Selective Regulation of Blood Pressure by Heme Oxygenase-1 in Hypertension

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Abstract—Heme oxygenase (HO) and carbon monoxide (CO) participate in the homeostatic control of cardiovascular functions, including the regulation of blood pressure (BP). Upregulation of the HO/CO system has been shown to lower BP in young (8 weeks) but not in adult (20 weeks) spontaneously hypertensive rats (SHR). The underlying mechanism for this selective effect, however, has been unknown and was investigated in the present study. The administration of hemin resulted in a marked decrease in BP (from 148.6±3.2 to 125.8±2.6 mm Hg, P<0.01) in young but not in prehypertensive (4 weeks) or adult SHR or Wistar-Kyoto rats at all ages. The inhibition of HO with chromium mesoporphyrin abrogated the BP-lowering effect of hemin. Significantly lower expression levels of HO-1 and soluble gyanylyl cyclase (sGC) as well as reduced cGMP content were detected in 8-week SHR but not in adult SHR or Wistar-Kyoto rats of all ages. These deficiencies were all corrected by hemin treatment. The expression of HO-2 protein was not different among all animal groups tested and not affected by hemin treatment. Desensitization of the sGC/cGMP pathway in adult SHR was demonstrated by the reduced vasorelaxant potency of the sGC activator 3-(5'-hydroxymethyl-2'-furlyl)-1-benzylindazole. Thus, in young and prehypertensive SHR, a defective HO/CO-sGC/cGMP system might constitute a pathogenic mechanism for the development of hypertension. The HO/CO-sGC/cGMP system appears normal in adult SHR, but desensitization of the downstream targets of the system to sGC/cGMP may endow SHR at this stage a persistent hypertension status. (Hypertension. 2002;40:315-321.)

Key Words: cyclic GMP ▪ hypertension, experimental ▪ rats, spontaneously hypertensive ▪ mesenteric arteries ▪ heme oxygenase

The homeostatic control of biological functions is maintained by a wide array of metabolic pathways capable of triggering the necessary corrective mechanisms to maintain cellular functions within physiological limits. Among these metabolic pathways is the catalytic breakdown of heme by heme oxygenase (HO) to carbon monoxide (CO), bilirubin, and iron. HO is a microsomal enzyme with three distinct isoforms, namely, inducible (HO-1) and constitutive forms (HO-2 and HO-3).1 HO-1 is a 32-kDa protein that is not constitutively present in cells but expressed after exposure of cells to different stimuli.2 This enzyme is believed to play a predominant role for CO generation during pathophysiological episodes. HO-2 is a 36-kDa protein that is normally expressed in many organs under physiological conditions.3 For example, HO-2 expressed in endothelial and smooth muscle layers of blood vessels generates CO that intrinsically modulates vascular tone.4 HO-3 (33 kDa) shares ~90% homology with HO-2.1 HO-3 is devoid of catalytic activity and considered important in the regulation of heme proteins.1

CO has been shown to induce relaxation of vascular smooth muscle cells (VSMC) by stimulating soluble gyanylyl cyclase (sGC), opening calcium-activated K+ channels, and acting on cytochrome P450.5 sGC is a heme-containing heterodimer composed of α and β subunits. α1 and β1 of the catalytic domain have molecular weights of 82 kDa and 70 kDa, respectively, whereas the regulatory domain is made up of α2 and β2 subunits. The activation of sGC converts GTP to cGMP. Intracellular cGMP regulates biological functions by activating cGMP-dependent protein kinases, directly gating ion channels,5,6 or regulating phosphodiesterase hydrolysis. The activation of the sGC/cGMP pathway is one of major mechanisms for CO-induced vasorelaxation. The synchronized activities of the HO/CO-sGC/cGMP system may constitute an important metabolic pathway in the modulation of blood pressure (BP).

The HO/CO metabolic pathway is believed to be involved in the regulation of basal tone of resistance blood vessels,7 growth of VSMC, and vascular remodeling.8 Many recent reports have shown that VSMC constitute a major site for HO-1 induction.9 Examples include hemin-induced dilatation of small arteries of the peritoneum10 and HO-1 induction in VSMC of human atherosclerotic lesions.11 Also, CO produced from heme metabolism in blood vessels12 was reported to elicit relaxation13 through the elevation of cGMP levels.14 Several studies have shown that upregulating the HO/CO system lowers BP in young15,16 (8 weeks) but not in adult (20 weeks).
weeks) spontaneously hypertensive rats (SHR).\textsuperscript{17,18} In young SHR, BP elevates and continues to increase with aging, whereas adult SHR have an established hypertension.\textsuperscript{17} If the HO/CO system were defective in young SHR, HO-1 inducers could enhance the activity of this system. Should the HO/CO system\textsuperscript{17} be already enhanced to certain level as a compensatory reaction, however, HO-1 inducers might not be able to upregulate this system any further.

To date, no systematic study has been carried out to correlate the actual expression levels of HO-1 and sGC proteins in VSMC as well as the HO-1–induced cGMP production to the developmental stages of hypertension. The present study was aimed at delineating the mechanisms underlying the selective effect of HO-1 inducers on the development of hypertension in young and adult SHR. To this end, the expression and function of the HO/CO-sGC/cGMP system in mesenteric artery beds from SHR and Wistar-Kyoto rats (WKY) at different stages of hypertension were examined, considering the important role of peripheral blood vessels such as the mesenteric artery\textsuperscript{19,20} in controlling the peripheral resistance in hypertension.

Methods

Animal and Tissue Preparation

Male SHR and WKY were obtained from Charles River Laboratories (Wilmington, Mass). They were housed, acclimated, and studied with appropriate approval as indicated previously.\textsuperscript{21} Hemin (Sigma) was administered to the animals (15 mg/kg per day IP) for 4 consecutive days as described in previous studies.\textsuperscript{15,16} In some animals, chromium mesoporphyrin (CrMP) (Porphyrin Products) was also administered (4 mg/kg per day IP)\textsuperscript{22} before hemin treatment daily. Hemin and CrMP were freshly dissolved in 0.1 mol/L NaOH, adjusted to pH 7.4 with 0.1 mol/L HCl, diluted 1:10 with phosphate buffer.\textsuperscript{16}

A total of 80 animals (40 WKY and 40 SHR) were used in this study. The 4-week old WKY weighed 111 ± 1 g and the age-matched SHR weighed 95 ± 3 g; the 8-week old WKY and age-matched SHR weighed 197 ± 6 g and 180 ± 4 g. The 20-week WKY and SHR weighed 340 ± 7 g and 300 ± 4 g, respectively.

Systolic blood BP was determined in conscious rats by means of the standard tail-cuff method (model-29 SSP NIB) after acclimatization for 2 consecutive days before and 1 day after the 4-day treatment regime with hemin alone or hemin plus CrMP.\textsuperscript{19} Animals were anesthetized with sodium pentobarbital (50 mg/kg body wt) before they were killed; the mesenteric artery was isolated in ice-cold PBS, cleaned, and snap-frozen in liquid nitrogen. The composition of PBS was as follows (mmol/L): NaCl 140, KCl 3, Na2HPO4 10, and KH2PO4 2.

The tension development of the isolated rat aortic tissues was measured as previously described.\textsuperscript{5,21} 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1) (Sigma) was dissolved in dimethyl sulfoxide and diluted with the bath solution. The cumulative dose-response curves of YC-1 on the phenylephrine-precontracted (0.3 μmol/L) tissues were constructed.

Western Immunoblotting for HO-1, HO-2, and sGC

Previously frozen mesenteric artery was homogenized in the presence of freshly added cocktail of protease inhibitors as previously described.\textsuperscript{23} The expression of HO-1, HO-2, and sGC proteins was assayed by following the procedure established previously.\textsuperscript{23}

Measurement of HO Activity and cGMP Content

HO activity of mesenteric artery tissues was measured with bilirubin production as reported by Taylor et al.\textsuperscript{23} The amount of bilirubin in each sample was determined spectrophotometrically at 560 nm, using a Total Bilirubin Kit (Sigma). The concentration of cGMP was determined by a radioimmunoassay kit (\textsuperscript{3}H-cGMP-RIA, Amersham International plc), following instructions from the manufacturer and the established protocols from the previous publications.\textsuperscript{23,26}

Statistical Analysis

All data were expressed as mean ± SEM from at least 3 independent experiments performed in duplicate. Statistical analyses were done with the unpaired Student’s t test, ANOVA in conjunction with the Newman-Keuls test, and ANOVA for repeated measures where appropriate. Differences at the level of $P<0.05$ were considered statistically significant.

Results

Effect of Hemin on BP of SHR and Age-Matched WKY

Blood pressure of 8-week young SHR than in age-matched WKY ($P<0.01$) but significantly lowered by hemin. CrMP increased BP of SHR but not of WKY. *$P<0.01$ vs untreated SHR. B, BP of adult SHR and WKY (20 weeks) remained unchanged after hemin treatment. †$P<0.01$ vs WKY. C, Hemin treatment had no effect on BP of prehypertensive (4 weeks) SHR. Each group contained 10 animals except that 5 were included in each group of CrMP treatment.

Figure 1. Systolic BP of SHR and aged-matched WKY with different treatments. Six different measurements were taken from each animal, and mean systolic BP was determined. A, BP was higher in 8-week young SHR than in age-matched WKY ($P<0.01$) but significantly lowered by hemin. CrMP increased BP of SHR but not of WKY. *$P<0.01$ vs untreated SHR. B, BP of adult SHR and WKY (20 weeks) remained unchanged after hemin treatment. †$P<0.01$ vs WKY. C, Hemin treatment had no effect on BP of prehypertensive (4 weeks) SHR. Each group contained 10 animals except that 5 were included in each group of CrMP treatment.

Effect of Hemin on BP of SHR and Age-Matched WKY

Blood pressure of 8-week SHR was significantly higher (148.6±3.2 mm Hg) than that of the age-matched WKY ($P<0.01$) (Figure 1A) but lower than that of 20-week SHR (182.5±4.6 mm Hg) ($P<0.05$) (Figure 1B). Hemin treatment reduced the high BP to normal level ($P<0.01$) in 8-week SHR (Figure 1A). There were no changes in BP of 8-week WKY (127.4±1.5 mm Hg) after the injection of hemin (125.7±2.5 mm Hg) (Figure 1A). CrMP treatment alone
increased BP in 8-week SHR but not in the age-matched WKY. Furthermore, the BP-lowering effect of hemin in 8-week SHR was abolished when the animals were simultaneously treated with CrMP (Figure 1A).

To understand whether the BP-lowering effect of hemin was related to the age and BP levels of animals, hemin treatment was also applied to other two groups of rats. Hemin treatment did not alter the high BP level in 20-week SHR (182.5 ± 4.6 versus 198.1 ± 2.7 mm Hg) (Figure 1B) nor the normal BP levels in 4-week SHR (Figure 1C). Similarly, BP of 4-week and 20-week WKY was not affected by hemin treatment.

Effects of Hemin on Expression of HO-1 and HO-2 in Mesenteric Artery From SHR and Age-Matched WKY

The basal level of HO-1 protein was much lower in young SHR than in adult SHR or WKY of all ages (Figure 2). The expression of HO-1 protein was significantly enhanced in the mesenteric artery of the hemin-treated young SHR in comparison to that of untreated young SHR. Hemin treatment did not increase the expression of HO-1 protein from adult SHR or WKY of all ages. On the other hand, HO-2 protein expression levels in the mesenteric artery were not different between SHR and WKY at all ages; neither did hemin treatment affect the expression of HO-2 in these animals (Figure 3).

Effect of Hemin on HO Activity in Mesenteric Artery From Young SHR and Age-Matched WKY

The basal HO activities in mesenteric arteries from 8-week SHR were similar to that of age-matched WKY (Figure 4).

The function of the HO-CO system depends on the product of the total amount of HO proteins and the activity of the unit HO protein. Thus, a lower expression level of total HO-1 protein in VSMC of young SHR than that of age-matched WKY (Figure 2) would still lead to a reduced function of the HO-CO system. Hemin treatment increased HO activity more significantly in tissues from SHR by 3.2 ± 0.3-fold than in tissues from WKY by 2.3 ± 0.28-fold ($P<0.01$) (Figure 4). Coadministration of CrMP with hemin to the animals annullled the increase in HO activity induced by hemin alone in both groups of rats (Figure 4).

Effect of Hemin on Expression of sGC Protein in Mesenteric Artery of SHR and Age-Matched WKY

The basal level of sGC protein expression in young SHR was significantly lower than that of the age-matched WKY,
whereas there was no difference between adult SHR and young or adult WKY (Figure 5). In prehypertensive 4-week SHR, the sGC expression level was also lower than that of the age-matched WKY (Figure 6). Hemin administration significantly increased the sGC expression by 60±3.2% and 76.5±4.5% in 8-week-old and 4-week-old SHR, respectively (P<0.05), but not in the age-matched WKY (Figures 5 and 6). In contrast, the expression levels of sGC in adult SHR and the age-matched WKY remained unaltered after hemin treatment (Figure 5).

**Effect of Hemin on cGMP Contents in Mesenteric Artery of SHR and Age-Matched WKY**

The basal cGMP contents from adult SHR and adult or young WKY were similar (Figure 7A). However, cGMP content in the mesenteric artery from 8-week SHR was significantly lower than the age-matched WKY. After treatment with hemin, this lower cGMP content was reversed to a level higher than all other groups (Figure 7A). Hemin treatment did not alter the cGMP contents of vascular tissues from adult SHR as well as young and adult WKY (Figure 7A). To explore if the low cGMP content in young SHR was secondary to an elevated BP level, we measured cGMP content in prehypertensive 4-week SHR and age-matched WKY that received similar treatment as the young and adult rats (Figure 7B). Basal cGMP content in 4-week SHR was lower than the age-matched WKY. Hemin treatment increased the cGMP contents of tissues from both SHR and WKY to the same elevated level, but the increase in tissues from 4-week SHR was much more significant (77.8±6.3%) than that from 4-week WKY (42.6±2.1%) (P<0.05).

**Vasorelaxant Effects of YC-1 on Vascular Tissues From SHR and Age-Matched WKY**

To examine the hypothesized desensitization of the sGC/cGMP pathway in VSMC of adult SHR, we used a CO-
NO-independent activator of sGC, \textsuperscript{27,28} YC-1, to relax the precontracted tissues. As shown in Figure 8A, the dose-response curve of YC-1 for 20-week SHR shifted to the right of that for 8-week SHR. In contrast, the dose-response curves of YC-1 of 8-week and 20-week WKY were superimposed (Figure 8B). Moreover, the maximal vasorelaxant effect of YC-1 of 8-week and 20-week WKY were superimposed on that for 8-week SHR. In contrast, the dose-response curves of YC-1 for 20-week SHR shifted to the right precontracted tissues. As shown in Figure 8A, the dose-response curve of YC-1 for 20-week SHR shifted to the right of that for 8-week SHR. In contrast, the dose-response curves of YC-1 of 8-week and 20-week WKY were superimposed (Figure 8B). Moreover, the maximal vasorelaxant effect of YC-1 was more pronounced in tissues from 8-week SHR than that from the age-matched WKY. These results indicate that the sGC/cGMP pathway in 8-week SHR is oversensitized but desensitized in 20-week SHR as compared with their relative age-matched WKY rats.

**Discussion**

The present investigation demonstrates that the basal expression levels of HO-1 and sGC proteins as well as cGMP contents were lower but BP was significantly higher in 8-week SHR than in the age-matched WKY. Our study also provided evidence that the malfunction of the HO/CO-sGC/cGMP system was not secondary to the increased BP because in 4-week SHR, of which BP was still within the normotensive range, sGC level and cGMP content were already lower than in the age-matched WKY rats (Figures 6 and 7). After a 4-day regimen of hemin treatment, BP of 8-week SHR was lowered to a normotensive (physiological) level. This normalized BP level was accompanied by elevated expressions of HO-1 and sGC proteins and increased cGMP contents in mesenteric arteries after hemin treatment. These observations intimate a link between a defective HO/CO-sGC/cGMP system and hypertension in 8-week young SHR. It is possible that hemin induced the expression of HO-1, leading to the increased HO activity and enhanced CO production. The latter in turn activated sGC to promote the conversion of GTP to cGMP. The increased cGMP content elicited vasorelaxation and lowered BP by multiple mechanisms including sequestering intracellular calcium concentration, \textsuperscript{13,29} altering the phosphorylation of cellular proteins involved in contraction, and inhibiting voltage-dependent calcium channels in VSMC. \textsuperscript{13,29} Interestingly, hemin treatment of 4-week prehypertensive SHR did not affect BP in these rats, although sGC level and cGMP content were significantly enhanced. The results from both 8-week and 4-week SHR demonstrated that the upregulation of the HO/CO-sGC/cGMP system by hemin is not dependent on BP level as long as the basal activity of this system is low. An intrinsic rather than hypertension-induced defective HO/CO-sGC/cGMP system in SHR is thus postulated to be one pathogenesis of hypertension in this animal model. The manifestation of the consequence of this deflection, that is, hypertension, may have a period of latency of at least 4 weeks after birth. Eventually, the impaired HO/CO-sGC/cGMP system outplays the endogenous compensatory mechanisms and hypertension emerges.

One novel observation of our study was that the lower sGC expression in 4- and 8-week SHR was enhanced by hemin treatment. This could be explained by a direct effect of hemin on the transcription and/or translation of sGC. Alternatively, CO generated as the result of the hemin-enhanced HO activity may affect the expression of sGC. The latter mechanism appears to be very attractive considering the fact that sGC is one of major targets of CO. To our knowledge, the regulation of sGC expression by CO has never been reported, though this may represent an important feedback mechanism by which CO can selectively control both the activity and expression of sGC, especially when the expression of sGC is impaired such as in young and juvenile SHR.

The direct implication of the HO/CO system in the BP-lowering effect of hemin could be challenged considering the multiple enzymes that can be regulated by hemin, such as the activation of thioredoxin gene. \textsuperscript{30} In our study, CrMP, a potent HO inhibitor without photoreactivity, \textsuperscript{22} was administered alone or in combination with hemin. CrMP alone increased BP significantly in 8-week SHR. Interestingly, BP of 8-week WKY was not affected by this long-term treatment with CrMP. Similar results of unaltered BP after administration of HO inhibitors have been reported in normotensive rats, such as Sprague-Dawley rats\textsuperscript{31,32} and Wistar rats. \textsuperscript{33} The mechanisms for this differential effect of HO inhibitors on BP of hypertensive and normotensive rats cannot be readily answered. Importantly, once the HO activity was abolished by CrMP in young SHR, hemin no longer had the capability to lower BP. Together with the observation that CrMP abolished the hemin-enhanced HO activity (Figure 4), one can conclude that the hemin-induced decrease in BP in young SHR is more likely to be the consequence of an upregulated expression of HO-1 and less likely to be due to the heme accumulation or the involvement of other pathways.

Our studies also indicate that the basal levels of HO-1 and sGC expression and cGMP contents in adult SHR were no different from those of age-matched WKY, although the former had hypertension but the latter was normotensive. This paradoxical observation could be explained if the basal...
levels of HO-1 and sGC proteins in adult SHR are significantly elevated from the previously lower level, as seen in juvenile and young SHR to compensate the elevated BP level. This compensatory increase in HO-1 and sGC expression as well as cGMP contents is not only insufficient to lower BP in adult SHR but also decreases the sensitivity of the HO/CO-sGC/cGMP system to the specific modulators. To echo this idea, hemin treatment serves well as exhibit A. Our data clearly showed that hemin treatment did not alter the expression levels of HO-1 and sGC proteins nor the cGMP content and BP in adult SHR. The desensitization of the downstream targets of cGMP, which constitutes an important mechanism in the pathogenesis of hypertension, can be called exhibit B. Our in vitro study revealed that the vasorelaxant effect of YC-1 was reduced in adult SHR. A rightward shift of the dose-response curve for 20-week SHR signified that a greater dose of YC-1 is necessary to evoke the same response as compared with young SHR and WKY of all ages. Thus, a defective and desensitized sGC/cGMP system in adult SHR could partially explain the inability of the sGC/cGMP metabolic pathway in adult SHR to lower BP. The involvement of a desensitized sGC/cGMP metabolic pathway has been extensively reported recently in VSMC from adult SHR and in cultured rat VSMCs after the exposure to IBMX or folskolin. Moreover, tissue-specific downregulation of elements of the sGC/cGMP metabolic pathway was suggested as the mechanism for the impairment of endothelium-independent but cGMP-mediated vasorelaxation in adult SHR. Similarly, genetic hypertension and age were associated with a defective relaxation of smooth muscle at the level or downstream of sGC. Taken together, a compensatory increase in the expression and activity of the HO/CO-sGC/cGMP system in the mesenteric artery of adult SHR (compared with young SHR) may result in a desensitization of the whole system. As such, a decreased vasorelaxation and consequently increased vascular tone would be encountered, although HO-1 level, CO production, and cGMP level might still be within a normal range.

Collectively, our results indicate that a functional HO/CO-sGC/cGMP system is closely related to the developmental stages of genetic hypertension. The HO/CO-sGC/cGMP system might have a lower functional status in young SHR, leading to the development of hypertension. In adult SHR, the HO/CO-sGC/cGMP system appears to be normal, but desensitization of downstream targets to cGMP may nullify the antihypertensive effects of this system. Thus, the activation of a functional HO/CO-sGC/cGMP system serves to lower BP if the cGMP targets remain normally sensitive to cGMP, and the failure of this system might yield lesser vasodilatory influence that counteracts hypertension.

Since CO, bilirubin, and iron are the end products of the HO enzymatic activity, it is conceivable that the antihypertensive effect of hemin, which upregulates HO expression, may be attributed to any of these products. In fact, our study has shown that bilirubin level was significantly elevated in mesenteric artery from hemin-treated animals (Figure 4). Being powerful antioxidants, bilirubin and biliverdin may reduce the severity of hypertension by lowering the elevated oxidative stress, inhibiting lipid peroxidation, and/or by increasing the half-life of nitric oxide. The evidence against the direct contribution of biliverdin was presented, however, because the injection of this antioxidant did not lower BP, whereas CO did.

The focus of our study was on the role of HO/CO-sGC/cGMP pathway in the pathogenesis of hypertension. However, this by no means excludes the involvement of other equally important modulatory systems that might be closed related to the function of HO/CO system. For instance, it has been well documented that HO inducers are implicated in the cytochrome P-450–dependent arachidonic acid metabolic pathway. Since heme constitutes the prosthetic moiety of many cytochrome enzymes, by degrading heme, HO controls the quantity of these enzymes, including cytochrome P-450 monooxygenase. Furthermore, CO has been reported to inhibit the activity of cytochrome P450 oxygenases. In this regard, the upregulated HO/CO system may well be accompanied by reduced formation of powerful vasoconstrictive substances such as 20-hydroxyeicosatetraenoic acids, thus ameliorating the development of hypertension.

Perspectives

The observed BP-lowering effect in young SHR of hemin might be indicative of a potential therapeutic window at the prehypertension or even early hypertension stages before the persistent hypertension is fully developed at the adulthood. The clinical implication of our results is remote but deserves comments. First, although our conclusion is based on the acute effect of hemin treatment, one study has shown that chronic treatment of 5-week SHR with another HO inducer, SnCl2, for 5 to 20 weeks prevented the hypertension development. Second, the application of HO-1 inducers in the treatment of hypertension depends on the determination of the specific “window” time. With the advanced knowledge of human genomics, it soon may be possible to diagnose essential hypertension well before the high blood pressure is fully manifested. This will set the stage for an early intervention with HO-1 inducers. Third, human studies need to be completed for a better understanding of the correlation of the HO-1 expression level with BP level in patients with essential hypertension. These data are currently unavailable but are essential for introducing HO-1 inducers in the clinical practice for the prevention and treatment of human hypertension.

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