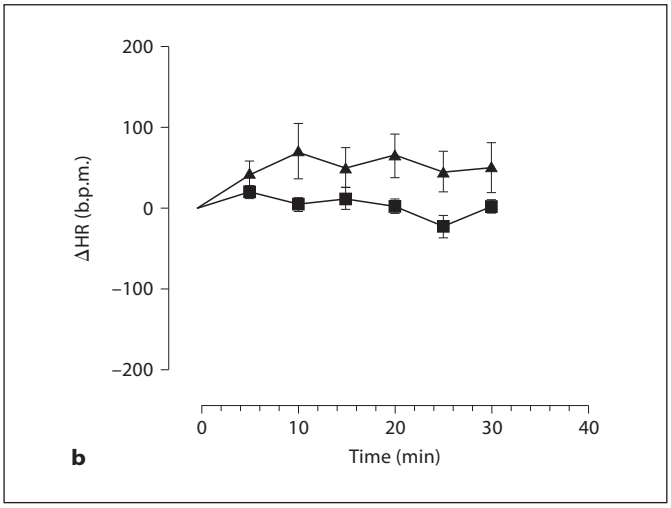


Fig. 1. Effects of the HO inhibitor ZnPP-IX, 50 μmol/kg, i.p., on changes in MAP (**a**) and HR (**b**) in sham-operated (▲) and hypertensive AC (■) rats. Changes in MAP and HR were described as the changes after intraperitoneal injection of ZnPP-IX. Values are the means ± SEM of multiple experiments (n = 7 in each group). * $p < 0.05$ versus before administration.

(0.02 mol/l Tris, 0.15 mol/l NaCl, pH 7.5, containing 1 g/l Tween 20) at room temperature for 1 h. The membranes were washed 3 times with T-TBS and incubated with the primary antibodies against eNOS and HO-1 overnight at 4°C. The polyclonal antibody against HO-1 was purchased from Abcam (Cambridge, Mass., USA), and the polyclonal antibody against eNOS was purchased from Cell Signaling Technology, Inc. (Danvers, Mass., USA). After washing 3 times with T-TBS, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit for eNOS and anti-mouse for HO-1) at room temperature for 2 h. Thereafter, the membranes were washed 5 times with T-TBS, developed using enhanced chemiluminescent reagents (Amersham Life Science, Arlington Heights, Ill., USA), and subjected to autoluminography for 1–5 min. Band intensities were quantified using the free ImageJ software (NIH). In all instances, the membranes were stained with Ponceau S stain to verify the uniformity of protein loading and transfer efficiency across the test samples. Immunoblotting with anti-β-actin (Sigma-Aldrich) was used as an internal control of protein loading. The intensity values were first normalized to β-actin and then expressed as relative protein expression, with the control lane being 1 unit.

Studies of Vascular Reactivity

Animals were sacrificed by cervical dislocation, and thoracic aortas were removed, dissected free of adventitia and cut into 3-mm ring segments in a Petri dish filled with physiological salt solution composed of (in mmol/l): 120 NaCl; 4.8 KCl; 1.2 KH_2PO_4; 1.6 $CaCl_2 \cdot 2H_2O$; 1.33 $MgSO_4$ and 10 dextrose. Thoracic aortas were mounted in an organ bath and connected to a Grass force transducer (Grass Instrument Co., Quincy, Mass., USA). The temperature of the bath was maintained at 37°C, and the bathing solution was bubbled with 95% O_2 and 5% CO_2. A tension of 2 g was applied to the thoracic aortas. The preparation was allowed to equilibrate for at least 45 min; during this step, it was washed every 15 min. Thereafter, a cumulative concentration-response curve to Ach (10^{-10}–10^{-4} mol/l) or nitroglycerin (10^{-9}–10^{-6} mol/l) was constructed after precontraction with phenylephrine (1×10^{-7} mol/l). The response was expressed as the reduction of the maximum increment in tension (precontraction) obtained by addition of phenylephrine.

The relaxation properties of Ach and nitroglycerin were assessed by estimation of the pEC_{50} and E_{max} of individual concentration-response curves using a nonlinear regression method that fit the data to a sigmoidal concentration-response curve.

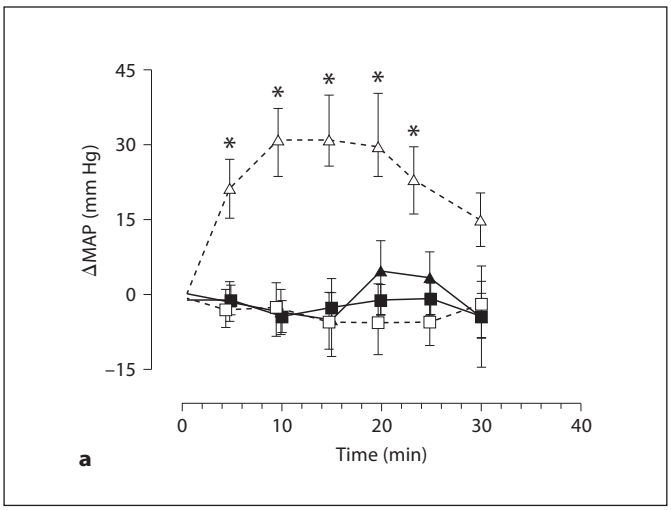
Statistics

Regression analyses and statistical tests were performed using standard software (GraphPad Prism v. 5.03 for Windows; GraphPad Software, San Diego, Calif., USA). Normal distributions of the data and variables were verified using the Kolmogorov-Smirnov test. Statistical analyses were performed using Student's t test, one-way ANOVA followed by Dunnets posttest or two-way ANOVA followed by Bonferroni posttest. The data were expressed as the mean ± SEM, and statistical significance was defined as $p < 0.05$.

Results

Effect of HO Inhibition on MAP and HR in SHAM-Operated and AC Rats

The basal levels of MAP and HR were 107 ± 13 mm Hg and 388 ± 21 b.p.m., respectively, in sham-operated rats (n = 7) and 162 ± 11 mm Hg ($p < 0.05$ vs. sham-operated rats) and 428 ± 40 b.p.m., respectively, in AC rats (n = 7). In sham-operated rats, acute inhibition of HO activity by ZnPP-IX administration increased the MAP starting at 3 min (23 mm Hg) and reached a peak (33 mm



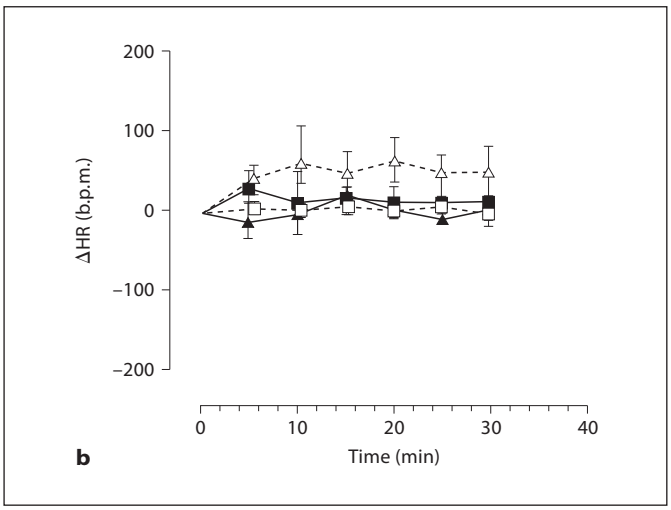


Fig. 2. Effects of the HO inhibitor ZnPP-IX, 50 μmol/kg, i.p., on changes in MAP (**a**) and HR (**b**) in sham-operated and hypertensive AC rats pretreated with L-NAME (75 mg/kg, i.v.) or vehicle (physiological solution, i.v.). Changes in MAP and HR were described as the changes intraperitoneal injection of ZnPP-IX. Values are the means ± SEM of multiple experiments (n = 7 in each group). * $p < 0.05$ versus before administration. △ = Sham-operated, vehicle-treated; ▲ = sham-operated, L-NAME-treated; □ = AC, vehicle-treated; ■ = AC, L-NAME-treated.

Hg) at 15 min (fig. 1a). Conversely, ZnPP-IX did not modify the MAP in AC rats at any time (fig. 1a). The HR was not changed by ZnPP-IX administration either in the sham-operated control group or in the AC rats (fig. 1b). Preadministration of L-NAME induced a basal pressor response in both sham-operated (148 ± 9 mm Hg) and AC (177 ± 7 mm Hg) rats without changes in HR (450 ± 37 and 407 ± 42 b.p.m. in sham-operated and AC rats, respectively). Pretreatment with L-NAME abolished the pressor response to ZnPP-IX in sham-operated rats (fig. 2a). However, the HR was unaltered in all groups (fig. 2b).

Effect of HO Induction on MAP and HR in Sham-Operated and AC Rats

Protein abundance of HO-1 was analyzed by Western blotting in sham-operated and AC rats before and after 4 days of treatment with hemin (fig. 3a). AC rats without treatment showed a 2.8-fold higher expression of HO-1 than sham-operated rats (fig. 3b). Hemin administration on 4 consecutive days increased the HO-1 protein abundance 3-fold in sham-operated rats and 3.9-fold in AC rats compared to sham-operated rats (fig. 3b). Although chronic hemin by intraperitoneal administration induced HO-1 overexpression, it did not modify MAP and HR in sham-operated and AC rats compared to those without treatment (fig. 4).

The effect of acute inhibition of HO was also evaluated in normotensive sham-operated and hypertensive AC rats treated with hemin. Basal levels of MAP and HR were 102 ± 12 mm Hg and 401 ± 21 b.p.m., respectively, in sham-operated rats (n = 7) and 167 ± 10 mm Hg ($p < 0.05$ vs. sham-operated rats) and 445 ± 35 b.p.m., respectively, in AC rats (n = 7). The pressor response induced by ZnPP-IX in sham-operated rats was similar to that of rats without hemin treatment (fig. 1a). As shown in figure 5a, the effect of HO inhibition on MAP in normotensive rats started at 3 min and reached a maximum of 30 mm Hg at 20 min. Conversely, ZnPP-IX did not modify the MAP of AC rats. In addition, the HR was not modified by ZnPP-IX in both experimental groups (fig. 5b).

Evaluation of eNOS Expression and Vascular Reactivity in Sham-Operated and AC Rats

To explore the mechanisms involved in the blunted response to HO inhibition in AC rats, we studied the expression of eNOS and the vascular reactivity of Ach, a NO-dependent relaxant, in the thoracic aortas from sham-operated and AC rats. A significant decrease in eNOS levels was found in AC rats compared to sham-operated rats (fig. 6b), suggesting impairment in eNOS synthesis in the hypertensive group. In addition, hemin treatment did not modify the protein levels in both sham-operated and AC groups compared to rats without treatment (fig. 6b).

At a functional level, aortic coarctation blunted the maximal response to Ach compared to sham-operated rats (fig. 6c), suggesting a defective endothelium-depen-

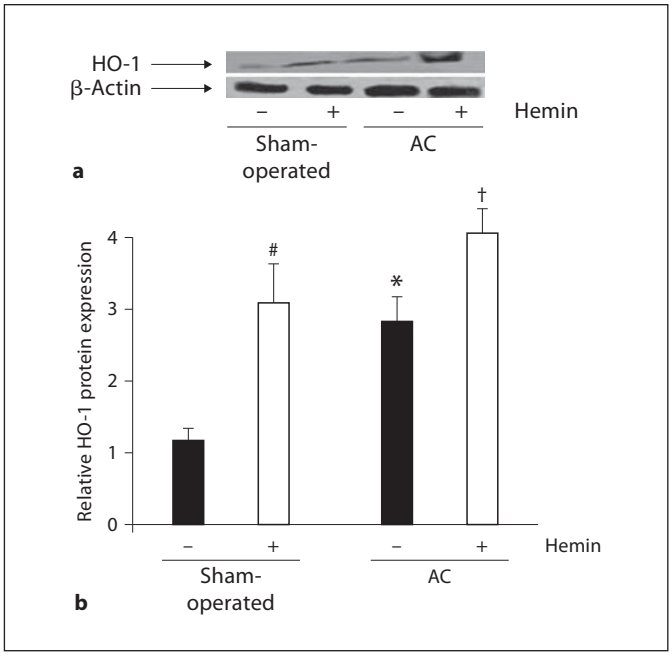


Fig. 3. HO-1 expression in the thoracic aortic homogenates of sham-operated and hypertensive AC rats with hemin (+; 15 mg/kg/day, i.p.) or vehicle pretreatment (–; 0.1 mol/l NaOH, titrated to pH 7.4 with 0.1 mol/l HCl and diluted 1:10 with phosphate buffer, pH 7.4, i.p.) for 4 consecutive days. **a** Representative Western blots. **b** Relative expression level of HO-1. HO-1 protein abundance was normalized using β-actin as control, and results are expressed as relative to a control of 1. # Significant differences ($p < 0.001$) between the hemin-treated sham-operated and vehicle-treated sham-operated groups. * Significant differences ($p < 0.001$) between the AC and vehicle-sham groups. † Significant differences ($p < 0.001$) between the hemin-treated AC and vehicle-treated sham-operated groups. Values are the means ± SEM of multiple experiments (n = 4 for each group).

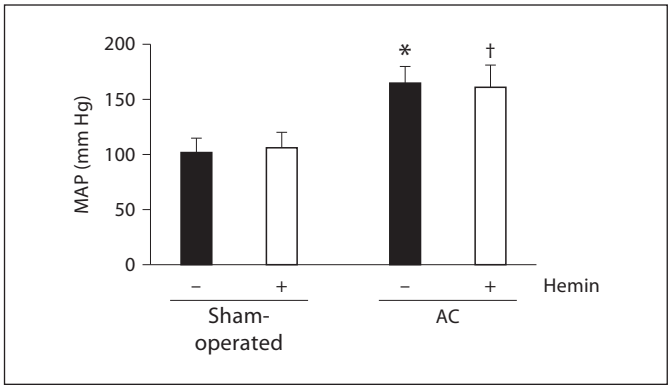


Fig. 4. MAP of sham-operated and AC rats with hemin pretreatment (+; 15 mg/kg/day, i.p.) or vehicle pretreatment (–; 0.1 mol/l NaOH, titrated to pH 7.4 with 0.1 mol/l HCl and diluted 1:10 with phosphate buffer, pH 7.4, i.p.) for 4 consecutive days. * Significant differences ($p < 0.001$) between the AC and vehicle-treated sham-operated groups. † Significant differences ($p < 0.001$) between the hemin-treated AC and vehicle-treated sham-operated groups. Values are the means ± SEM of multiple experiments (n = 7 for each group).

dent relaxation in the arterial vessels of AC rats. These results were confirmed by the fact that the vasorelaxant properties of nitroglycerine, an non-endothelium-dependent vasodilator, were not affected in AC rats compared to the sham-operated rats (fig. 6d).

Discussion

In the present study, we demonstrated that HO regulates blood pressure when the NOS pathway is fully functional. In addition, we demonstrated that HO fails to attenuate hypertension in AC rats, probably due to the impairment of the NOS system in peripheral vascular vessels in this experimental model.

HO is an important enzyme involved in numerous secondary enzymatic functions, including those of antioxidant enzymes and heat shock proteins. CO, one of the products of HO, seems to exert an important role on blood pressure regulation, considering its direct vasodilator action is through the activation of sGC and the subsequent production of cGMP [25, 26]. Because CO is produced endogenously as a by-product of heme catabolism by HO, the inhibition of HO may result in an increase in blood pressure. This hypothesis was supported by the elegant study of Johnson et al. [27], who showed that HO inhibitors such as ZnPP-IX increase the systemic arterial pressure with an upsurge in peripheral resistance. A reduction in HO-catalyzed CO production, rather than that of biliverdin and iron, is believed to underlie the pressor response to HO inhibition, as biliverdin and iron per se do not induce vasorelaxation [27]. Metalloporphyrins, including ZnPP-IX, have been reported to inhibit NOS and sGC activities in addition to HO inhibition [28, 29]. However, the specific effect of ZnPP-IX on HO activity has been reported at lower concentrations [30, 31]. For example, several studies showed that the intraperitoneal administration of ZnPP-IX (50 μmol/kg) does not alter sGC activity [31, 32]. Furthermore, ZnPP-IX, at the same concentration, specifically inhibits aortic HO activity without affecting sGC and NOS activity [33]. In agreement with Johnson et al. [27], we showed that the acute inhibition of HO by ZnPP-

IX administration produced a fast increase in MAP without changing the HR in normotensive rats. Furthermore, we evaluated the mechanism involved in this pressor response. It has been shown that CO can directly stimulate sGC in many different cell types [34–40]. For instance, this signaling cascade is largely responsible for the CO-induced activation of neurotransmission and vasodilation [27, 34]. By activating sGC, CO also inhibits platelet aggregation [34] and can increase the activity of purified bovine lung sGC several-fold [26, 41] although its effect is 30–100 times less than that of NO [35]. The relatively low potency of CO in activating sGC does not seem to diminish the physiological importance of CO, as certain putative endogenous substances may greatly boost the stimulatory effect of CO on sGC [41]. Nevertheless, a number of recent studies have shown that CO may activate NOS, and the subsequently synthesized and released NO stimulates sGC [42]. Therefore, CO could be an important modulator of NO signaling. Considering this interrelation between CO and NO, we hypothesized that inhibition of NO production could prevent the pressor response found by ZnPP-IX administration in the normotensive group. In this manner, L-NAME pretreatment induced a hypertensive response in sham-operated rats, and HO inhibition with ZnPP-IX did not further increase MAP in those rats. This last result could be attributed to the endothelial isoform of NOS, considering that ZnPP-IX does not cross the brain barrier and NO only acts in the peripheral tissue together with the fact that L-NAME, at the selected dose, is specific for eNOS. These findings suggest that the CO generated by HO activity interacts with the NO pathway to exert its regulatory action.

On the other hand, HO/CO seems to play an important role in the pathogenesis of experimental hypertension [20]. In this regard, it has been shown in young spontaneously hypertensive rats that the activation of the sGC/cGMP pathway through the upregulation of the HO/CO system could have a beneficial effect not only on hypertension but also on many cardiovascular complications that arise from elevated blood pressure, but only when the NOS/sGC/cGMP system is not impaired [42]. Thus, the integrity of the sGC/cGMP pathway is indispensable to the homeostatic control of the contractile machinery of vascular smooth muscle cells by CO [43], and alterations in the CO/sGC/cGMP pathway result in the development of hypertension [44]. Therefore, an interesting question is whether the hypotensive effect of HO induction observed in spontaneously hypertensive rats could be extrapolated to other models of experimental hypertension.

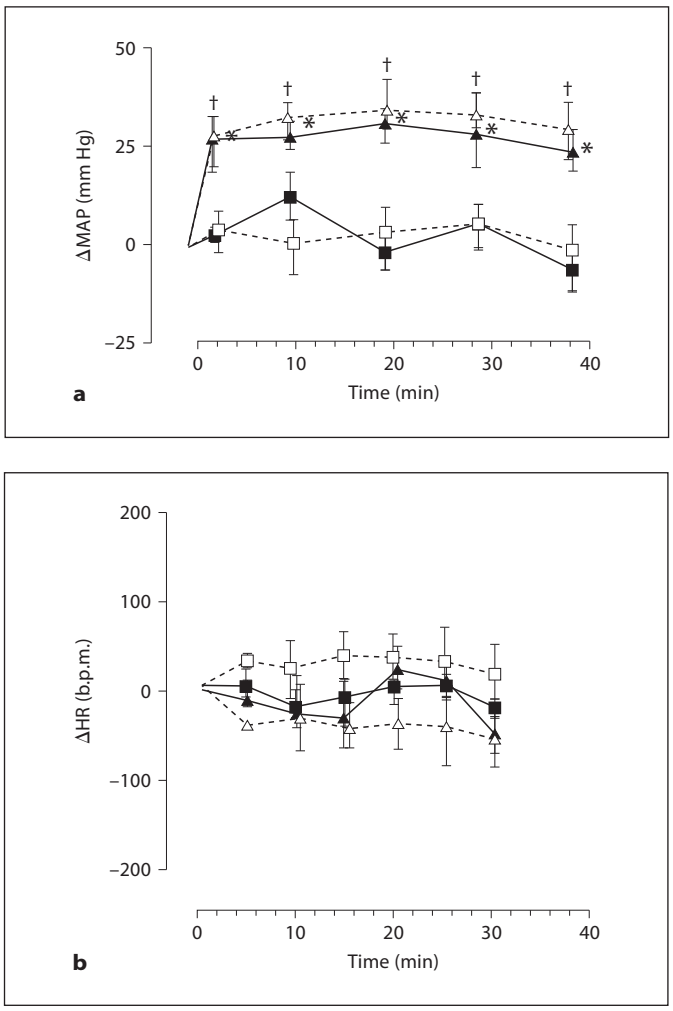


Fig. 5. Effects of the HO inhibitor ZnPP-IX, 50 μmol/kg, i.p., on changes in MAP (**a**) and HR (**b**) in sham-operated and AC hypertensive rats pretreated with vehicle (0.1 mol/l NaOH, titrated to pH 7.4 with 0.1 mol/l HCl and diluted 1:10 with phosphate buffer, pH 7.4, i.p.) or hemin (15 mg/ kg/day, i.p.) for 4 consecutive days. Changes inMAP were described as the changes after intraperitoneal injection of ZnPP-IX. Values are the mean ± SEM of multiple experiments (n = 7 for each group). † $p < 0.05$ versus before administration in the sham-operated vehicle-treated group. * Before administration in the sham-operated hemin-treated group. △ = Sham-operated, vehicle-treated; ▲ = sham-operated, hemin-treated; □ = AC, vehicle-treated; ■ = AC, hemin-treated.

Experimental hypertension is frequently induced by stimulation of the renin–angiotensin system by chronic infusion of angiotensin II , overexpression of renin and angiotensinogen genes, or coarctation of the renal artery with or without uninephrectomy [45]. Aortic coarctation is a renovascular model of hypertension that depends on angiotensin II function [46]. In this model, we previously

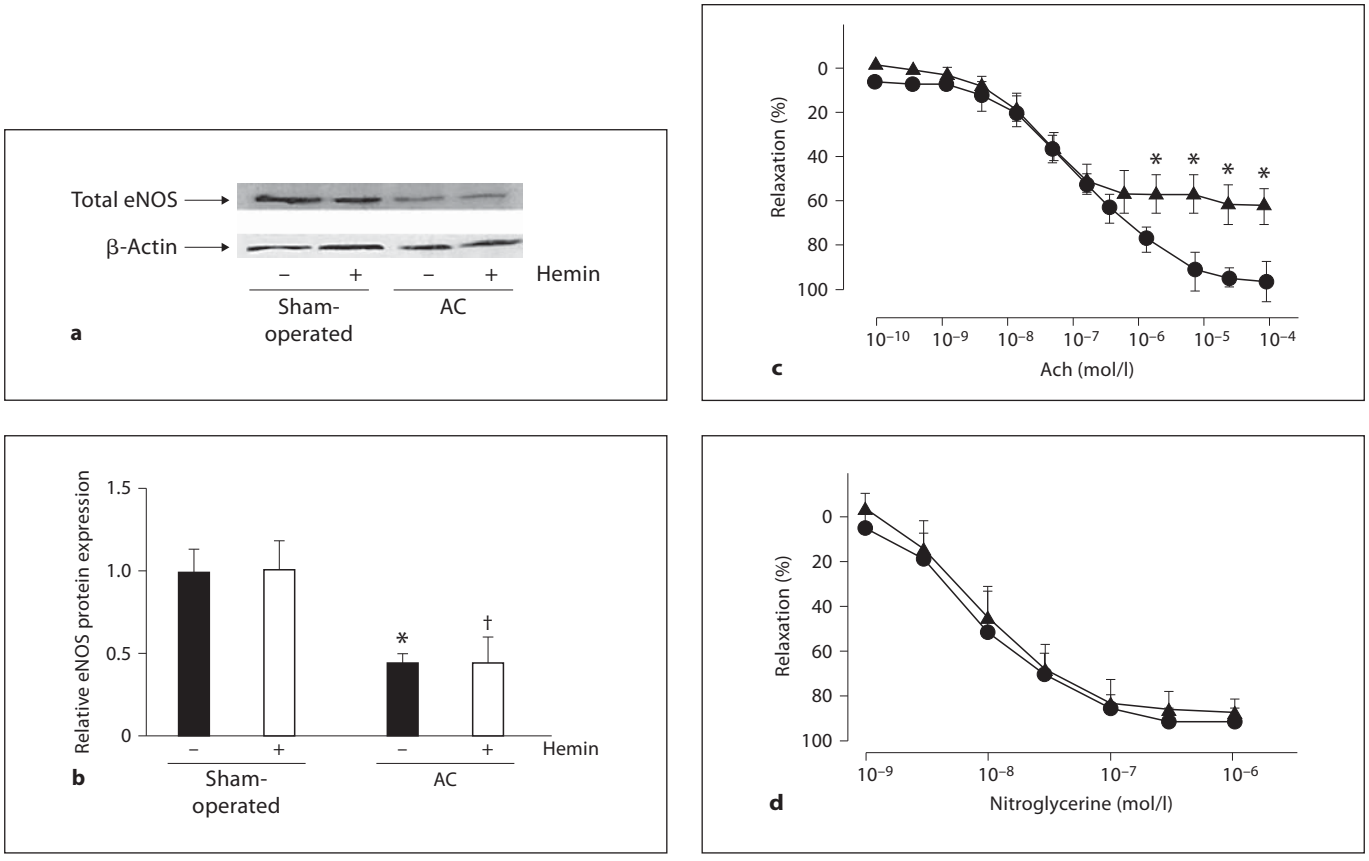


Fig. 6. eNOS expression in the thoracic aortic homogenates of sham-operated and hypertensive AC rats pretreated with vehicle (–; 0.1 mol/l NaOH, titrated to pH 7.4 with 0.1 mol/l HCl and diluted 1:10 with phosphate buffer, pH 7.4, i.p.) or hemin (+; 15 mg/kg/day, i.p.) for 4 consecutive days. **a** Representative Western blots. **b** Relative expression level of eNOS. Total eNOS protein abundance was normalized using β-actin as a control, and the results were expressed relative to a control of 1. * Significant differences ($p < 0.05$) between the AC versus sham groups. † Significant differences ($p < 0.001$) between the hemin-treated AC and vehicle-treated sham-operated groups (n = 4 for each group). Vascular reactivity to the endothelium-dependent agonist Ach (**c**) or endothelium-independent NO donor nitroglycerin (**d**) in aortic rings from sham-operated (●) and AC rats (▲). Rings were preconstricted with phenylephrine (1×10^{-7} mol/l). * Significant differences ($p < 0.05$) between the AC versus the sham-operated groups.

showed a redox imbalance in the aorta [9], heart [8] and kidney [21] and oxidative stress generation that contributed in the development of the hypertensive stage. As a consequence, HO-1 was overexpressed in these tissues and exerted a protective response against reactive oxygen species [8, 9]. Taking into account these previous findings, the main objective of the present work was to establish the role of the HO system in blood pressure regulation in AC rats. Although HO expression is upregulated 3-fold in these rats compared to sham-operated rats, we did not find any increase in MAP by acute administration of ZnPP-IX in this experimental group. This observation suggests that HO hypotensive activity is abolished in AC rats. Moreover, we performed a set of experiments with hemin, a potent inducer of HO-1, to determine whether HO-1 upregulation led to a drop in MAP in AC rats. We did not find any antihypertensive effect through the upregulation of HO-1 in AC rats. It seems that, depending on the model of hypertension, the pathogenic contributions from reduced or increased expression and activity of HO-1, lowered or increased CO production and dysfunctional signaling pathways mediated by CO will be different [45].

As L-NAME blocked the pressor response to ZnPP-IX in normotensive rats, we explored the expression levels and functionality of eNOS in this model of hypertension. With regard to sham-operated rats, AC rats showed a greatly reduced expression of eNOS and a blunted re-

sponse to endothelium-dependent relaxation induced by Ach, suggesting a functional impairment of this system. Taken together, these data suggest that NOS mediates the biological action of CO, and impairment of this system, as we found in AC rats, may prevent its action. Furthermore, we found that the pharmacological induction of HO-1 by hemin in sham-operated rats increased the MAP similarly to the group without treatment. We hypothesized that this equivalent response is due to the fact that eNOS could have a limiting role in that effect because the expression of this enzyme was equal in both the sham-operated and hemin-treated sham-operated groups, strengthening our conclusion that the CO derived from HO activity exerts its biological action through the NOS pathway.

In summary, our results indicate that the CO derived from HO activity exerts its biological action through the NOS pathway. Thus, it seems that the successful HO therapeutic strategy for hypertension is closely related to the mechanisms involved in the pathogenesis of the hypertensive stage. Furthermore, as in adult spontaneously hypertensive rats, HO-1 overexpression in AC rats failed to attenuate hypertension when the NOS system was not fully operative.

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Disclosure Statement

The authors report no conflicts of interest.

References

1 Maines MD, Traskhel GM, Kutty RK: Characterization of two constitutive forms of rat microsomal heme oxygenase: only one molecular species of the enzyme is inducible. J Biol Chem 1986;261:411–419.
2 Maines MD: Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. FASEB J 1988;2:2557–2568.
3 Tomaro ML, Frydman J, Frydman RB: Heme oxygenase induction by $CoCl_2$, Co-protoporphyrin IX, phenylhydrazine, and diamide: evidence for oxidative stress involvement. Arch Biochem Biophys 1991;226:610–617.
4 Ossola JO, Tomaro ML: Heme oxygenase induction by cadmium chloride: evidence for oxidative stress involvement. Toxicology 1995;104:141–147.
5 Ewing JE, Maines MD: Glutathione depletion induces heme oxygenase-1 (HSP32) mRNA and protein in rat brain. J Neurochem 1993;60:1512–1519.
6 Keyse SM, Tyrrell RM: Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. Proc Natl Acad Sci USA 1989;86:99–103.
7 Clark JE, Foresti R, Sarathchandra P, Kaur H, Green C, Motterlini R: Heme oxygenase-1-derived bilirubin ameliorates post ischemic myocardial dysfunction. Am J Physiol Heart Circ Physiol 2000;278:H643–H651.
8 Polizio AH, Balestrasse KB, Yannarelli GG, Noriega GO, Gorzalczany S, Taira C, Tomaro ML: Angiotensin II regulates cardiac hypertrophy via oxidative stress but not antioxidant enzyme activities in experimental renovascular hypertension. Hypertens Res 2008;31:325–334.
9 Polizio AH, Gorzalczany SB, Tomaro ML: Lowering arterial pressure delays the oxidative stress generation in a renal experimental model of hypertension. J Cardiovasc Pharmacol 2009;54:348–354.
10 Kaide JI, Zhang F, Wei Y, Jiang H, Yu C, Wang WH, Balazy M, Abraham NG, Nasjletti A: Carbon monoxide of vascular origin attenuates the sensitivity of renal arterial vessels to vasoconstrictors. J Clin Invest 2001;107:1163–1171.
11 Wang R, Wang Z, Wu L: Carbon monoxide-induced vasorelaxation and the underlying mechanisms. Br J Pharmacol 1997;121:927–934.
12 Morita T, Perrella M A, Lee M, Kourembanas S: Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. Proc Natl Acad Sci USA 1995;92:1475–1479.
13 Foresti R, Hammad J, Clark JE, Johnson TR, Mann BE, Friebe A, Green CJ, Motterlini R: Vasoactive properties of CORM-3, a novel water-soluble carbon monoxide-releasing molecule. Br J Pharmacol 2004;142:453–460.
14 Sammut IA, Foresti R, Clark JE, Exon DJ, Vesely MJ, Sarathchandra P, Green CJ, Motterlini R: Carbon monoxide is a major contributor to the regulation of vascular tone in aortas expressing high levels of haeme oxygenase-1. Br J Pharmacol 1998;125:1437–1444.
15 Motterlini R, Mann BE, Johnson TR, Clark JE, Foresti R, Green CJ: Bioactivity and pharmacological actions of carbon monoxide-releasing molecules. Curr Pharm Des 2003;9:2525–2539.
16 Abraham NG, Quan S, Mieyal PA, Yang L, Burke-Wolin T, Mingone CJ, Goodman AI, Nasjletti A, Wolin MS: Modulation of cyclic GMP by retrovirus-mediated human hemeoxgyenase-1 gene transfer in microvessel endothelial cells. Am J Physiol 2002;283:L1117–L1124.
17 Abraham NG, Lin JH, Schwartzman ML, Levere RD, Shibahara S: The physiological significance of heme oxygenase. Int J Biochem 1988;20:543–558.
18 Ndisang JF, Wang R: Alterations in heme oxygenase/carbon monoxide system in pulmonary arteries in hypertension. Exp Biol Med (Maywood) 2003;228:557–563.
19 Levere RD, Martasek P, Escalante B, Schwartzman ML, Abraham NG: Effect of heme arginate administration on blood pressure in spontaneously hypertensive rats. J Clin Invest 1990;86:213–219.

20 Sacerdoti D, Escalante B, Abraham NG, McGiff JC, Levere RD, Schwartzman ML: Treatment with tin prevents the development of hypertension in spontaneously hypertensive rats. Science 1989;243:388–390.
21 Polizio AH, Balestrasse KB, Gornalusse GG, Gorzalczany SB, Santa-Cruz DM, Yannarelli GG, Peña C, Tomaro ML: Losartan exerts renoprotection through NAD(P)H oxidase downregulation in a renovascular model of hypertension. Regul Pept 2009;156:28–33.
22 Rojo-Ortega JM, Genest J: A method for production of experimental hypertension in rats. Can J Physiol Pharmacol 1968;46:883–885.
23 Gaballa MA, Raya TE, Hoover CA, Goldman S: Effects of endothelial and inducible nitric oxide synthases inhibition on circulatory function. Cardiovasc Res 1999;42:627–635.
24 Gonzales S, Erario MA, Tomaro ML: Heme oxygenase-1 induction and dependent increase in ferritin. A protective antioxidant stratagem in hemin-treated rat brain. Dev Neurosci 2002;24:161–168.
25 Christodoulides N, Durante W, Kroll MH, Schafer AI: Vascular smooth muscle cell heme oxygenases generate guanylyl cyclase-stimulatory carbon monoxide. Circulation 1995;91:2306–2309.
26 Kharitonov VG, Sharma VS, Pilz RB, Magde D, Koesling D: Basis of guanylate cyclase activation by carbon monoxide. Proc Natl Acad Sci USA 1995;92:2568–2571.
27 Johnson RA, Lavesa M, Askari B, Abraham NG, Nasjletti A: A heme oxygenase product, presumably carbon monoxide, mediates a vasodepressor function in rats. Hypertension 1995;25:166–169.
28 Grundemar L, Ny L: Pitfalls using metalloporphyrins in carbon monoxide research. Trends Pharmacol Sci 1997;18:193–195.
29 Lou D, Vincent SR: Metalloporphyrins inhibit nitric oxide-dependent cGMP formation in vivo. Eur J Pharmacol 1994;267:263–267.
30 Zakhary R, Gaine SP, Dinerman JL, Ruat M, Flavahan NA, Snyder SH: Heme oxygenase 2: endothelial and neuronal localization and role in endothelium-dependent relaxation. Proc Natl Acad Sci USA 1996;93:795–798.
31 Ndisang JF, Wu L, Zhao W, Wang R: Induction of heme oxygenase-1 and stimulation of cGMP production by hemin in aortic tissues from hypertensive rats. Blood 2003;101:3893–3900.
32 Ndisang JF, Wang R, Vannacci A, Marzocca C, Fantappiè O, Mazzanti R, Mannaioni PF, Masini E: Haeme oxygenase-1 and cardiac anaphylaxis. Br J Pharmacol 2001;134:1689–1696.
33 Motterlini R, Gonzales A, Foresti R, Clark JE, Green CJ, Winslow RM: Heme oxygenase-1-derived carbon monoxide contributes to the suppression of acute. Circ Res 1998;83:568–577.
34 Brune B, Ullrich V: Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. Mol Pharmacol 1987;32:497–504.
35 Furchgott RF, Jothianandan D: Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. Blood Vessels 1991;28:52–61.
36 Karlsson JO, Axelsson KL, Andersson RG: Effects of hydroxyl radical scavengers KCN and CO on ultraviolet light- induced activation of crude soluble guanylate cyclase. J Cyclic Nucleotide Protein Phosphor Res 1985;10:309–315.
37 Morita T, Mitsialis SA, Koike H, Liu Y, Kourembanas S: Carbon monoxide controls the proliferation of hypoxic vascular smooth muscle cells. J Biol Chem 1997;272:32804–32809.
38 Ingi T, Cheng J, Ronnett GV: Carbon monoxide: an endogenous modulator of the nitric oxide-cyclic GMP signaling system. Neuron 1996;16:835–842.
39 Verma A, Hirsch DJ, Glatt CE, Ronnett GV, Snyder SH: Carbon monoxide: a putative neural messenger. Science 1993;259:381–384.
40 Xue L, Farrugia G, Miller SM, Ferris CD, Snyder SH, Szurszewski JH: Carbon monoxide and nitric oxide as coneurotransmitters in the enteric nervous system: evidence from genomic deletion of biosynthetic enzymes. Proc Natl Acad Sci USA 2000;97:1851–1855.
41 Stone JR, Marletta MA: Soluble guanylate cyclase from bovine lung activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. Biochemistry 1994;33:5636–5640.
42 Wu L, Wang R: Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. Pharmacol Rev 2005;57:585–630.
43 Silva CL, Noel F, Barreiro EJ: Cyclic GMP-dependent vasodilatory properties of LASSBio 294 in rat aorta. Br J Pharmacol 2002;135:293–298.
44 Ruetten H, Zabel U, Linz W, Schmidt HH: Downregulation of soluble guanylyl cyclase in young and aging spontaneously hypertensive rats. Circ Res 1999;85:534–541.
45 Lassegue B, Griendling KK: Reactive oxygen species in hypertension. Am J Hypertens 2004;17,852–860.
46 Gironacci MM, Brosnihan KB, Ferrario CM, Gorzalczany S, Verrilli MA, Pascual M, Taira C, Peña C: Increased hypothalamic angiotensin-(1–7) levels in rats with aortic coarctation-induced hypertension. Peptides 2007;28:1580–1585.