Heme Oxygenase-1–Derived Carbon Monoxide Contributes to the Suppression of Acute Hypertensive Responses In Vivo

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Abstract—The enzyme heme oxygenase, which exists in inducible (HO-1) and constitutive (HO-2) isoforms, catalyzes the degradation of heme to biliverdin and CO in mammalian tissues. CO has been implicated in the control of vascular tone in a manner similar to that for NO. In the present study, we investigated the contribution of the heme oxygenase/CO pathway to the modulation of acute hypertensive responses in vivo induced by (1) ααHb, a chemically modified hemoglobin known to scavenge NO, and (2) N\textsubscript{G}-nitro-L-arginine methyl ester (L-NAME), a competitive NOS inhibitor. Experiments were carried out in conscious rats in which femoral arteries and veins were surgically catheterized 1 or 5 days before treatment with the vasoconstrictor agents. Intravenous infusion of ααHb (8% solution) or L-NAME (30 mmol/kg) produced an acute and significant increase in mean arterial pressure (P<0.05) in rats at 5 days after catheter implantation. In contrast, no change in blood pressure was observed when ααHb or L-NAME was infused 1 day after the surgical intervention. The suppression of the hypertensive response observed at 1 day after surgery correlated with a significant (P<0.05) HO-1 expression in aorta, heart, and liver as well as increased aortic CO production and cGMP levels. At 1 day after surgery, pretreatment of animals with the heme oxygenase inhibitor zinc protoporphyrin IX (50 μmol/kg IP) markedly decreased aortic CO and cGMP levels and completely restored the vasoconstrictor effects of both ααHb and L-NAME. These results provide evidence for a crucial role of the heme oxygenase/CO pathway in the regulation of blood pressure under stress conditions in vivo. (Circ Res. 1998;83:568-577.)

Key Words: surgical stress ▪ hemoglobin-based blood substitute ▪ carbon monoxide ▪ bilirubin ▪ cGMP

The initial degradation of heme in mammalian species is catalyzed by microsomal heme oxygenase. This is the rate-limiting enzyme in heme catabolism and involves the loss of iron, the formation of biliverdin, and the elimination of the α-methene carbon bridge of the porphyrin ring as CO.1 The subsequent step in heme catabolism occurs in the cytosol with the conversion of biliverdin to bilirubin by the enzyme biliverdin reductase. Heme oxygenase is a ubiquitous protein and exists in 2 different isoforms that are products of 2 different genes.2 The heme oxygenase-2 (HO-2) isoform is constitutively expressed and is found at high levels primarily in the brain and testes.1 In contrast, the heme oxygenase-1 (HO-1) isoform is inducible and is regarded as a heat shock protein (HSP32), the expression of which is markedly up-regulated by stress-related conditions that alter the redox status of the cellular milieu. Several pro-oxidant factors and agents, including heat shock,3 endotoxin,4 heavy metal ions,5 and ultraviolet irradiation6 have been reported to increase HO-1 expression and activity in a variety of tissues. The precise physiological significance of HO-1 induction under stress conditions still remains to be elucidated. The hypothesis has been postulated that the expression of this gene is part of the defensive mechanism that cells and tissues are capable of mounting against different stress stimuli.5,7 Consistent with this notion are the findings that biliverdin and bilirubin, end products of heme catabolism, possess antioxidant properties8 and that CO seems to mimic many NO functions.9

NO is enzymatically synthesized from L-arginine by NO synthase (NOS), which, similar to heme oxygenase, exists in constitutive (cNOS and nNOS) and inducible (iNOS) isoforms.10 NO is a free radical gas that possesses multiple biological functions and has been identified as endothelium-derived relaxant factor.11,12 The basal release of NO, observed during resting conditions in vascular endothelial cells, mediates the relaxation of smooth muscle via activation of a heme-dependent guanylate cyclase that catalyzes the conversion of GTP to the second-messenger cGMP. This transduction mechanism mediated by NO is responsible for the maintenance of vascular tone and is fundamental to the regulation of blood pressure in mammals.13,14 CO has also been reported to activate guanylate cyclase in smooth muscle cells, although its affinity for the heme moiety of guanylate cyclase is much lower than that associated with NO.15 Similar to NO, CO derived from heme oxygenase has been postulated to act as a neurotransmitter,16,17 and recent findings have demonstrated the participation of CO in the regulation of...
vascular tone in hepatic sinusoidal cells, suggesting that NO and CO could share the control of relaxation processes. CO has been demonstrated to function as an endogenous modulator of the NO-cGMP signaling system in the brain. In cultured cerebellar granule cells, the NO-mediated cGMP increase is blocked by augmenting endogenous CO production and potentiated by inhibitors of the heme oxygenase pathway. In vitro and in vivo studies conducted in our laboratory and other laboratories have demonstrated that NO is also capable of increasing HO-1 expression and activity in vascular and liver tissues, emphasizing the importance of heme oxygenase as a target/stimulator of signal transduction mechanisms.

Vascular tone and blood pressure are controlled at various levels by a series of systems that involve a complex interaction between homeostatic factors, including the renin-angiotensin system, the autonomic nervous system, and, as mentioned above, the release of local mediators such as endothelium-derived relaxing factor/NO. Although the administration of inhibitors of NOS activity, such as Nω-nitro-L-arginine methyl ester (L-NAME), has been extensively used to produce vasoconstrictor effects in various species in vivo, only limited information is available concerning the possible role of the heme oxygenase/CO pathway in the control of blood pressure. Johnson et al have recently reported that administration of zinc protoporphyrin IX (ZnPPIX), a potent heme oxygenase inhibitor, causes an increase in arterial pressure in rats. This effect was attributed to blockade of the inhibitory action of endogenous CO on a pressor mechanism that is mediated by the autonomic nervous system. However, no direct link has been established between the level of tissue heme oxygenase, its activity in terms of CO production, and its role in the regulation of pressor responses in animal models.

The present study was designed to investigate the potential contribution of stress-mediated HO-1 induction to the modulation of hypertensive responses caused by inhibitors of the NOS/NO system in conscious rats. We report herein that increased CO release after upregulation of tissue HO-1 is a determinant factor in the control of blood pressure under stress conditions in vivo. The biochemical and physiological relevance of these findings will be discussed.

Materials and Methods

Materials
Hemoglobin cross-linked between α chains at α-Lys (αHb) was obtained from Letterman Army Institute of Research (San Francisco, Calif) and was prepared by reacting human stroma-free hemoglobin with bis(3,5-dibromosalicyl)fumarate as previously described. αHb was stored as oxyhemoglobin in sealed foil containers at −80°C until use. Bovine hemoglobin was obtained from Sigma Chemical Co, and deoxyhemoglobin was prepared by using sodium dithionite as previously described. ZnPPIX was purchased from Porphin Products and dissolved in 50 mmol/L sodium bicarbonate immediately before use. L-NNAME was from Sigma, and SIN-1 was kindly provided by Dr R. Henning (Cassella A.G., Frankfurt, Germany). Both L-NNAME and SIN-1 were dissolved in 0.9% saline immediately before intravenous injection. [2-14C]Glycine was purchased from ICN. All other reagents used were from Sigma unless otherwise specified.

Animal Preparation
Male Sprague-Dawley rats (300 to 350 g) were purchased from Harlan Sprague Dawley Laboratories, Inc (San Diego, Calif) and housed at constant temperature (23°C) in rooms that provided automatic lighting with a 12-hour on-off cycle. Rats were acclimated for 2 to 3 days in plastic cages and were given free access to water and food. On the day of surgery, animals were anesthetized with an intramuscular injection of the following mixture: 0.5% ketamine/0.4% acepromazine/1% xylazine (0.2 mL/100 g body wt). Specially designed femoral artery and venous catheters were then surgically implanted as previously described. Rats were then returned to the plastic cages and allowed to recover from the surgical procedure for the time indicated in the experimental protocol. Both femoral arteries and one femoral vein were cannulated in rats undergoing isovolemic exchange transfusion with αHb, whereas animals receiving bolus injections of NOS inhibitor/NO donor had only one arterial and one venous catheter implanted in opposite legs. The basic design for this chronic catheter implantation technique was optimized to improve longevity and patency in studies requiring vascular access for up to 7 days after cannulation.

Experimental Protocols
All in vivo experiments were performed on previously instrumented and fully conscious rats that were given a brief (1-day) or prolonged (5-day) postsurgical recovery period. On the day of experimentation, awake rats were placed in a restrainer (Braintree Scientific Inc), and one arterial line was connected to a pressure transducer (UFI model 1050) for continuous mean arterial pressure monitoring, and data were recorded using a Biopac MP100 system with AcqKnowledge software (BIOPAC System Inc). In the first and second group of animals (1 and 5 days after surgery, respectively; n = 5 each), a 50% isovolemic exchange transfusion with αHb was carried out using a 2-channel peristaltic pump (Labconco Corp). αHb was a chemically modified hemoglobin that is currently being investigated as a potential oxygen carrier and has been shown to induce acute systemic hypertension in a similar animal stress model. Removal of blood from the arterial line and intravenous infusion of αHb solution (8%) were performed simultaneously at identical flow rates (0.5 mL/min). The αHb solution was sterilized before infusion by filtration through a 0.2-μm filter (Millipore) and warmed to 37°C in a water bath. In a third and fourth group of experiments, animals (1 and 5 days after surgery, respectively; n = 5 each) were given an intravenous injection of 30 mmol/kg L-NNAME, followed 1 hour later by administration of SIN-1 (30 mmol/kg) for 5 hours, an organic compound that spontaneously releases NO, hence causing vasorelaxation. In complementary experiments conducted in rats at 1 day after surgery, a selective inhibitor of heme oxygenase activity (ZnPPIX, 50 μmol/kg IP) was injected intraperitoneally 10 minutes before infusion of αHb or L-NNAME (n = 5 for each group). A control group received ZnPPIX (50 μmol/kg IP) alone. Mean arterial pressure was continuously monitored before and after injection with the various materials. Because ZnPPIX is light sensitive and has been reported to have effects unrelated to heme oxygenase inhibition when exposed to light, we carefully protected this drug from light during its preparation.

Determination of Hepatic and Renal Heme Oxygenase Activity
In another set of experiments, heme oxygenase activity was determined in rat kidney and liver microsomes at different time points (1.5 and 12 hours and 1, 2, 3, 5, and 7 days) after catheter implantation and compared with the activity in control rats not subjected to any surgical intervention (time 0) (n = 5 for each group). Microsomes were prepared by ultracentrifugation as previously described. A portion of the microsomal fraction was lyzed by the addition of PBS buffer containing 1% Triton X-100 and stored at −80°C for Western blot analysis. The remaining microsomes were resuspended in 1 mL of 0.1 mmol/L potassium phosphate buffer, pH 7.4, containing 2 mmol/L MgCl and analyzed for heme oxygenase activity by a spectrophotometric assay that measures the formation of...
bibilirubin, the end product of heme degradation, as previously described.23

Western Blot Analysis
Liver microsomes were prepared as reported above. Aorta and heart tissues were homogenized in 1 mL of lysis buffer (50 mmol/L HEPES, 5 mmol/L EDTA, 50 mmol/L NaCl, and 1% Triton X-100, pH 7.5) containing Complete protease inhibitor (Boehringer Mannheim). Samples were kept on ice for 1 hour and then centrifuged (4°C) for 30 minutes at 12 000g. After the precipitated unsolubilized fraction was discarded, the protein concentration was determined in the supernatant by the Lowry method. Aliquots (30 μg) of protein from each sample were electrophoresed on a 12% SDS-polyacrylamide gel using a Mini Protean II system (Bio-Rad). The protein samples were transferred overnight onto a nitrocellulose membrane. Nonspecific antibody binding was blocked with 3% nonfat dried milk in PBS, pH 7.4, for 2 hours at room temperature. The membrane was then probed with polyclonal rabbit anti-hemoglobin H-1 antibody (stressGen) (1:1000 dilution in Tris-buffered saline, pH 7.4) for 2 hours at room temperature. After 3 washes with PBS containing 0.05% (vol/vol) Tween 20, blots were visualized with the use of an amplified alkaline phosphatase kit (extra-3A, Sigma). The relative band densities were analyzed with the use of an imaging densitometer (model GS-700) with Molecular Analyst software (Bio-Rad).

Measurement of ααααHb Disappearance in Plasma
Plasma heme concentration was used as an indirect index of the rate of heme catabolism in tissues after exchange transfusion with ααααHb. Arterial blood (50 to 100 μL) was collected in heparinized microhematocrit tubes (n=4 or 5 for each group) at 1, 2, and 3 hours after the exchange transfusion in animals at 1 and 5 days after surgery. Samples were centrifuged, and total hemoglobin content was determined in the plasma fraction by using a spectrophotometric method that measures azide-methemoglobin complexes.24 The absorbance was read with the use of a hemoglobin photometer (Hemocue AB) at 2 different wavelengths (570 and 880 nm) in order to compensate for turbidity. The rate of heme metabolism from plasma was calculated by measuring the change in total plasma hemoglobin concentration over time and determining the slope of the resultant linear plot. The data were expressed as a fraction of the initial concentration of total plasma hemoglobin taken at 1 hour after the beginning of ααααHb infusion, since 30 to 40 minutes was required to complete the exchange transfusion. To determine the direct involvement of tissue heme oxygenase in hemoglobin catabolism, plasma hemoglobin concentration was also measured after transfusion with ααααHb in animals (1 day after surgery) pretreated with the heme oxygenase inhibitor ZnPPIX (50 μmol/kg).

Measurement of Bilirubin in Urine
Bilirubin levels were determined spectrophotometrically with the use of a diagnostic kit (550-A, Sigma). Surgically catheterized rats were placed in metallic cages, and urine was collected at different times after surgery. The rate of urinary bilirubin produced over time was expressed in milligrams per hour.

FourFourFourFourCO Production in Aortic Tissue
We specifically designed an appropriate device for measuring CO released from aortic tissue (R. Motterlini, unpublished data). Two plastic syringes were positioned vertically facing each other and were connected by a 0.4-μm filter (Millipore) to create 2 separate chambers. The lower chamber contained 5 μCi of [2-14C]-glycine, a heme precursor, and the upper chamber contained a solution of deoxyhemoglobin (15 μmol/L), which is known to avidly bind CO. Aortas were removed from rats at various times after surgery, trimmed of adventitial tissue, and transferred into the lower chamber containing [2-14C]-glycine in oxygenated Krebs-Henseleit buffer (1:20 [vol/vol]) containing (mmol/L) NaCl 118, KCl 4.7, KH2PO4 1.2, MgSO4 2.5, NaHCO3 22, glucose 11, potassium EDTA 0.03, and CaCl2 2.5, pH 7.4. With the use of a piston syringe, a 1-cm air space was left in the lower chamber between the solution containing the radiolabeled material and the filter. This enabled the 2 solutions to be kept physically separated and, at the same time, allowed the diffusion of gas (CO) between the chambers. The lower chamber was then immersed in a water bath at 37°C for 9 hours to allow incorporation of [2-14C]-glycine into the aortic tissue. At the end of the incubation period, [14C]bound to hemoglobin was measured in the upper chamber by scintillation counting (Beckman liquid scintillation counter, model LS6500). The radioactivity incorporated in the tissue was also measured after repeated washes of aortas with distilled water. [14C]Production was measured in counts per minute (cpm) and expressed as a fraction of the total [14C]incorporated in the aorta by using the following formula: ([14C]cpm in the upper chamber/([14C]+[2-14C]-glycine)cpm in aorta) ×10^-7/mg tissue.

Determination of cGMP Levels
Levels of cGMP were measured in tissue extracts with the use of a commercial radioimmunoassay [125I] kit (Du Pont) and ELISA kit (Amersham). Tissues were collected from the following groups of animals: control animals (no surgery); animals at 1 and 5 days after surgery; and animals at 1 day after surgery treated with L-NAME (30 mmol/kg body wt, bolus injection), ZnPPIX (50 μmol/kg), or L-NAME plus ZnPPIX 10 minutes before tissue harvest. At the end of each protocol, samples were immediately freeze-clamped in liquid nitrogen and stored at −80°C until the cGMP assay was carried out. Duplicate measurements were performed on all samples (n=4 or 5 per group), including the standard curve.

Statistical Analysis
All values are expressed as mean±SE. Differences in the data among the groups were analyzed by 1-way ANOVA combined with the Bonferroni test. A value of P<0.05 was considered significant.

Results
Effect of Surgical Stress on the Hypertensive Response Induced by Hemoglobin and L-NAME
Hemoglobin, a well-established scavenger of NO, and L-NAME, a competitive inhibitor of cNOS activity, have been consistently reported to increase systemic blood pressure when infused in vivo in instrumented animals.14 In our experiments, however, we observed that not all animals responded equally to L-NAME and ααααHb treatment and that, in some circumstances, the expected hypertensive effect of these 2 vasoconstrictor agents was completely absent. After a series of preliminary experiments, we found that this variability in the pressor response was dependent on the time at which the experiment was carried out after catheter implantation. As shown in Figure 1, 50% exchange transfusion with ααααHb (Figure 1A) or infusion of L-NAME (Figure 1B) in rats at 5 days after surgery resulted in a significant increase in mean arterial pressure from 140±3.6 to 165±5.7 mm Hg and from 129±11 to 166±12 mm Hg (P<0.05, respectively). This increase was rapid and sustained for at least 2 hours after the treatment. Conversely, transfusion with ααααHb or L-NAME treatment did not significantly change mean arterial pressure when experiments were conducted in rats that were given only a 1-day postsurgical recovery period (130±3.1 versus 132±4.5 mm Hg and 122±3.5 versus 138±8.3 mm Hg, respectively). These findings suggest that “postsurgical stress” may influence local pressor mechanisms involved in the regulation of systemic blood pressure. It is interesting to note that 1 hour after L-NAME treatment a bolus injection of SIN-1, an organic NO-releasing agent, produced a marked vasodilatory effect in both groups, indicating that NO-
dependent mechanisms that control vessel tone were not impaired by surgical stress. Moreover, the suppression of pressor responses was not observed immediately after surgery (0-day surgery). In experiments conducted at 1.5 hours after surgery in fully conscious rats, administration of \( \text{aaHb} \) or \( \text{L-NAME} \) produced an increase in blood pressure from 93.6 ± 3.9 to 135.4 ± 2.7 mm Hg and from 90.8 ± 4.2 to 150.5 ± 1.4 mm Hg, respectively. It needs to be pointed out that the baseline mean arterial pressure in these groups was 40 mm Hg lower than the baseline blood pressure in animals at 1 day after surgery. This is due to the fact that immediately after surgery the animals, although fully awake, were still under the effect of anesthesia.

**Surgical Stress Increases Tissue Heme Oxygenase Activity and HO-1 Expression**

The finding that the pressor response to \( \text{aaHb} \) or \( \text{L-NAME} \) infusion varied considerably at different times after surgery (1 and 5 days) prompted us to search for a possible mechanism in the regulation of blood pressure mediated by surgical stress. On the basis of previous reports showing that surgical procedures cause specific and simultaneous heat shock protein (HSP) induction in various tissues, we investigated whether the catheter implantation technique used in our protocol could affect the activity and expression of HO-1 protein in liver and kidney determined in the microsomal fraction with the use of a spectrophotometric assay that measures the formation of the end product bilirubin. Data represent the mean ± SE of 5 or 6 experiments. \(*P < 0.05\) compared with baseline.
implantation; the activity was maximal at 1 day after surgery (2191 ± 73 pmol bilirubin/mg protein per hour, P < 0.05) and started to diminish gradually at 2 days after surgery (1540 ± 141 pmol bilirubin/mg protein per hour, P < 0.05). Heme oxygenase activity returned to control levels by 3 days after surgery (907 ± 76 pmol bilirubin/mg protein per hour) and remained unchanged up to 7 days after catheter implantation.

No significant changes in renal heme oxygenase activity were detected after surgery at the time points considered. A possible effect of the anesthetic in the modulation of the stress response (HO-1 induction) can be excluded a priori, since anesthetized rats not subjected to surgery or immediately after surgery (1.5 hours) showed no increase in hepatic heme oxygenase activity (data not shown). The increase in hepatic heme oxygenase activity after surgery was reflected in an increase in the inducible isoform of this protein (HO-1) in liver, heart, and aorta, as shown by Western immunoblot analysis (Figure 2B and 2C). A recent observation showed that transfusion of ααHb in a similar animal model produced an increase in mean arterial pressure at 1 day after surgery. However, a direct comparison with the present study is difficult to make, since in that study no measurements of heme oxygenase activity and HO-1 expression were performed in any tissue after surgery.

Effect of ZnPPIX on Blood Pressure at 1 Day After Surgery

We used ZnPPIX, a selective inhibitor of heme oxygenase, to establish the direct involvement of HO-1 induction in the control of pressor responses after surgical stress. Injection of ZnPPIX before transfusion with ααHb or L-NAME treatment resulted in a rapid increase in blood pressure in rats at 1 day after surgery (from 121 ± 5.7 to 149 ± 2.7 mm Hg and from 122 ± 4.3 to 162 ± 5.7 mm Hg, respectively; P < 0.05) (Figure 3A and 3B), whereas ααHb or L-NAME alone did not produce a significant vasoconstrictor effect (Figure 1A). The hypertensive effect mediated by L-NAME was sustained for 1 hour and then completely reversed by a bolus injection of the NO-releasing agent SIN-1, as shown by a significant drop in mean arterial pressure from 159 ± 3.3 to 87 ± 3.1 mm Hg (P < 0.05) (Figure 3B). It has to be noted that 50 μmol/kg of ZnPPIX alone did not have any significant effect on mean arterial pressure in rats at 1 day after surgery, when heme oxygenase activity and HO-1 protein were at maximal levels (Figure 3A).

Heme Catabolism Is Accelerated After HO-1 Induction by Surgical Stress

Since the rates of autoxidation and release of heme from ααHb are much higher than from unmodified hemoglobin, we assumed that the presence of ααHb in plasma would augment heme absorption and catabolism by vascular and organ tissues. If that is the case, then the half-life of plasma hemoglobin in the systemic circulation would be expected to vary as a function of the activity of tissue heme oxygenase, the enzyme responsible for heme breakdown. Total plasma hemoglobin concentration was measured at various time points after transfusion with ααHb in rats at 1 and 5 days after surgery. The half-life of ααHb in plasma (time at which the concentration of ααHb is 50% of its initial value) has been previously determined by using a similar animal stress model and has been reported to be in the range of 4.5 to 5.5 hours. The rate of metabolism of heme circulating in plasma in the various groups is represented in Figure 4, and the half-life of hemoglobin disappearance was calculated from the slope of each linear plot. Plasma hemoglobin disappearance was significantly (P < 0.05) faster in rats that were transfused at 1 day after catheter implantation (half-life of 3.4 hours) compared with rats transfused at 5 day after surgery (half-life of 4.4 hours). In experiments conducted at 1 day after surgery, pretreatment of animals with ZnPPIX prolonged the retention time of ααHb in the circulation, and the half-life increased from 3.4 to 4.1 hours. It is interesting to note that the rate of heme metabolism in plasma directly correlated with the level of tissue heme oxygenase, since heme oxygenase activity and protein expression (HO-1) were significantly higher at 1 day after surgery than at 5 days after surgery.
Surgical Stress Increases Urinary Bilirubin Production and CO Formation in Aorta

Surgical stress–mediated induction of HO-1 in rat tissues was associated with a high level of urinary bilirubin, the end product of heme catabolism. As shown in Figure 5A, the rate of bilirubin production in urine markedly increased (*P*, 0.05) at 1 day after surgery from 0.13 ± 0.01 to 0.31 ± 0.03 mg/h and gradually returned to control values at 5 days after surgery (0.14 ± 0.01 mg/h). Accordingly, the level of aortic CO, another product of heme catabolism, directly correlated with the induction of HO-1 mediated by surgery (Figure 5B). The release of 14CO from aortas significantly increased at 1 day after surgery (*P*, 0.05) but was completely blocked by pretreatment of animals with ZnPPIX. The increase in CO production after surgery was temporary and returned to control levels at 5 days after surgery.

Changes in cGMP Levels in Response to Surgical Stress

Recent evidence suggests that heme oxygenase–derived CO interacts with the heme moiety of guanylate cyclase in a manner similar to NO and may thereby contribute to the regulation of vascular tone by increasing intracellular levels of the second messenger cGMP.18 We therefore investigated whether cGMP is increased after surgical stress–mediated activation of tissue heme oxygenase. As shown in Figure 6, cGMP significantly increased at 1 day after surgery in aortic tissue (from 5.6 ± 1.1 to 20.2 ± 2.8 fmol/mg tissue, *P* < 0.05) and returned to control levels at 5 days after surgery (6.9 ± 1.7 fmol/mg tissue). At 1 day after surgery, treatment of animals with L-NAME alone produced a significant decrease in cGMP to control levels (8.0 ± 0.8 fmol/mg tissue). Similarly, ZnPPIX suppressed the increase in cGMP, which did not fall below control levels (8.6 ± 0.4 fmol/mg tissue), suggesting a specific inhibitory action of ZnPPIX on heme oxygenase activity rather than on soluble guanylate cyclase activity. Interestingly, only when the 2 inhibitors were used simultaneously did aortic cGMP levels markedly diminish below the control values (1.65 ± 0.1 fmol/mg tissue, *P* < 0.05). In contrast to aortic tissue, hepatic cGMP significantly decreased at 1 day after surgery and returned to basal levels at 5 days after surgery (Figure 7). Furthermore, when the NOS inhibitor L-NAME was injected intravenously in rats at 1 day after surgery…
for the first time that under stress conditions, heme oxygenase–derived CO significantly contributes to the control of pressor responses in vivo. We showed that upregulation of inducible heme oxygenase (HO-1) by surgical stress in vascular and organ tissues correlates with the suppression of hypertensive responses mediated by hemoglobin and L-NAME, 2 agents that are commonly used in in vivo and ex vivo models to block NO functions. The increase in tissue HO-1 expression and activity correlated with increased CO production from aorta and urinary bilirubin excretion, indicating a functional link between tissue HO-1, its active function as a heme degrading protein, and its physiological role in blood pressure regulation.

It is known that hemoglobin avidly binds NO, the endothelium-derived relaxing factor, with an affinity much higher than its affinity for oxygen and can be effectively used as a NO scavenger. In our experimental protocol, we used αHb, a hemoglobin cross-linked between α chains, which is currently investigated as a potential red blood cell substitute. Similar to infusion of unmodified hemoglobin, infusion of αHb in ex vivo and in vivo systems has been shown to produce coronary vasoconstriction and increase in arterial blood pressure. Resuscitation with αHb during treatment of hemorrhagic shock in a water-deprived swine model resulted in systemic and pulmonary hypertension. This effect was mimicked by the NOS inhibitor L-NAME but not by cyanomethemoglobin, which does not bind NO. In the present study, we unexpectedly found that a 50% isovolemic exchange transfusion with αHb in rats did not cause any change in mean arterial pressure. This is apparently in contrast with a previous report by Keipert et al showing that in the same animal model, transfusion with αHb produced an acute increase in systemic blood pressure. In search of a possible explanation for these contradictory results, we noticed that Keipert et al conducted their experiments ≥5 days after femoral artery and venous catheters were implanted to allow αHb infusion and blood pressure monitoring, whereas in our experiments the transfusion was performed 1 day after the surgical intervention. If animals were allowed at least 5 days of postsurgical recovery period, we could then observe a significant increase in blood pressure after the administration of αHb, as Keipert et al did. This suggests that the time after surgical procedures is a critical determinant in the manifestation of the acute hypertensive effect of αHb. Moreover, the fact that αHb, which avidly binds NO with an affinity much higher compared with O₂ or CO, did not produce an increase in mean arterial pressure at 1 day after surgery indicates that NO does not significantly contribute to the suppression of the αHb-mediated hypertensive effect. This was confirmed by experiments with L-NAME showing that administration of the NOS inhibitor did not produce any increase in arterial blood pressure in rats at 1 day after surgery. These findings indicate that under the particular conditions used in our protocol, local effector molecules other than NO play a critical role in the maintenance of the pressor tone. In view of these data, our experimental model of stress provided a tool for investigating the biochemical pathway(s), in addition to the NOS pathway, that may be directly involved in the suppression of hypertensive responses in vivo.
We reasoned that the exquisitely sensitive stress protein HO-1 (HSP32) could be affected in rat tissues by the surgical procedure used for catheter implantation. In fact, we found that surgical stress significantly increased the levels of heme oxygenase in aorta, heart, and liver. The time course of heme oxygenase activity was paralleled by the time course of HO-1 expression, with maximum induction of this stress protein being observed at 24 hours after surgery and gradually decreasing thereafter. This finding is consistent with a 40-fold increase in HO-1 (HSP32) mRNA expression observed in all rat organs after brief exposure of animals to heat shock, another commonly used type of stress.3 We also found that pretreatment of animals with ZnPPIX alone (or L-NAME alone) suppressed the increase in aortic cGMP levels at 1 day after surgery. A recent report showed that in normal conditions, ZnPPIX may have a nonselective effect in inhibiting the activity of NOS enzymes and soluble guanylate cyclase.40 In our experiments conducted at 1 day after surgery, when HO-1 is highly upregulated, ZnPPIX alone (or L-NAME alone) prevented the increase in cGMP levels. However, cGMP did not fall below control values; accordingly, arterial blood pressure remained unchanged after the treatments. These results would suggest that under our experimental conditions, ZnPPIX (50 μmol/kg) inhibits preferentially heme oxygenase activity rather than guanylate cyclase.

It is reasonable to suggest that HO-1-derived CO plays a fundamental role in the regulation of pressor responses after surgical stress. Recent reports have demonstrated that CO serves as an important cellular signaling molecule in major organs, such as brain, liver, and heart,16,18,43 and that the HO-1/CO system may be upregulated in vascular endothelial and smooth muscle cells when appropriately stimulated.21,22,44 Similar to NO, CO is an activator of guanylate cyclase and may function as a vasodilator by regulating the intracellular level of cGMP in smooth muscle cells.13 The main endogenous source of CO in mammalian tissues derives from the degradation of heme by constitutive heme oxygenase (HO-2), and this accounts for almost 95% of the total CO produced in normal conditions.45 This amount can be increased greatly when inducible HO-1 is upregulated in response to a variety of stressful agents that challenge a particular organ or tissue.3 The results of the present study are consistent with the hypothesis that HO-1, once stimulated, can actively use heme as a substrate for CO production. In fact, we found that stress-mediated induction of tissue heme oxygenase was associated with (1) increased CO and cGMP production in aortic tissues, (2) accelerated metabolism of heme, as measured by an increased rate of plasma hemoglobin disappearance, and (3) increased excretion of urinary bilirubin, the end product of heme catabolism. The increase in aortic CO and the augmented catabolism of heme were significantly attenuated by pretreatment of animals with ZnPPIX. In addition, both CO release and aortic cGMP as well as urinary bilirubin returned to basal levels at 5 days after surgery (when heme oxygenase activity was also back to normal values). It needs to be pointed out that the actual amount of CO released from aortas after surgical stress may be higher than the values measured. In fact, it is possible that not all of the [2-14C]-glycine was used for heme synthesis and converted to 14CO, because of a possible incorporation of this amino acid into proteins.

We also found that pretreatment of animals with ZnPPIX alone (or L-NAME alone) suppressed the increase in aortic cGMP levels at 1 day after surgery. A recent report showed that in normal conditions, ZnPPIX may have a nonselective effect in inhibiting the activity of NOS enzymes and soluble guanylate cyclase.40 In our experiments conducted at 1 day after surgery, when HO-1 is highly upregulated, ZnPPIX alone (or L-NAME alone) prevented the increase in cGMP levels. However, cGMP did not fall below control values; accordingly, arterial blood pressure remained unchanged after the treatments. These results would suggest that under our experimental conditions, ZnPPIX (50 μmol/kg) inhibits preferentially heme oxygenase activity rather than guanylate cyclase. It has to be noted that at 1 day after surgery, only the simultaneous inhibition of the cNOS and heme oxygenase pathways (ie, L-NAME+ZnPPIX or hemoglobin+ZnPPIX treatment) resulted in a significant decrease in aortic cGMP below control levels; this effect was accompanied by a substantial increase in mean arterial pressure. These findings together would suggest the existence of a complementary action between NO and CO in the control of vascular tone; in other words, under conditions of elevated HO-1 expression, CO can compensate for decreased NO availability (ie, L-NAME or hemoglobin treatment) in activating guanylate cyclase, whereas by blocking heme oxygenase activity (ie, ZnPPIX treatment), intracellular cGMP levels would be nevertheless maintained by the continuous production of vascular NO. We also observed a lack of correlation between HO-1 induction and intracellular cGMP in hepatic and heart tissues; although these findings remain to be further elucidated, changes in cGMP content in those tissues may not be relevant to explain mechanisms controlling vasomotor tone.

The results reported in the present study imply that regulation of heme oxygenase activity and CO production in vascular and organ tissues may be as important as the modulation of the NO pathways, particularly in clinical situations characterized by a pronounced tissue stress response, such as hemorrhagic and septic shock. A functional link between increased hepatic HO-1 expression after hemorrhage/resuscitation in anesthetized rats and vascular control in the hepatic portal circulation is in line with the work presented here.47 It is also of relevance that increased HO-1 expression has been detected in large blood vessels and small resistance arterioles after treatment of animals with endotoxin.48 This upregulation of HO-1 was present in both endothe-
lial and smooth muscle cells and was associated with a significant in vivo hypotensive effect, which was reversed by administration of ZnPPIX. Recent studies from our group have also established that treatment of aortic rings with a potent inducer of HO-1 resulted in suppression of the contractile response to phenylephrine, an effect that was reversed by inhibitors of heme oxygenase but was unaffected by an inhibitor of cNOS.49

In summary, we provide in the present study the first in vivo evidence that mechanisms of blood pressure regulation after surgical stress involve the HO-1/CO pathway, emphasizing the importance of a versatile physiological role for heme oxygenase in addition to its established function in heme catabolism.

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