Blood Vessels

Upregulation of Heme Oxygenase 1 by Hemin Impairs Endothelium-Dependent Contractions in the Aorta of the Spontaneously Hypertensive Rat

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See Editorial Commentary, pp 772–773

Abstract—Heme oxygenase converts heme to carbon monoxide, biliverdin (subsequently converted to bilirubin), and free iron. Pharmacological induction of heme oxygenase 1 has an antihypertensive effect in the spontaneously hypertensive rat. The present study investigated whether upregulation of heme oxygenase 1 by hemin reduces endothelial dysfunction in this animal. Thirty-six-week-old rats were divided into a heme treatment (50 mg/kg, IP injection, once) and a control group. Aortas were isolated for the measurement of isometric tension, production of reactive oxygen species, and heme oxygenase activity, as well as gene and protein expressions. Hemin treatment augmented the expression and activity of heme oxygenase 1. This in vivo induction of heme oxygenase 1, but not in vitro incubation with the heme oxygenase products carbon monoxide or bilirubin, led to an improvement of endothelial function in that acetylcholine-induced relaxations were potentiated and acetylcholine- and calcium ionophore-induced contractions were attenuated. Free radical production was suppressed by heme treatment, judging from the results of 2′,7’-dichlorodihydrofluorescein diacetate staining, dihydroethidium staining, and lucigenin chemiluminescence, which was explained by the decreased expressions of NADPH oxidase 2 and cyclooxygenase 1. The production of prostacyclin was decreased by heme oxygenase 1 induction, which was explained by a lower expression of cyclooxygenase 1. Contractions to vasoconstrictor concentrations of prostacyclin and its mimetic iloprost were attenuated, suggesting that the responsiveness of thromboxane-prostanoid receptors to prostacyclin was decreased in hemin-treated rats. The suppressed production of free radicals and prostacyclin and the decrease of thromboxane-prostanoid receptors sensitivity concur to explain the impairment of endothelium-dependent contractions caused by heme oxygenase 1 induction by hemin. (Hypertension. 2011;58:926-934.) * Online Data Supplement

Key Words: endothelial dysfunction ■ endothelium-dependent contractions ■ heme oxygenase 1 ■ hemin ■ prostacyclin ■ reactive oxygen species ■ spontaneously hypertensive rat ■ thromboxane-prostanoid receptor

The endothelial cell layer contributes to the regulation of vascular tone by releasing vasodilators, in particular NO.1 However, the endothelium becomes dysfunctional in aging, hypertension, and diabetes mellitus.2 Endothelial dysfunction is characterized by a decreased production of NO, as well as an increased generation of vasoconstrictors, in particular cyclooxygenase (COX)-derived prostanoids and reactive oxygen species (ROS), termed endothelium-dependent contracting factors (EDCFs).3 The production of EDCFs is a hallmark of endothelial dysfunction in the spontaneously hypertensive rat (SHR).3 Indeed, in the endothelium of the SHR aorta, there is an increased expression of COX-1, increased intracellular calcium concentration, and enhanced production of ROS, endoperoxides, and other prostanoids, which underlies the greater occurrence of endothelium-dependent contractions.3 The increase of endothelial cytosolic calcium, in response to agonists like acetylcholine, initiates the activation of calcium-dependent phospholipase A2, which produces the arachidonic acid needed by endothelial COX-1 to produce prostanoids.3 ROS activate COX-1 in the smooth muscle to further produce prostanoids.3–5 Those prostanoids, among which prostacyclin is the most abundant,6 stimulate thromboxane-prostanoid (TP) receptors located on the smooth muscle to evoke contractions.3 In the SHR aorta, the loss of responsiveness of prostaglandin I2 (IP) receptors directs prostacyclin toward TP receptors so that it no longer causes relaxations but rather contractions.3,6,7

Heme oxygenase (HO) is the rate-limiting enzyme in heme degradation, catalyzing the cleavage of heme ring to produce carbon monoxide (CO), biliverdin (subsequently converted to
bile pigments, including bilirubin, and the release of free iron. Two isoforms of HO exist: the inducible isofrom HO-1 and the constitutive isoform HO-2. HO-1 can be induced as a general response to a number of physical (eg, oxidative stress, heat shock, and hypoxia) and chemical (eg, hemin and metalloporphyrins) stimuli.8,9 Uprogulation of HO-1 lowers arterial blood pressure in the SHR.10–13 However, it is still unknown whether this antihypertensive effect is accompanied by an improvement in endothelial function. The present study was designed to investigate whether HO-1 induction, using the pharmacological agent hemin, improves endothelial function in the SHR. In view of the critical role of EDCF in endothelial function, we focused on the effect of HO-1 induction on endothelium-dependent contractions.

Materials and Methods

Animals and Tissue Preparation

The experiments were conducted in 36- to 42-week-old male SHRs (380–450 g). All of the animal experimental procedures were approved by the University of Hong Kong Committee on the Use of Live Animals for Teaching and Research and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (publication No. 85–23, revised 1996). The SHRs were randomly divided into 2 groups: the treated group received a single IP injection of hemin (50 mg·kg⁻¹, given IP) and euthanized. The thoracic aortas of the animals were dissected as described in the online Data Supplement (please see http://hyper.ahajournals.org).

Isometric Tension Measurement

The aortic rings were suspended in organ chambers for isometric tension recording. Endothelium-dependent relaxations, endothelium-dependent contractions, and contractions to TP receptor agonist 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin F₂α (U46619), the prostacyclin mimetic iloprost and prostacyclin, were studied as described in the online Data Supplement, which also lists the pharmacological agents tested and their source.

RT-PCR

mRNA expression levels of HO-1, TP receptors, and IP receptors in aortas of the animals were detected by RT-PCR (see the online Data Supplement).

Western Immunoblotting

Protein expression levels of HO-1, HO-2, COX-1, COX-2, prosta-cyclin synthase, Nox1, Nox2, Nox4, TP receptor, phospho-endothelial NO synthase (eNOS), and total eNOS were detected by Western blotting (see the online Data Supplement).

Measurement of HO Activity

HO activity was estimated by the amount of bilirubin (the stable end product of HO, including HO-1 and HO-2). Details can be found in the online Data Supplement.

Immunohistochemistry

The presence of HO-1 in aortic frozen sections was determined by immunohistochemical staining, as described in the online Data Supplement.

Measurement of ROS

The endothelium-derived ROS were measured with 2′,7′-dichlorodihydrofluorescin diacetate (DCF) with confocal microscopy. To measure superoxide production, frozen cross-sections of aortas (10 μm) were stained with dihydroethidium by using a previously validated method.14 The production of superoxide-dependent on NADPH oxidase in the rat aorta was measured by lucigenin chemiluminescence.15–17 Details can be found in the online Data Supplement.

Release of 6-Keto Prostaglandin F₁α, Prostaglandin F₂α, and Thromboxane B₂

The amounts of 6-keto prostaglandin F₁α, prostaglandin F₂α, and thromboxane B₂ were measured by enzyme immunoassay, as described in the online Data Supplement.

Figure 1. A, RT-PCR analysis for mRNA expression of HO-1 and (B) Western blotting analysis for protein expression of HO-1 in the aortas of control and hemin-treated rats. C, HO activity analysis in the aortas of control and hemin-treated rats. D, Immunohistochemical staining for HO-1 (green, shown by arrows) and 4′,6-diamidino-2-phenylindole (DAPI, blue) in the aortic frozen sections of control and hemin-treated rats. “L” stands for lumen. Data are shown as mean±SEM; *P<0.05 vs the control rats. n=5 for PCR; n=5 for Western blotting; n=6 for HO activity; n=4 for immunohistochemistry.
Data Analysis
Data are presented as mean±SEM; n refers to the number of individual observations in preparations from different rats. Statistical analysis was performed using 1- or 2-way ANOVA or Student t test. P values ≤0.05 were considered to indicate statistically significant differences. More details are available in the online Data Supplement.

Results
HO-1 Expression and Activity in Hemin Treatment
The in vivo treatment with hemin caused, after 24 hours, a significant upregulation of the expression of HO-1 both at the mRNA and protein levels (Figure 1A and 1B) but did not alter the protein expression of HO-2 (Figure S1, available in the online Data Supplement). Hemin treatment increased HO activity by almost 5-fold (Figure 1C). HO-1 protein was present in both the endothelial cells and the smooth muscle cells and showed a stronger fluorescent signal in aortas of hemin-treated rats, as indicated by immunohistochemical staining (Figure 1D).

Endothelium-Dependent Relaxations
In SHR rings contracted with phenylephrine (10^-6.0 M), the concentration-response curve to acetylcholine was triphasic (concentration-dependent relaxation from 10^-10.0 to 10^-7.0 M, followed by a secondary contraction between 10^-6.5 and 10^-5.0 M, and then a tertiary relaxation from 10^-5.0 M onward). In aortas of hemin-treated SHR, the secondary contractile phase of the acetylcholine concentration-response curve was reduced significantly, whereas the 2 relaxation phases showed no significant differences compared with those in preparations from control rats (Figure 2A).

In vitro incubation with the CO-releasing molecule (10^-9 to 10^-5 M) or bilirubin (10^-8 to 10^-6 M) for 40 minutes did not significantly alter endothelium-dependent relaxations induced by acetylcholine in aortas of untreated SHRs (effect of CO-releasing molecule 10^-6 M and bilirubin 10^-6 M was shown in Figure 3A; data of other concentrations were not shown). In aortic rings of in vivo hemin-treated rats, acute incubation with the HO inhibitor ZnDPBG (10^-5 M) did not reverse the effect of hemin treatment on endothelium-dependent relaxations (Figure 3B).

Endothelium-Dependent Contractions
In the presence of N^G-nitro-L-arginine methyl ester (10^-4 M), acetylcholine, or the calcium ionophore, A23187 induced endothelium-dependent contractions in quiescent rings of SHR aortas. Hemin treatment significantly attenuated the contractions to the 2 agonists (Figure 2B).

In vitro treatment with either the CO donor CO-releasing molecule or bilirubin did not alter contractions in response to acetylcholine or A23187 in aortas of untreated SHRs (Figure 3C). Likewise, acute in vitro incubation with the HO inhibitor ZnDPBG (10^-5 M) did not reverse the effect of hemin treatment on endothelium-dependent contractions (Figure 3D).

Expression of eNOS
The protein expression levels of both the total and phosphorylated eNOS (Ser1177) were comparable in aortas from control and hemin-treated rats (Figure S2).

Production of ROS
To evaluate the production of endothelium-derived ROS, fluorescence of the nonspecific ROS dye DCF was detected with confocal microscopy focusing on the endothelial layer. The basal level of ROS intensity was not different between aortas of control and hemin-treated animals (data not shown). The ROS fluorescence signal on stimulation with acetylcholine was reduced significantly by the hemin treatment compared with control (Figure 4A and 4B).

Vascular ROS intensity was determined in situ in rat aortic sections using dihydroethidium dye. The ROS intensity was significantly decreased in the aortas of hemin-treated rats, not
only in the endothelial layer, but also in the vascular smooth muscle cells (Figure 4C and 4D).

The production of superoxide anions by NADPH oxidase was measured by lucigenin chemiluminescence. A significantly lower luminescence was detected in aortas from hemin-treated rats (Figure 4E).

Expression of Nox1, Nox2, and Nox4
The protein level of Nox2 (Figure 5), but not those of either Nox1 (Figure S3A) or Nox4 (Figure S3B), was significantly reduced in aortas of hemin-treated rats compared with controls.

Production of Prostanoids
A significantly lower release of 6-keto prostaglandin \( \text{F}_{1\alpha} \), the major stable metabolite of prostacyclin, was observed in preparations from the hemin treatment group compared with controls. The release of prostaglandin \( \text{E}_{2} \) and thromboxane \( \text{B}_{2} \) was not significantly different in aortas of the 2 groups (Figure 6A).

Expression of COX-1, COX-2, and Prostacyclin Synthase
Hemin treatment significantly attenuated the protein expression of COX-1 but did not alter that of COX-2. The expression of prostacyclin synthase was not changed significantly by the hemin treatment (Figure 6B).

Contractions to U46619, Iloprost, and Prostaglandin \( \text{I}_{2} \)
The full TP receptor agonist U46619 caused comparable contractions in aortic rings without endothelium from control and hemin-treated rats (Figure 7A). However, contractions elicited by higher concentrations of prostacyclin and the

**Figure 3.** Effect of CO-releasing molecule (CORM) and bilirubin on (A) acetylcholine-induced relaxations; (C) acetylcholine- and A23187-induced contractions in the aortas of control rats; effect of ZnDPBG on (B) acetylcholine-induced relaxations; (D) acetylcholine- and A23187-induced contractions in the aortas of hemin-treated rats. Data are shown as mean±SEM; \( n=6 \).
prostacyclin mimetic iloprost were reduced significantly in preparations from the hemin-treated group compared with controls (Figure 7B and 7C). In preparations with endothelium contracted with phenylephrine (10⁻⁶ M), no relaxations were obtained with prostacyclin or iloprost in preparations of either control or hemin-treated SHRs (data not shown).

Expression of TP and IP Receptors
The expression levels of TP and IP receptors were not significantly different in aortas from control and hemin-treated rats (Figure S4A through S4C).

Discussion
Twenty-four hours after IP injection of hemin, a pharmacological inducer of HO-1, the mRNA and protein expression of HO-1 in the SHR aorta were augmented. This increased expression of HO-1 occurred in both endothelium and smooth muscle. In the present study, the activity of HO (to judge from the production of bilirubin) was enhanced, which can be attributed to the increased expression of HO-1, because that of HO-2 was unchanged by the hemin treatment. These observations demonstrate that the enzyme was upregulated successfully by the hemin treatment used. This HO-1 upregulation resulted in an improvement of endothelial function, because vascular relaxations elicited by the muscarinic receptor agonist acetylcholine were potentiated by the hemin treatment.

This improvement of endothelial function in the SHR cannot be attributed to an increased bioavailability of NO, judging from the following observations: the initial part of the

Figure 4. A, Images of 2',7'-dichlorodihydrofluorescein diacetate (DCF) fluorescence staining taken by confocal microscopy showing reactive oxygen species (ROS) in the endothelial cells of the aortas of control and hemin-treated rats. B, Bar graph showing DCF fluorescence intensity. C, Images of dihydroethidium (DHE) and 4',6-diamidino-2-phenylindole (DAPI) fluorescence staining in the cross-sections of the aortas of control and hemin-treated rats. DHE-stained signals are in red, DAPI-stained nuclei are in blue, and the merged signals are in purple. D, Bar graph showing DHE fluorescence intensity per positive nuclei. E, Lucigenin chemiluminescence of ROS in aortas of control and hemin-treated rats. Data are shown as mean±SEM; *P<0.05 vs the control rats. n=5 for DCF staining; n=6 for DHE staining; n=6 for lucigenin chemiluminescence.

Figure 5. Western blotting analysis for protein expression of Nox2 in the aortas of control and hemin-treated rats. Data are shown as mean±SEM; *P<0.05 vs the control rats. n=4 for control rats; n=5 for hemin-treated rats.
acetylcholine concentration-response curve, which indicates NO-mediated relaxations, was not changed by the hemin treatment; and the expression level of either phosphorylated eNOS (Ser1177) or total eNOS was not altered by the hemin treatment. These observations are in line with the finding that induction of HO-1 did not improve NO- or CO-mediated vascular relaxations in angiotensin II–induced hypertensive mice, although it caused a reduction in arterial blood pressure. However, the present findings diverge from previous observations, indicating that upregulation of HO-1 may

![Figure 6. A. Release of 6-keto prostaglandin F1α, prostaglandin F2α, and thromboxane B2 in response to acetylcholine in aortas of control and hemin-treated rats. Data are shown as mean±SEM; *P<0.05 vs the control rats. n=12. B. Western blotting analysis for protein expression of cyclooxygenase (COX)-1, COX-2, and prostacyclin synthase (PGIS) in the aortas of control and hemin-treated rats. Data are shown as mean±SEM; *P<0.05 vs the control rats. n=5.]

![Figure 7. Contractions induced by (A) U46619, (B) iloprost, and (C) prostaglandin I2 (PGI2) in the aortas without endothelium of control and hemin-treated rats; data are shown as mean±SEM; *P<0.05 vs the control rats; n=6.]

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preserve NO bioavailability by increasing eNOS expression in streptozocin-induced diabetic,\textsuperscript{20} angiotensin II–induced hypertensive,\textsuperscript{21} and old rats.\textsuperscript{22} They also do not support findings suggesting that overproduction of CO caused by overexpression of HO-1 in vascular smooth muscle cells blunts vasodilatation in response to NO.\textsuperscript{23} The present results do not permit further speculation on the reasons for those apparent discrepancies.

The improvement of endothelial function cannot be attributed to the direct acute effects of the HO products CO and bilirubin. This conclusion is based on the following observations: in vitro incubation with a CO donor or bilirubin did not alter acetylcholine-induced relaxations; and acute inhibition of HO in vitro with ZnDPBG did not reverse the effect of hemin treatment on the response to the muscarinic agonist. CO can cause vasodilatation by stimulation of the production of cGMP\textsuperscript{24} and/or the activation of large conductance calcium-activated potassium channels.\textsuperscript{25,26} However, the modest potency of CO in increasing cGMP,\textsuperscript{24} as well as the loss of functional large conductance calcium-activated potassium channels in the hypertensive vasculature,\textsuperscript{27} may help to explain why CO does not contribute to the improved endothelial function after hemin treatment. Bilirubin, although it can act as an antioxidant,\textsuperscript{8,28} also failed to directly augment endothelium-dependent relaxations in the SHR aorta, as indicated by the present studies.

The most likely interpretation for the improvement of endothelial function by hemin treatment is that it curtails endothelium-dependent contractions. This conclusion is supported by the following observations: when hemin treatment potentiated acetylcholine-induced relaxations, it did so in particular by attenuating the secondary contractile phase of the concentration-response curve, which has been attributed to the generation of EDCFs\textsuperscript{18,29}; and contractions of quiescent aortic rings with endothelium induced by acetylcholine and the calcium ionophore A23187 were impaired in preparations of the hemin-treated rats compared with controls. This impairment of endothelium-dependent contractions cannot be attributed to direct effects of HO products and was not reversed by acute HO inhibition. This thus prompts the conclusion that the impairment of endothelium-dependent contractions by hemin results from genomic changes after hemin treatment. Bilirubin, although it suppresses prostacyclin production contributes greatly to the impairment of endothelium-dependent contractions.\textsuperscript{5} Thus, the observation of a lower production of endothelial ROS is consistent with the impairment of endothelium-dependent contractions. In line with these findings, dihydroethidium staining in aortic cross-sections confirmed a reduced presence of ROS in the endothelial layer in hemin-treated rats but also suggests a lower ROS intensity in vascular smooth muscle, which also may contribute to the impaired EDCF-mediated response.\textsuperscript{5}

The reduced ROS production can be attributed to suppression of NADPH oxidase by HO-1. This conclusion is based on the lower expression of Nox2 after hemin treatment, as well as on the lower production of ROS dependent on NADPH oxidase measured by lucigenin chemiluminescence. Nox1, Nox2, and Nox4 are the major isoforms of NADPH oxidase expressed in blood vessels.\textsuperscript{30} Because Nox1 and Nox2 are the most crucial ROS-generating sources in the aorta of the aged SHR,\textsuperscript{31} suppression of Nox2 may contribute importantly to the impaired ROS production resulting from HO-1 induction. Inhibition of NADPH oxidase (mainly Nox2) by HO-1 induction is probably attributed to the subsequent generation of bilirubin.\textsuperscript{32,33} Bilirubin interferes with the membrane binding of p47phox associated with NADPH oxidase activation with the possible involvement of protein kinase C.\textsuperscript{33,34} In line with the present observation that Nox2 is the main target of HO-1 induction, bilirubin probably inhibits Nox1 or Nox2, because regulation by protein kinase C-p47phox is likely to activate NADPH oxidases containing Nox1 or Nox2 but not the Nox4 subunits.\textsuperscript{34,35} In addition, inhibition of Nox2 by HO-1 induction can also probably be attributed to heme degradation, with a resulting limited availability of heme for the maturation of Nox2.\textsuperscript{36}

In the SHR aorta, COX is another potential target of HO-1 induction to impair ROS production. In that preparation, the endothelial ROS production in response to acetylcholine and A23187 depends on the activation of COX.\textsuperscript{4,5} When activated, COX co-oxidizes substances such as NADPH to become a source of superoxide anions.\textsuperscript{7,37} The present observations of the reduced presence of COX-1 may help to explain, at least in part, the impairment of ROS production after the induction of HO-1.

The production of prostacyclin is decreased selectively in response to upregulation of HO-1. This conclusion is based on the observation of a lower release of 6-keto prostaglandin F\textsubscript{1α} (the major stable metabolite of prostacyclin), but not prostaglandin F\textsubscript{2α} or thromboxane B\textsubscript{2} (major stable metabolite of thromboxane A\textsubscript{2}), in aortas of hemin-treated rats. Prostacyclin is the principal metabolite of arachidonic acid and the major contributor to endothelium-dependent contractions in the SHR aorta.\textsuperscript{6,7} The present findings thus imply that suppression of prostacyclin production contributes greatly to the impairment of endothelium-dependent contractions by HO-1 induction. A decrease in prostacyclin production may result from the reduced expressions or activities of COX or prostacyclin synthase (enzyme responsible for the synthesis of prostacyclin downstream of COX activation). The present results with Western blotting revealed a lower expression of COX-1, but not COX-2 or prostacyclin synthase, in preparations of hemin-treated rats. They thus imply that the decreased production of prostacyclin can be attributed to the reduced presence/activity of COX-1. This is in line with previous
findings that COX-1 is the major COX isoenzyme contributing to the generation of prostacyclin in the SHR aorta.3,38,39 Although CO may inhibit cytochrome P450,40,41 a super family that includes prostacyclin synthase, the present results exclude the possibility that the production of prostacyclin is suppressed because of a lower expression of prostacyclin synthase. Because ROS can stimulate COX-1 to produce prostanoids,3,5 the ability of HO-1 to attenuate ROS production may contribute to the suppression of COX-1 activity.

TP receptors are the effectors of endothelium-dependent contractions.5 In view of this critical role of TP receptors in endothelium-dependent contractions, their expression and responsiveness were compared in preparations from control and hemin-treated rats. Based on the PCR and Western blotting results obtained, the mRNA and protein expression levels appear to be comparable in the 2 groups. Likewise, contractions induced by the full TP receptor agonist U46619 were not altered by the hemin treatment, whereas those evoked by prostacyclin (or its mimetic iloprost) were attenuated in preparations of hemin-treated rats. One possible explanation for this finding would be that HO-1 increases the responsiveness of IP receptors, which serve as the functional competitors to TP receptors and cause relaxations of vascular smooth muscle. However, this possibility is excluded, because the mRNA expression of the IP receptor was not altered, and prostacyclin or iloprost did not induce relaxations in aortas of the hemin-treated group. Thus, the likely explanation is that the responsiveness of TP receptors to partial agonists, such as prostacyclin, is impaired by HO-1 upregulation. The lower responsiveness of TP receptors leads to reduced responses to the major EDCF prostacyclin, therefore contributing to impaired endothelium-dependent contractions. How HO-1 affects the responsiveness of TP receptors is uncertain. This may be associated with the antioxidant effect of HO-1, because ROS enhance the stability and increase the density of functional TP receptors at the cell membrane.7,42,43

Limitations
The present study demonstrated the impairment by hemin of endothelium-dependent contractions only in the aorta of the SHR. This is the standard preparation for studying EDCF in aortas but also in smaller arteries.45–47 Studies on human forearm microcirculation in healthy subjects and hypertensive patients further provide evidence that the production of EDCF contributes to the endothelial dysfunction with aging and hypertension.48,49 These findings presumably permit the extrapolation of the effect of hemin observed in the present study on the endothelium of the aorta to that of the resistance arteries.

Perspectives
The main finding of the present study is that upregulation of HO-1 by hemin improves endothelial function in the aorta of SHRs by impairing endothelium-dependent contractions. This beneficial effect of HO-1 induction can be attributed to a suppression of EDCF (ROS and prostacyclin) and a decrease of TP receptor responsiveness. The present findings suggest that if selective pharmacological induction of endothelial HO-1 could be achieved, this may be a potential therapeutic strategy for treating endothelial dysfunction in hypertension.

Sources of Funding
This research is supported by the Hong Kong Research Grant Council (University of Hong Kong, No. 780410M); by Research Centre of Heart, Brain, Hormone and Healthy Aging of the University of Hong Kong; and by the World Class University program (R31-20029) funded by the Ministry of Education, Science, and Technology, South Korea.

Disclosures
None.

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Hypertension. 2011;58:926-934; originally published online September 26, 2011;
doi: 10.1161/HYPERTENSIONAHA.111.173807

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In the *Hypertension* article by Li et al (Li Z, Wang Y, Vanhoutte PM. Upregulation of Heme Oxygenase 1 by Hemin Impairs Endothelium-Dependent Contractions in the Aorta of the Spontaneously Hypertensive Rat. *Hypertension*. 2011;58:926–934), an error was made in the Sources of Funding. The acknowledgment to the Hong Kong Research Grant Council listed a wrong grant number. The correct grant number is 780410M.

The authors regret this error.

These corrections have been made to the current online version of the article, which is available at http://hyper.ahajournals.org/cgi/content/full/58/5/926.
Online Data Supplement

Up-regulation of Heme oxygenase-1 by hemin impairs endothelium-dependent contractions in the aorta of the spontaneously hypertensive rat

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Materials and Methods

Tissue preparation

The thoracic aortae of the animals were dissected free and placed in Krebs-Ringer buffer of the following composition (mM): NaCl 120, KCl 4.76, CaCl$_2$ 2.5, MgSO$_4$ 1.18, NaH$_2$PO$_4$ 1.18, NaHCO$_3$ 25 and glucose 5.5 (control solution). The aortae were cut into rings (3-4 mm length). In some preparations, the endothelium was removed by perfusing the blood vessel with 0.5 ml of Triton (0.5%, 1 ml/min) prior to cutting the rings.

Isometric tension measurement

The aortic rings were suspended in organ chambers, filled with 5 ml of warmed (37°C), aerated (95% O$_2$, 5% CO$_2$) control solution, and were connected to force transducers (ADInstruments, Sydney, Australia) for isometric tension recording (PowerLab, ADInstrument). The rings were stretched to an optimal resting tension of 2.5 g (determined in previous experiments; data not shown) and allowed to equilibrate for 90 minutes. After stabilization, they were exposed twice to 60 mM KCl to obtain a reference contraction. All further increases in tension were expressed in percentage of this reference response. To study relaxations, rings were contracted with $10^{-6}$ M phenylephrine, followed by cumulative addition of acetylcholine ($10^{-10}$ M - $10^{-4}$ M) to obtain concentration-relaxation curves. Relaxations were expressed as percentage of contractions to phenylephrine. To elicit endothelium-dependent contractions, rings were incubated with the eNOS inhibitor N$^\omega$-nitro-L-arginine methyl ester (L-NAME, $10^{-4}$ M; to optimize EDCF-mediated responses) for 40 minutes before the cumulative application of acetylcholine ($10^{-8}$ M - $10^{-5}$ M) or the calcium ionophore 5-[(methylamino)-2-[(2R, 3R, 6S, 8S, 9R, 11R)-3,9,11-trimethyl-8-[(1S)-1-methyl-2-oxo-2-(1H-pyrrol-2-yl)-ethyl]-1,7-dioxaspiro[5.5]undec-2-yl)methyl]-4-benzoxazolcarboxylic acid (A23187; $10^{-8}$ M - $10^{-6}$ M). In some experiments, aortic rings of rats from the control group were incubated with the carbon monoxide releasing molecule tricarbonyldichlororuthenium(II) dimer (CORM, $10^{-9}$ to $10^{-5}$ M) or bilirubin ($10^{-8}$ to $10^{-6}$ M) for 40 minutes before evoking relaxations or contractions; while rings of the hemin-treated rats were incubated with the heme oxygenase inhibitor Zn(II) Deuteroporphyrin IX-2,4-bis-ethylene glycol (ZnDPBG, $10^{-5}$ M). Contractions to the TP receptor agonist 9,11-dideoxy-9$\alpha$,11$\alpha$-methanoepoxy prostaglandin F$_{2\alpha}$ (U46619, $10^{-9}$ M - $10^{-6}$ M), the prostacyclin mimetic iloprost ($10^{-7}$ M - $10^{-5}$ M) or prostacyclin [Prostaglandin I$_2$ (PGI$_2$), $10^{-6}$ M - $10^{-5}$ M] in rings without endothelium were compared in both groups.

RNA extraction and Reverse Transcription-PCR

Aortae were cut into small pieces and exposed to 1 ml of TRIZOL® Reagent for isolation of total RNA according to the manufacturer’s instruction. The RNA was then dissolved in RNase free diethylpyrocarbonate (DEPC)-treated water and quantified using a GeneQuant II spectrophotometer (Pharmacia Biotech, Cambridge, UK). Reverse transcription was carried out in a total volume of 20 μl, comprising 2 μg of total RNA, 4 μl of 5x first strand buffer, 2 μl dithiothreitol (DTT, 0.1 M), 1 μl
deoxynucleoside triphosphate (dNTP, 10 mM), 0.4 μl oligo dT (50 ng/μl), 0.5 μl ribonuclease inhibitor (RNase, 40 U/μl) and 0.2 μl moloney murine leukemia virus reverse transcriptase (M-MLV RT, 200 U/μl). DEPC-treated water was used to make up the total volume. The mixture was left at room temperature for ten minutes followed by incubation at 37°C for 60 minutes. The reaction was stopped by incubation at 95°C for two minutes and the cDNA was stored at -80°C until use. Primers for PCR were HO-1 (155 bp) sense (5'-AGG GAA GGC TTT AAG CTG GT-3') and antisense (5'-AAG GCC ATG TCC TGC TCT A-3'), TP receptor (187 bp) sense (5'-TGG TGC TTC TTG ACT CTT GG-3') and antisense (5'-CCA TCA TCT CCA CCT CAC AG-3'), IP receptor (191 bp) sense (5'-CTGTGTGACACTTTCGCTT-3') and antisense (5'-GGCAGTGAGCAGAAGACA-3'), GAPDH (100 bp) sense (5'-AAT GAC CCC TTC ATT GAC CTC C-3') and antisense (5'-GCT TCC CAT TCT CAG CCT TGA C-3'). The PCR assay was carried out by using 45 μl PCR SuperMix, 1 μl of each primer (10 μM) and 2 μl of the cDNA using the GeneAmp® PCR system 9700 (Applied Biosystems, Warrington, UK). The reaction conditions were as follows: 95°C for 30 seconds for denaturing, 55°C for 30 seconds for annealing and 72°C for one minute for Taq activity. The PCR was performed for 35 cycles with a final ten minutes extension step. PCR products were run on 1% agarose gel containing 0.5 μg/ml gel red in 0.5X Tris/Borate/EDTA (TBE) buffer at 100 V for one hour. The amplified DNA bands were visualized under UV illumination using Gel Doc 1000 (BioRad, Hercules, CA, USA). Densitometric analysis was normalized to the immunoreactive GAPDH band.

**Protein extraction and Western immunoblotting**

Aorta were collected and cut into small pieces and homogenized on ice in lysis buffer (20 mM Tris-HCl, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate) supplemented with a cocktail of protease inhibitors [phenylmethylsulfonyl fluoride (100 mM), trypsin inhibitor (10 μg/ml), leupeptin (1 mg/ml) and pepstatinA (2μg/ml)]. The mixture was sonicated and then centrifuged at 5000 rpm for three minutes at 4°C and the supernatant was kept at -80°C until use. The protein concentration was determined spectrophotometrically using the Bradford protein assay reagent with bovine serum albumin as the standard. Tissue homogenate containing 10 μg protein (for COX-1 and PGIS) or 40 μg protein (for HO-1, HO-2, phospho-eNOS, total eNOS, Nox1, Nox2, Nox4, COX-2 and TP receptor) were mixed with 1x NuPAGE SDS Sample Buffer, 1x reducing agent and ultrapure water to a total volume of 20 μl. They were boiled for five minutes at 95°C and subsequently separated in 10% SDS-PAGE at 100 V, 500 mA for 100 minutes. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes at 300 mA for two hours. Next, the membranes were blocked with Tris buffered saline (TBS) with 5% dry milk for one hour at room temperature, followed by washing in 0.5% Tween TBS buffer (TTBS) three times for ten minutes. The membranes were then incubated with primary antibody (1:1000 HO-1 monoclonal, 1:500 HO-2 polyclonal, 1:1000 phosphor-eNOS (Ser1177) monoclonal, 1:1000 total eNOS monoclonal,
1:300 COX-1 monoclonal, 1:200 COX-2 polyclonal, 1:1000 PGIS polyclonal, 1:250 
Nox1 monoclonal, 1:1000 Nox2 polyclonal, 1:500 Nox4 polyclonal 1:500 TP receptor 
polyclonal, and 1:3000 β-actin monoclonal) in TBS under gentle agitation overnight. 
Membranes were washed three times for ten minutes in TTBS before adding 
secondary antibody. They were then incubated with HRP-conjugated secondary 
anti-rabbit antibody (1:5000) or anti-mouse antibody (1:3000) in TBS for two hours at 
room temperature followed by three washes (ten minutes each) with TTBS. The 
bound secondary antibody was visualized by chemiluminescence using Amersham™ 
ECL™ Western Blotting Detection Reagent (GE Healthcare) and subsequently the 
membranes were exposed to X-ray film (Fuji Super RX medical X-ray film; Fuji photo 
Film, Dusseldorf, Germany). A computer package [MultiAnalysis (BioRad, Hercules, 
CA, USA)] was used to analyze the optical densities of the protein bands. 
Densitometric analysis was normalized to the immunoreactive β-actin band.

Measurement of HO activity
Tissue segments were homogenized (4 ml/g wet weight) in 250 mM sucrose 
containing 50 mM Tris-HCl (pH 7.5). The homogenates were centrifuged at 10,000 g 
for ten minutes at 4°C. The supernatant was then used for measuring HO activity and 
protein amount. HO activity was assessed by bilirubin, the end product of heme 
degradation. Two hundred μg of sample protein were then incubated for 40 minutes 
at 37°C in the dark in a final volume of 200 μl 0.1 M phosphate buffer (pH7.4) 
containing 5 mM MgCl2, 1 mM NADPH, 2 mM glucose 6-phosphate, 1U 
glucose-6-phosphate dehydrogenase, 0.025 mg/ml hemin, and 2 mg of biliverdin 
reductase (prepared from rat liver homogenates by centrifugation with 100000g, for 
one hour at 4°C). Following this, the reaction was stopped by placement of the test 
tube on ice and chloroform was used to extract bilirubin for detection. Bilirubin was 
determined spectrophotometrically (MRX Microplate Reader; Dynex Technologies, 
Chantilly, VA, USA) using the difference in absorbance at wavelength from λ460 to 
λ530nm with an extinction coefficient of 40 mM/cm.

Immunohistochemistry
Frozen sections of aortae were fixed with cold acetone for 10 seconds. Following 
incubation of the slides with blocking reagent (Vectastain®), the sections were 
incubated with a primary antibody against HO-1 (1:100) overnight at 4°C. After 
washing in phosphate buffer solution containing 0.01% Triton X-100 for three times, 
the sections were incubated for 2 hours in anti-mouse IgG-FITC secondary antibody 
(1:100). After washing, the sections were treated with antifade reagent [20μl/slide, 
ProLong® Gold, with DAPI (4’,6-diamidino-2-phenylindole)] and then mounted on a 
cover slide. Immunofluorescence of the sections was visualized using a fluorescent 
microscope (Olympus, Japan).

Measurement of ROS
DCF fluorescence under confocal microscopy. Aortic rings were incubated for 15 
minutes at room temperature with 4×10⁻⁵ M DCF in buffer with 0.02% (v/v) pluronic
acid. After rinsing three times with control solution, the rings were opened, placed on a glass slide and the dye-loaded aortae examined with a laser confocal microscope [Nikon Eclipse TE300 microscope, confocal argon ion radiance-2100 laser scanning unit (Bio-Rad, Hertfordshire, England)]. The 488 nm argon ion laser line and one of the photomultiplier tubes with a band pass filter (510-525 nm) were for fluorescence detection. The parameters of the confocal laser scanning were as follow: image field = 256*256, zoom factor=4, intensity=36.4%, pinhole size=1.2, gain=20%. Because DCF is not resistant to excess laser exposure, ROS were quantified using single still images. The mean intensity of the fluorescence images was obtained three minutes after the application of acetylcholine (10⁻⁵ M).

**DHE fluorescence.** In the presence of superoxide anions, DHE is converted to the fluorescent molecule ethidium, which can then label nuclei by intercalating with DNA⁷. Sections from control rats and hemin-treated rats were exposed to DHE (10 μM, ten minutes, 37°C) in the dark in a humidified chamber, briefly washed, treated with antifade reagent (20μl/slide, ProLong® Gold) and then mounted on a cover slide. The sections were also counterstained with 4',6-diamidino-2-phenylindole (DAPI, a fluorescence stain of nuclei, 0.2μg/slide). These sections were quickly imaged with a fluorescent microscope (Olympus, Japan) keeping the same exposure for every section. The images of DHE (red) and DAPI (blue) fluorescence were obtained by setting the same color threshold for each image. The imaging software, Image J, was utilized to quantify relative fluorescence intensity of acquired digital images. DHE fluorescence was normalized as percentage of DAPI fluorescence intensity.

**Lucigenin chemiluminescence.** Lucigenin-enhanced chemiluminescence has been used as an indicator of superoxide production by superoxide generating systems such as NADPH oxidase ⁸-¹⁰. NADPH oxidase transfers electrons from NADPH and couples these electrons to molecular oxygen to produce the superoxide. Using NADPH as a substrate, the production of superoxide dependent on NADPH oxidase in the rat aorta was measured in the present study. Aortic rings were placed into 96-well-plate wells containing 190 μl control solution and 10 μl lucigenin (100 μM). The reaction was started by the addition of 1.5μl NADPH (75 μM). Luminescence was measured (for 0.1 seconds per each reading, one minute per cycle for a total of 35 cycles) in a luminometer (GloMax Multi Detection System; Promega Corporation, Sunnyvale, CA, USA). Values were normalized to dry tissue weight.

**Release of 6-keto prostaglandin F₁α, prostaglandin F₂α and thromboxane B₂**
The amount of the prostanoid end-up products 6-keto prostaglandin F₁α, prostaglandin F₂α and thromboxane B₂ were measured using enzyme immunoassay (EIA) kits (Cayman Chemical Company, Ann Arbor, MI, USA). Aortic rings of rats from both groups were placed in organ chambers containing warmed and aerated control solution. After equilibration for 90 minutes, the preparations were incubated with acetylcholine (10⁻⁵ M) for five minutes to stimulate the generation of prostanoids. Then, 0.5 ml of the bath solution was collected for enzyme immunoassay analysis.
For 6-keto prostaglandin F\(_{1\alpha}\) and prostaglandin F\(_{2\alpha}\) measurement, the samples were diluted 100 times and 10 times, respectively. The samples were assayed in duplicate. Values were normalized to dry tissue weight.

**Data analysis**
Concentration-response curves are shown to compare the responses to various agonists in the two groups. Data are presented as means ± SEM; \(n\) refers to the number of individual observations in preparations from different rats. Statistical analysis was performed using one or two-way ANOVA (Prism version 4, GraphPad Software, San Diego, CA). The intensity of PCR images and Western blot images was calculated with a computerized program (Multi-Analyst version 1.1; Bio-Rad Laboratories, Inc; Hercules, CA, USA). Data are expressed as means ± SEM. Statistical analysis was performed using Student’s t-test. P-values equal to or less than 0.05 were considered to indicate statistically significant differences.

**Materials and drugs**
Hemin, L-NAME, acetylcholine, A23187, phenylephrine, CORM, bilirubin, DCF, DHE, NADPH, lucigenin and anti-mouse IgG-FITC (goat) secondary antibody were purchased from Sigma-Aldrich® Chemical Company (St Louis, MO, USA). ZnDPBG was purchased from Frontier Scientific (Logan, Utah, USA). U46619 was purchased from Biomol (Plymouth Meeting, PA, USA). Iloprost, PGI2, anti-TP receptor antibody, anti-PGIS antibody, COX-1 monoclonal antibody, COX-2 polyclonal antibody and Anti-β-actin were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Anti-phospho-eNOS (Ser1177) and total eNOS antibody were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Anti-Nox1, Nox2 and Nox4 antibody were purchase from Abcam (Cambridge, UK). Anti-HO-1 and anti-HO-2 antibody were purchased from Assay Design (Plymouth Meeting, PA, USA). DAPI was purchased from Invitrogen (Carlsbad, CA, USA). Hemin was prepared in normal saline containing 5% 0.5 N NaOH buffer. U46619 was prepared in ethanol. A23187 and DCF were dissolved in DMSO. DHE was dissolved in phosphate buffered saline (PBS) buffer. All other compounds were prepared in deionized water.

**Supplementary reference**
4. Koneru P, Leffler CW. Role of cGMP in carbon monoxide-induced cerebral


Supplementary figures

Figure S1. HO-2 expression level was not altered by hemin treatment. Western blotting analysis for protein expression of HO-2 in the aortae of control and hemin-treated rats. Data are shown as means±S.E.M. n=4.
Figure S2. Protein expressions of phosphorylated eNOS and total eNOS were not altered by hemin treatment. Western blotting analysis for protein expression of phosphorylated eNOS and total eNOS in aortae of control and hemin-treated rats. Data are shown as means±S.E.M. n=6.
Figure S3. Protein expression of Nox1 and Nox4 were not altered by hemin treatment. Western blotting analysis for protein expression of (A) Nox1, (B) Nox4 in the aortae of control and hemin-treated rats. Nox4 was detected as two bands (67kDa and 31.8kDa). Data are shown as means±S.E.M. n=4 for control rats; n=5 for hemin-treated rats.
Figure S4. Expressions of TP and IP receptors were not altered by hemin treatment. (A) Reverse Transcription-PCR analysis for mRNA expression of TP receptors, (B) Western blotting analysis for protein expression of TP receptors, and (C) Reverse Transcription-PCR analysis for mRNA expression of IP receptors, in the aortae of control and hemin-treated rats; data are shown as means±S.E.M. n=5.