Upregulation of heme oxygenase-1 potentiates EDH-type relaxations in the mesenteric artery of the spontaneously hypertensive rat

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Li Z, Wang Y, Man RY, Vanhoutte PM. Upregulation of heme oxygenase-1 potentiates EDH-type relaxations in the mesenteric artery of the spontaneously hypertensive rat. Am J Physiol Heart Circ Physiol 305: H1471–H1483, 2013. First published September 6, 2013; doi:10.1152/ajpheart.00962.2012.—Heme oxygenase (HO) converts heme to carbon monoxide, bilirubin, and free iron. The present study investigated whether or not HO-1 induction improves vascular relaxations attributable to endothelium-dependent hyperpolarization (EDH). Thirty-six-week-old spontaneously hypertensive rats were treated with the HO-1 inducer hemin, the HO inhibitor zinc protoporphyrin IX (II) (ZnPP), the antioxidant apocynin, or combinations of these compounds. Isolated mesenteric arteries were prepared for measurement of isometric tension, protein presence, and production of reactive oxygen species (ROS). Hemin potentiated acetylcholine-evoked EDH-type relaxations in the presence of N\textsuperscript+\textendash,nitro-l-arginine methyl ester (l-NNAME) and indomethacin, while the combined treatment with ZnPP plus hemin prevented these improvements. The intermediate conductance Ca\textsuperscript2+-activated K\textsuperscript+ channel (IKCa) blocker TRAM-34 and the Na\textsuperscript+\textendashK\textsuperscript+\textendashATPase blocker ouabain significantly impaired these hemin-potentiated relaxations. NS309-induced TRAM-34- and ouabain-sensitive relaxations were enhanced by hemin. K\textsuperscript+\textendashinduced ouabain-sensitive relaxations and the expression of Na\textsuperscript+\textendashK\textsuperscript+\textendashATPase were increased by hemin. Thus HO-1 induction improves EDH-type relaxations by augmented activation of IKCa and the downstream Na\textsuperscript+\textendashK\textsuperscript+\textendashATPase. Treatment with apocynin showed a similar effect as hemin in impairing ROS production, enhancing K\textsuperscript+\textendashinduced relaxations, and increasing Na\textsuperscript+\textendashK\textsuperscript+\textendashATPase expression, without affecting the expression of HO-1. The effects of hemin and apocynin were not additive. These observations suggest that the effect of HO-1 induction on EDH-type relaxations is possibly due to its antioxidant properties. In vitro treatment with bilirubin, but not carbon monoxide, enhanced EDH-type relaxations and K\textsuperscript+\textendashinduced ouabain-sensitive relaxations, suggesting that the production of bilirubin may be also involved. The present findings reveal that HO-1 may be a potential vascular-specific therapeutic strategy for endothelial dysfunction in hypertension.

bilirubin; endothelium-dependent hyperpolarization; heme oxygenase-1; hemin; Na\textsuperscript+\textendashK\textsuperscript+\textendashATPase

HEME OXYGENASE (HO) is a critical cytoprotective enzyme responding to cellular stress and organ damage, through its ability to degrade cytotoxic free heme and produce the potent vasodilator carbon monoxide (CO) and the antioxidant bilirubin. Upregulation of the inducible isoform HO-1 lowers arterial blood pressure in the spontaneously hypertensive rat (SHR), probably by elevating sGC and cGMP levels (47), or by diminishing cytochrome P-450-dependent formation of arachidonic acid metabolites (37, 51). Previous findings of the laboratory revealed that HO-1 induction can improve endothelial function in the SHR (38). The improvement of endothelial function is due to an impairment of endothelium-dependent contractions without changes in the production of nitric oxide (NO) (38). However, it is still unknown whether or not overexpression of HO-1 affects relaxations attributed to endothelium-dependent hyperpolarization (EDH-type relaxations), an important mechanism distinct from NO and prostacyclin in the local regulation of vascular tone. Thus the present study was designed to test the hypothesis in SHR that upregulation of HO-1 by the pharmacological inducer hemin potentiates EDH-type relaxations and to investigate the possible underlying mechanisms.

EDH has a complex nature involving various ion channels and endothelium-derived vasoactive substances. It can be initiated by activation of small- and intermediate-conductance Ca\textsuperscript2+-activated K\textsuperscript+ channels (SKCa and IKCa) in the endothelial cells, which lead to an efflux of K\textsuperscript+ and endothelial hyperpolarization (6, 16). The K\textsuperscript+ activates the ouabain-sensitive Na\textsuperscript+\textendashK\textsuperscript+\textendashATPase and Ba\textsuperscript2+-sensitive inwardly-rectifying K\textsuperscript+ channels (KIR), subsequently generating hyperpolarization (16, 56, 66). The endothelial hyperpolarization can be propagated to the vascular smooth muscle cells through myoendothelial gap junctions (15, 26) or be mediated by the endothelial release of NO (44), nitroxyl (HNO) (3), hydrogen peroxide (H2O2) (30, 43), cytochrome P-450-dependent epoxygenase satrienoic acids (EET) (7, 8), and hydrogen sulfide (H2S) (68). These substances stimulate various K\textsuperscript+ channels on the smooth muscle cells, predominantly large conductance Ca\textsuperscript2+-activated K\textsuperscript+ channel (BKCa) and/or ATP-sensitive K\textsuperscript+ channel (KATP), in turn causing hyperpolarization of the latter. In the present study, known inhibitors of these channels or substances were used to evaluate their possible involvement in EDH-type relaxations.

Since oxidative stress can impair the activity of several ion channels (39, 53), and since HO-1 induction suppresses the production of reactive oxygen species (ROS), once the potentiating EDH-type relaxations of hemin was demonstrated, the hypothesis was tested that this improvement can be attributed to its antioxidant effect.

Since the HO product CO can hyperpolarize myocytes by stimulating BKCa channels (63, 64), the involvement of CO and bilirubin in EDH-type relaxations was assessed also.

MATERIALS AND METHODS

Animals and tissue preparation. The experiments were conducted in 36- to 42-wk-old male SHRs or Wistar-Kyoto (WKY) rats (380–450 g). All the animal experimental procedures were approved by the
Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85–23, revised 1996). The SHRs were randomly divided into the following groups: control [0.9% sodium chloride solution, intraperitoneal injection (ip)], hemin (50 mg/kg ip) (10, 38), zinc protoporphyrin IX (ZnPP; 30 mg/kg ip) (52), hemin plus ZnPP, apocynin (50 mg/kg ip) (34), and hemin plus apocynin. The WKY were divided into a control and a hemin-treatment group. Twenty-four hours after the treatment, animals of all groups were anesthetized with pentobarbital sodium (70 mg/ml/kg) and killed.

The mesenteric arteries of the animals were dissected free and placed in Krebs-Ringer buffer of the following composition (mM): 120 NaCl, 4.76 KCl, 2.5 CaCl₂, 1.18 MgSO₄, 1.18 NaH₂PO₄, 25 NaHCO₃, and 5.5 glucose. The first-order branches of mesenteric arteries were cut into rings (3- to 4-mm length). In some preparations, the endothelium was removed by gently rubbing the intimal surface with a wooden stick (22).

Blood pressure measurement. The blood pressure was measured by the invasive arterial blood pressure monitoring technique. A catheter was placed in the carotid artery with the connection to a pressure transducer (P23 ID; Gould Statham, Oxnard, CA). Blood pressure was recorded by a physiography (MK-IV; Narco Bio-Systems).

Isometric tension measurement. The rings were suspended in organ chambers [filled with 5 ml of warmed (37°C), aerated (95% O₂ and 5% CO₂) control solution] and were connected to force transducers (ADInstruments, Sydney, Australia) for isometric tension recording (PowerLab, ADInstruments). The rings were stretched to an optimal resting tension of 1.0 g (determined in previous experiments; data not shown) and allowed to equilibrate for 90 min. After stabilization, they were exposed twice to 60 mM KCl to test for tissue viability.

To study acetylcholine-induced endothelium-dependent relaxations, the rings were contracted with 10⁻⁶–10⁻⁵ M phenylephrine to a level approximating 80% of the reference contraction to 60 mM KCl, followed by the cumulative addition of acetylcholine (10⁻¹⁰–10⁻⁶ M) to obtain concentration-response curves. This was done after incubation (40 min) with Nω-nitro-L-arginine methyl ester (L-NAME, nitric oxide synthase inhibitor, 10⁻⁴ M) plus indomethacin (cyclooxygenase inhibitor, 10⁻⁵ M) to obtain EDH-mediated response (16), with indomethacin plus TRAM-34 and UCL 1684 (10⁻⁶ M, IKCa and SKCa inhibitors, respectively) to study NO-mediated relaxations (19, 25) or with L-NAME plus TRAM-34 and UCL 1684 to study prostanoid-induced responses. In some experiments, the rings were incubated with 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ, inhibitor of soluble guanylyl cyclase, 10⁻⁵ M), together with indomethacin. In some experiments, acetylcholine-induced relaxations in the presence of L-NAME and indomethacin were obtained in mesenteric rings of rats from the hemin treatment group incubated with TRAM-34, UCL 1684, apamin (10⁻⁷ M) (16), charybdotoxin (10⁻⁷ M) (16), ibotenic acid (10⁻⁷ M) (16), ouabain (5 × 10⁻⁷ M) (16, 65), barium chloride (BaCl₂, 10⁻⁵ M) (58), glibenclamide (10⁻⁵ M) (30), catalase (800 unit/ml) (42), 37,43GAP27 plus 43GAP26 plus 40GAP27 (3 × 10⁻⁴ M each) (41, 60) or 14,15-eicosanoid acid (14,15-EEZE, 10⁻⁵ M) (24). In other experiments, arteries were incubated with the carbon monoxide releasing molecule tricarbonyldichlororuthenium (II) dimer (CORM, 10⁻⁶ M) (36) or bilirubin (10⁻⁶ M) (57). Finally, rings of rats from the apocynin treatment group were incubated with bilirubin (10⁻⁶ M) for 40 min before evoking relaxations.

To study acetylcholine-induced, endothelium-dependent contractions, quiescent rings were incubated with L-NAME plus TRAM-34 and UCL 1684 for 40 min before the cumulative application of acetylcholine (10⁻⁸ M–10⁻⁵ M).

To study endothelium-independent relaxations induced by sodium nitroprusside, cumulative concentration (10⁻¹⁰–10⁻⁶ M)-response curves to the NO donor were obtained during sustained contractions to phenylephrine.

To test K⁺-induced relaxations (16, 67), mesenteric rings without endothelium were incubated in a low K⁺ Krebs-Ringer buffer (in mM: 120 NaCl, 1.2 KCl, 2.5 CaCl₂, 1.18 MgSO₄, 1.18 NaH₂PO₄, 25 NaHCO₃, and 5.5 glucose) after the two exposure to 60 mM KCl, followed by incubation with L-NAME and indomethacin. Rings were contracted with 10⁻⁶ M phenylephrine, followed by a single addition of 10 mM KCl. In some experiments, the preparations incubated for 40 min with ouabain (10⁻³ M) and BaCl₂ (10⁻⁴.5 M) or with bilirubin (10⁻⁶ M) before obtaining the contraction to phenylephrine and testing the relaxations to K⁺.

To study relaxations to the SKCa and IKCa channel activator NS309 (32), the rings were contracted with phenylephrine, followed by the cumulative addition (10⁻⁷–10⁻³ M) of the compound. In some

![Fig. 1. A: Western blotting demonstrating the presence of heme oxygenase-1 (HO-1) in the mesenteric arteries with endothelium of spontaneously hypertensive rat (SHR) control, hemin, zinc protoporphyrin IX (ZnPP), and hemin plus ZnPP groups. Bar graphs show the expression level, presented as percentage of β-actin expression. B: HO activity in the aortas of control, hemin-treated rats, and ZnPP-treated rats and in rats treated with hemin plus ZnPP. Data are shown as means ± SE. *P < 0.05 compared with control. #P < 0.05 compared with the hemin group. n = 5.](http://ajpheart.physiology.org/)
experiments, the preparations were incubated for 40 min with TRAM-34, UCL 1684, ouabain, and BaCl₂ before obtaining the contraction to phenylephrine.

Protein extraction and Western immunoblotting. The first-order branches of mesenteric arteries were collected, 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 [phenylmethanesulfonyl fluoride (100 mM), trypsin inhibitor (10 μg/ml), leupeptin (1 mg/ml) and pepstatin A (2 μg/ml)]. The mixture was sonicated and then centrifuged at 5,000 rpm for 3 min 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 40 μg protein (for HO-1), or 50 μg protein (for α2-Na⁺-K⁺-ATPase), was mixed with 1 × NuPAGE SDS Sample Buffer, 1 × reducing agent and ultrapure water to a total volume of 20 μl. They were boiled for 5 min at 95°C and subsequently separated in 10% SDS-PAGE at 100 V, 500 mA for 100 min. The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes at 300 mA for 2 h. Next, the membranes were blocked three times for 10 min. The membranes were then incubated with primary antibody [1:1,000 HO-1 monoclonal (38), 1:500 α2-Na⁺-K⁺-ATPase polyclonal (4), and 1:3,000 β-actin monoclonal] in TBS under gentle agitation overnight. Membranes were washed three times for 10 min in TTBS before adding secondary antibody. They were then incubated with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibody (1:5,000) or anti-mouse antibody (1:3,000) in TBS for 2 h at room temperature followed by three washes (10 min each) with TTBS. The bound secondary antibody was visualized by chemiluminescence using ECL Western Blotting Detection Reagent (Amersham, 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)] 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. HO activity was estimated by the amount of bilirubin [the stable end-product of HO, whether HO-1 or HO-2 (2)]. Aorta segments were homogenized (4 ml/g wet wt) in 250 mM sucrose containing 50 mM Tris-HCl (pH 7.5). The homogenates were centrifuged at 10,000 g for 10 min at 4°C. The supernatant was then used for measuring HO activity and protein amount. Two hundred micrograms of sample protein was incubated for 40 min at 37°C in the dark in a final volume of 200 μl 0.1 M phosphate buffer (pH 7.4) containing 5 mM MgCl₂, 1 mM NADPH, 2 mM glucose 6-phosphate, 1 U glucose-6-phosphate dehydrogenase, 0.025 mg/ml 6-phosphate, 1 U glucose-6-phosphate dehydrogenase, 0.025 mg/ml

![Fig. 2. A: concentration-dependent relaxations to acetylcholine in mesenteric arteries [with endothelium, contracted by phenylephrine (10⁻⁶–10⁻⁵ M)] of SHR control, hemin group, ZnPP group, and hemin plus ZnPP group. B: concentration-dependent relaxations to acetylcholine in mesenteric arteries [with endothelium, contracted by phenylephrine (10⁻⁶–10⁻⁵ M)] of SHR control, hemin group, ZnPP group, and hemin plus ZnPP group, in the presence of indomethacin (10⁻⁸ M) plus N°-nitro-L-arginine methyl ester (l-NAME; 10⁻³ M) or ODQ (10⁻⁸ M). C: concentration-dependent relaxations to sodium nitroprusside in mesenteric arteries [with endothelium, contracted by phenylephrine (10⁻⁶–10⁻³ M)] of control and hemin-treated rats. D: concentration-dependent relaxations to acetylcholine in the presence of indomethacin (10⁻³ M) + TRAM-34 (10⁻⁶ M) + UCL 1684 (10⁻⁶ M) in mesenteric arteries [with endothelium, contracted by phenylephrine (10⁻⁶–10⁻⁵ M)] of control and hemin-treated rats. Data expressed as changes in tension as percentage of contraction to phenylephrine and are shown as means ± SE. *P < 0.05 compared with the control, #P < 0.05 compared with the hemin group. n = 4 for A and D; n = 9 for B and C.](http://ajpheart.physiology.org/)

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hemin, and 2 mg of biliverdin reductase (prepared from rat liver homogenates by centrifugation with 100,000 g, for 1 h 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) using the difference in absorbance at wavelength from λ = 460 to λ = 530 nm with an extinction coefficient of 40 mM/cm.

**Measurement of ROS intensity.** To measure the intensity of ROS, frozen cross sections of mesenteric arteries (10 μm) were stained with dihydroethidium (DHE) by using a previously validated method (5). Sections from different groups, including control, hemin, apocynin, hemin plus apocynin, ZnPP, and hemin plus ZnPP, were exposed to DHE (10 μM, 10 min, 37°C) in the dark in a humidified chamber, briefly washed, treated with antifade reagent (20 μl/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, 0.2 μg/slide). These sections were quickly imaged with a fluorescent microscope (Olympus) 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, ImageJ, was utilized to quantify relative fluorescence intensity of acquired digital images. DHE fluorescence was normalized as percentage of DAPI fluorescence intensity.

**Measurement of bilirubin content.** Bilirubin content in blood serum was measured by bilirubin assay kit. Blood was obtained from anesthetized rats by inserting a needle into the carotid artery. After collection of the whole blood, it was allowed to clot by leaving it undisturbed at room temperature for 20 min. The clot was removed by spinning at 1,000 g for 10 min in a refrigerated centrifuge. Serum was stored at 4°C until use. Total serum bilirubin was measured using a bilirubin assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Bilirubin content in mesenteric arteries was measured by HPLC according to a previously reported method (11). Mesenteric arteries of three rats were pooled and homogenized in 500 μl of 250 mM sucrose and 50 mM Tris-HCl (pH 7.4), mixed with 200 μl chloroform, and shaken vigorously for 5 min. The chloroform phase containing the extracted bilirubin was separated from the aqueous phase by centrifugation at 1,000 g for 20 min. This extraction step was repeated twice, and the extracts were pooled and evaporated under a stream of nitrogen gas. Once fully dried, the residue was resuspended in 100 μl DMSO and stored at 4°C until analysis. All the extraction procedures.

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**Fig. 3.** Effect of TRAM-34 (10⁻⁶ M), charybdotinin (10⁻⁷ M), ouabain (5 × 10⁻⁷ M), UCL 1684 (10⁻⁶ M), apamin (10⁻⁷ M), barium chloride (10⁻⁶ M), iberiotoxin (10⁻⁷ M), glibenclamide (10⁻⁵ M), catalase (800 unit/ml), gap peptides 37,43Gap27 plus 43gap26 plus 40gap27 (3 × 10⁻⁴ M each) and 14,15-EEZE (10⁻⁵ M) on acetylcholine-induced relaxations in mesenteric arteries [with endothelium, in the presence of L-NAME (10⁻⁴ M) and indomethacin (10⁻⁵ M), contracted by phenylephrine (10⁻⁶–10⁻⁵ M)] of hemin-treated SHRs. Data are expressed as changes in tension as percentage of contraction to phenylephrine and are shown as means ± SE. *P < 0.05 compared with vehicle. n = 2–9.
were conducted in the dark. HPLC analysis was performed on Waters HPLC equipment (Waters, Milford, MA), with a dual pump, an auto-sampler, and a DAD detector. Chromatographic separations of bilirubin were performed using a ZORBAX SB C18 column (4.6 mm × 150 mm, 5 μm). The mobile phases consisted of 0.5% acetic acid and methanol running a gradient elution at a flow rate of 1 ml/min. The determining absorbance spectrum was set at 450 nm.

Data analysis. Data are presented as means ± SE; 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 5, GraphPad Software, San Diego, CA) or Student’s t-test. The intensity of Western blot images was calculated with a computerized program (Multi-Analyst version 1.1; Bio-Rad Laboratories, Hercules, CA). P values equal to or less than 0.05 were considered to indicate statistically significant differences.

Materials and drugs. Hemin, l-NNAME, acetylcholine, phenylephrine, KCI, TRAM-34, UCL 1684, apamin, iberiotoxin, charybdotoxin, ouabain, BaCl2, glibenclamide, catalase, NS309, CORM, bilirubin, sodium nitroprusside, ODQ, DHE, SOD-PEG, and anti-β-actin antibodies (cat. no. A1978) were purchased from Sigma-Aldrich® Chemicals (St Louis, MO). Apocynin was purchased from Avocado Research Chemicals (Morecambe, Lancashire, UK). Anti-α2-NA+-K+ ATPase polyclonal antibody (cat. no. 07-674) was purchased from Millipore (Billerica, MA). Anti-HO-1 antibody (cat. no. OSA-110F) was purchased from Cayman Chemical (Billerica, MA). Anti-HO-1 antibody was purchased from Avocado Research Chemicals (Morecambe, Lancashire, UK). Anti-α2-NA+-K+ ATPase polyclonal antibody (cat. no. 07-674) was purchased from Millipore (Billerica, MA). Anti-HO-1 antibody (cat. no. OSA-110F) was purchased from Cayman Chemical (Ann Arbor, MI). The gap peptides, 37,43Gap27 (H-Ser-Arg-Pro-Thr-Glu-Lys-Thr-Ile-Phe-Ile-Val-Val-Val-Val-Val-Val-OH), 40Gap27 (H-Ser-Arg-Pro-Thr-Glu-Lys-Asn-Val-Phe-Ile-Val-OH), and 43Gap26 (H-Val-Cys-Tyr-Asp-Lys-Ser-Phe-Pro-Ile-Ser-His-Arg-OH) were purchased from Biomatik (Wilmington, DE). Hemin and ZnPP were prepared in normal saline containing 5% 0.5 NaOH buffer. Apocynin was prepared in 95% ethyl alcohol. A stock solution of indomethacin was prepared in a sodium bicarbonate (5 × 10−3 M) solution. The stock solutions of TRAM-34 (10−6 M), UCL 1684 (10−6 M), sodium nitroprusside (10−3 M), ODQ (10−3 M), and NS309 (10−2 M) were prepared in dimethylsulfoxide (DMSO). All other compounds were prepared in deionized water.

RESULTS

Mean arterial blood pressure. Twenty-four hours after the in vivo treatment with hemin, the mean arterial blood pressure of 36-wk-old SHR was significantly reduced from 182 ± 12 to 153 ± 3 mmHg. Treatment with ZnPP (190 ± 8 mmHg) or the combination treatment of hemin plus ZnPP (190 ± 10 mmHg) did not affect mean arterial blood pressure compared with control (182 ± 12 mmHg).

Protein presence of HO-1. Hemin caused a significant up-regulation of the protein presence of HO-1 in the mesenteric arteries of SHR. In vivo treatment with the HO inhibitor ZnPP did not alter the protein level of the enzyme. After combined treatment with hemin plus ZnPP, the presence of HO-1 was not significantly different from control (Fig. 1A).

HO activity. In vivo treatment with hemin, but not with the HO inhibitor ZnPP or with the combination of hemin plus ZnPP, significantly augmented the activity of HO in the aortas of the SHR (Fig. 1B).

Acetylcholine-induced relaxations. In SHR mesenteric arteries with endothelium contracted with phenylephrine, acetylcholine-induced relaxations were significantly potentiated by hemin, but were not altered by ZnPP. The combined treatment with hemin plus ZnPP significantly reduced the enhancement of the relaxations in response to hemin (Fig. 2A; see Supplementary Material and Methods).
Hemin significantly potentiated acetylcholine-induced relaxations in the presence of L-NAME (10^{-4} M) and indomethacin (10^{-5} M) in mesenteric arteries of SHR (Fig. 2B; see Supplemental Table 1). A similar potentiation was observed in arteries of hemin-treated SHRs in the presence of ODQ (10^{-5} M) plus indomethacin (Fig. 2B; Supplemental Table 1). Treatment with ZnPP did not change the relaxations to acetylcholine in the presence of L-NAME and indomethacin, while the combined treatment with ZnPP plus hemin significantly prevented the improvement of the relaxations by the latter (Fig. 2B; Supplemental Table 1).

Relaxations induced by the NO donor sodium nitroprusside were not significantly different between arteries of control and hemin-treated SHRs (Fig. 2C; Supplemental Table 1). In the presence of indomethacin and TRAM-34 (10^{-6} M) plus UCL1864 (10^{-6} M), acetylcholine-induced relaxations were comparable between rings from control and hemin-treated SHRs (Fig. 2D; Supplemental Table 1); the presence of L-NAME and TRAM-34 plus UCL1864 abolished relaxations to acetylcholine in both groups (data not shown).

The hemin-improved relaxations in SHRs were reduced significantly by the IKCa channel blocker TRAM-34 (10^{-6} M), the nonspecific BKCa and IKCa channel blocker charybdotoxin (10^{-7} M), and the Na^{+}-K^{+}-ATPase blocker ouabain (5\times10^{-7} M) (Fig. 3; Supplemental Table 2). However, these relaxations were not affected significantly by the SKCa channel blockers UCL 1684 (10^{-6} M) or apamin (10^{-7} M), the nonspecific KIR channel blocker BaCl_2 (10^{-6} M), the BKCa channel blocker iberiotoxin (10^{-7} M), the KATP channel blocker glibenclamide (10^{-5} M), the H_2O_2 scavenger catalase (800 unit/ml), the gap peptides 37,43Gap27 plus 43gap26 plus 40gap27 (3\times10^{-4} M each), or the EETs inhibitor 14,15-EEZE (10^{-5} M) (Fig. 3; Supplemental Table 2).

Acetylcholine-induced relaxations in the presence of L-NAME and indomethacin were significantly impaired in the mesenteric arteries of SHR compared with WKY (Fig. 4; Supplemental Table 3). Hemin did not significantly affect relaxations to acetylcholine in WKY mesenteric arteries in the presence of L-NAME and indomethacin (Fig. 4; Supplemental Table 3).

Acetylcholine-induced contractions. Hemin attenuated acetylcholine-induced contractions in quiescent preparations with endothelium incubated with a combination of L-NAME, TRAM-34, and UCL1864 (blockage of NO-mediated relaxations and EDH-type relaxations) (Fig. 5; Supplemental Table 4).

NS309-induced relaxations. NS309, the activator of SKCa and IKCa channels (32), induced concentration-dependent relaxations in mesenteric rings with endothelium. Hemin significantly potentiated these relaxations (Fig. 6; Supplemental Table 5). The HO inhibitor ZnPP did not alter the NS309-induced relaxations, but treatment with ZnPP plus hemin reversed the effect of the latter (Fig. 6; Supplemental Table 5).

**Fig. 7.** In the absence or presence of TRAM-34 (10^{-6} M), ouabain (5\times10^{-7} M), UCL1684 (10^{-6} M), and BaCl_2 (10^{-6} M), concentration-dependent relaxations to NS309 in mesenteric arteries with endothelium, in the presence of L-NAME (10^{-4} M) and indomethacin (10^{-5} M), contracted by phenylephrine (10^{-6}–10^{-5} M) of control (A) and hemin-treated rats (B). Data are expressed as changes in tension as percentage of contraction to phenylephrine and are shown as means ± SE. *P < 0.05 compared with vehicle, n = 4.
In arteries of the control rats, NS309-induced relaxations were not altered by TRAM-34, ouabain, UCL 1684, or BaCl₂ (Fig. 7A; Supplemental Table 5). In preparations from hemin-treated rats, NS309-induced relaxations were attenuated in the presence of TRAM-34, ouabain, and of the combination of TRAM-34 and ouabain, but were not affected by UCL 1684, BaCl₂ or their combination (Fig. 7B; Supplemental Table 5).

**K⁺-induced relaxations.** In preparations without endothelium, a relaxation was induced when the K⁺ concentration in the bathing solution was raised from 1.2 mM to 11.2 mM (Fig. 8A). Hemin significantly potentiated the K⁺-induced relaxations, increasing both their maximum and their duration. Treatment with ZnPP or hemin plus ZnPP did not alter either the maximum or the duration of K⁺-induced relaxations (Fig. 8, B and C).

In mesenteric arteries from both control and hemin-treated SHRs, ouabain (5 x 10⁻⁷ M) and ouabain plus BaCl₂ (10⁻⁶ M), but not BaCl₂ alone, significantly inhibited the K⁺-induced relaxations (Fig. 8D).

**Apocynin treatment.** In mesenteric arteries with endothelium of SHR treated with apocynin (50 mg/kg ip), the relaxations to acetylcholine in the presence of L-NAME and indomethacin were potentiated significantly (Fig. 9A; Supplemental Table 6). The degree of potentiation by apocynin was comparable to that observed after hemin. Treatment with hemin plus apocynin had no additive effect (Fig. 9A; Supplemental Table 6).

In preparations without endothelium, the amplitude and duration of K⁺-induced relaxations were potentiated to a...
comparable extent by treatment with apocynin or hemin plus apocynin (Fig. 9, B and C).

Protein presence of Na\(^{+}\)-K\(^{+}\)-ATPase. Hemin treatment significantly augmented the protein presence of α2-Na\(^{+}\)-K\(^{+}\)-ATPase in the mesenteric arteries of SHR (Fig. 10). Treatment with apocynin and hemin plus apocynin increased the protein presence of α2-Na\(^{+}\)-K\(^{+}\)-ATPase to the same extent as hemin treatment (Fig. 10).

ROS intensity. Vascular ROS intensity was determined in situ in sections of mesenteric arteries using DHE dye. Superoxide dismutase-polyethylene glycol (SOD-PEG) was administered ex vivo to arteries of control rats as a positive control. The DHE fluorescence signal was significantly decreased in the mesenteric arteries of rats treated with hemin, apocynin, or hemin plus apocynin compared with the control rats. Treatment with ZnPP or hemin plus ZnPP did not alter the vascular ROS intensity (Fig. 11).

In vitro treatment with CORM or bilirubin. In mesenteric arteries with endothelium, in vitro treatment with bilirubin (10\(^{-6}\) M), but not CORM (10\(^{-6}\) M), significantly potentiated acetylcholine-induced relaxations in the presence of L-NAME and indomethacin (Fig. 12A; Supplemental Table 7). In preparations of SHR treated with apocynin, in vitro exposure to 10\(^{-6}\) M bilirubin did not significantly alter acetylcholine-induced relaxations (Fig. 12B; Supplemental Table 7).

In arteries without endothelium, incubation with bilirubin significantly potentiated K\(^{+}\)-induced relaxations, both in terms of amplitude (Fig. 12C) and duration (Fig. 12D). These potentiated K\(^{+}\)-induced relaxations were abolished by ouabain (data not shown).

Bilirubin content. The bilirubin content in serum was comparable in control and hemin-treated rats (0.032 ± 0.003 mg/ml and 0.032 ± 0.002 mg/ml, respectively). The bilirubin content in SHR mesenteric arteries was below detection level (data not shown).

DISCUSSION

Hemin is a potent pharmacological inducer of HO-1 (2, 50). Twenty four hours after intraperitoneal injection of hemin, the protein expression of HO-1 in the SHR mesenteric arteries was augmented, and this was accompanied by an increase of HO activity. The effect of hemin on HO-1 induction was prevented by the HO inhibitor ZnPP. These observations demonstrate that HO-1 was upregulated successfully by the hemin treatment. This acute HO-1 upregulation resulted in a reduction in arterial blood pressure, which is in line with the observations that chronic hemin treatment lowers blood pressure in the same hypertensive animal model (54, 62). This antihypertensive effect is accompanied by an improvement of endothelium-
dependent relaxations, since the decreases in tension elicited by the muscarinic receptor agonist acetylcholine were potentiated by hemin in the mesenteric arteries, and since concomitant treatment with the HO inhibitor ZnPP prevented the effect of hemin. The improvement persisted after blockade of eNOS and cyclooxygenase, suggesting that the potentiated relaxations are distinct from those mediated by NO or prostacyclin (17, 23), and thus can be probably attributed to an improvement of EDH-type relaxations (9, 21). Such potentiation was not observed in preparations of normotensive WKY rats, confirming that HO-1 induction improves endothelium-dependent responses only when the endothelial cells are dysfunctional (38).

An involvement of NO in these hemin-potentiated relaxations is unlikely, to judge from the following observations: 1) acetylcholine-induced relaxations were comparable in arteries of control and hemin-treated animals after blockade of cyclooxygenase and EDH; 2) there was no difference in the response to the NO donor sodium nitroprusside between arteries of control and hemin-treated SHR; and 3) ODQ, the inhibitor of the NO effector soluble guanylyl cyclase, had a similar effect as the eNOS inhibitor l-NAME on acetylcholine-induced relaxations in preparations of hemin-treated rats, excluding the involvement of residual NO (69).

Hemin impaired acetylcholine-induced endothelium-dependent relaxations (obtained after blockade of eNOS and EDH) in the SHR mesenteric artery as it does in the aorta of the same strain (38). This impairment can be attributed to the suppressed production of vasoconstrictor prostanoids (38). However, this impairment of endothelium-dependent contractions did not contribute to the potentiated relaxations induced by acetylcholine in arteries of the hemin-treated rats since they were observed after incubation with indomethacin. Since in the presence of inhibitors of eNOS and EDH, no relaxations to acetylcholine were obtained in phenylephrine-contracted arteri- es, hemin obviously does not potentiate endothelium-dependent relaxations due to vasodilator prostanoids, in particular prostacyclin. The lack of contribution of endothelium-derived NO and prostacyclin in the hemin-induced potentiation of the response to acetylcholine further emphasizes that upregulation of HO-1 improves EDH-mediated relaxations.

The hemin-potentiated EDH-type relaxations involve the activation of IKCa and Na\(^{+}\)-K\(^{+}\)-ATPase. This conclusion is based on the observations that ouabain (Na\(^{+}\)-K\(^{+}\)-ATPase blocker), TRAM-34 (specific inhibitor of IKCa), and charybdotoxin (blocker of BKCa and IKCa channel), but not iberiotoxin (specific inhibitor of BKCa), attenuated the relaxations.

**Fig. 11.** Images of DHE and DAPI fluorescence staining in the cross sections of mesenteric arteries of control, hemin, apocynin, hemin plus apocynin, ZnPP, and hemin plus ZnPP groups. SOD-PEG was administered ex vivo to cross sections of control rats as a positive control. DHE-stained signals are in red, DAPI-stained nuclei are in blue. Bar graph shows DHE fluorescence intensity per positive nuclei. Data are shown as means ± SE. *P < 0.05 compared with the control. n = 5.
Relaxations to NS309 involve an endothelium-dependent response that is due to activation of SKCa and IKCa channels, as well as an endothelium-independent component (32). NS309-induced endothelium-dependent relaxations were impaired in the 36-wk-old SHR, which has developed serious endothelial dysfunction, judged from the observations that NS309-induced relaxations were not altered by TRAM-34 or UCL 1684 in the control rats. NS309-induced relaxations were potentiated by hemin, but were not affected by ZnPP or hemin plus ZnPP. Since the hemin-improved NS309-induced relaxations were reversed by TRAM-34, but not by UCL 1684, the EDH-type relaxations due to activation of IKCa channels must be increased by hemin (14, 17, 29).

A small increase in extracellular K\(^+\) acts as a local, physiological regulator of blood flow to certain vascular beds (18, 28). Activation of SKCa and/or IKCa channels can cause an efflux of K\(^+\), which stimulates Na\(^+\)-K\(^+\)-ATPase and/or KIR to cause hyperpolarization (16). By studying K\(^+\)-induced relaxations in the absence or presence of ouabain or BaCl\(_2\), the involvement of Na\(^+\)-K\(^+\)-ATPase and KIR was further evaluated (16). Since hemin potentiated K\(^+\)-induced relaxations, and since this potentiation was blocked by ouabain but not affected by BaCl\(_2\), the augmented expression of HO-1 must result in increased activation of Na\(^+\)-K\(^+\)-ATPase.

SKCa and KIR channels are not involved in the potentiation by hemin of the EDH-type response to acetylcholine. This conclusion is based on the following observations: 1) the SKCa inhibitors apamin and UCL 1684, as well as KIR blocker BaCl\(_2\), did not alter the relaxations; 2) UCL 1684 and BaCl\(_2\) did not alter NS309-induced relaxations; and 3) BaCl\(_2\) did not affect K\(^+\)-induced relaxations in the mesenteric arteries of apocynin-treated SHRs. In hypertension, the expression of endothelial SKCa channels is reduced (33, 65), and the downstream hyperpolarizing pathway amplified by KIR channel opening is compromised (65). The exclusion of a contribution of SKCa and KIR in the hemin-improved EDH type-relaxations thus suggests that the HO-1 inducer did not restore the impaired SKCa and KIR channel function, but rather augmented the ability of the IKCa-Na\(^+\)-K\(^+\)-ATPase to compensate for the dysfunctional relaxations.

The Western blotting results indicate that the protein level of Na\(^+\)-K\(^+\)-ATPase \(\alpha_2\) subunit, which is responsible for activation of this Na\(^+\)/K\(^+\) pump during EDH responses in the rat mesenteric artery (40, 66), was increased by the hemin treatment. Thus the increased activation of IKCa and downstream Na\(^+\)-K\(^+\)-ATPase caused by hemin can be attributed to the increased presence of Na\(^+\)-K\(^+\)-ATPase.

Reproduced from H1480 HO-1 IMPROVES EDH-TYPE RELAXATIONS AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00962.2012 • www.ajpheart.org by 10.220.33.4 on October 15, 2017 http://ajpheart.physiology.org/ Downloaded from
The present results rule out the involvement of BKCa, KATP, H2O2, EETs, and gap junctions in the hemin-improved EDH-type relaxations. Although both H2O2 (30, 43) and EETs (7, 8) can induce hyperpolarization by stimulating BKCa and/or KATP on the smooth muscle cells and are regarded as EDH factors, their participation can be ruled out since hemin-potentiated relaxations were resistant to the H2O2 scavenger catalase, the EET inhibitor 14,15-EEZE, the BKCa blocker iberiotoxin, and the KATP blocker glibenclamide. Gap junctions facilitate communication between endothelial cells, between myocytes, and between endothelial cells and myocytes (13). Hyperpolarizing current can be transferred from endothelial to smooth muscle cells through myoendothelial gap junctions (15, 17, 26). However, it is unlikely that hemin changes the function of gap junctions, since relaxations potentiated by hemin were not altered by the gap peptides 37,43Gap27 plus 43Gap26 plus 40Gap27, inhibitors of Cx37, Cx43, and Cx40 gap junctional channels (41, 60).

The improvement of EDH-type relaxations by HO-1 induction is most likely attributable to the antioxidant properties of the enzyme (12, 59). Indeed, treatment with hemin, but not with ZnPP or hemin plus ZnPP, attenuated the production of vascular ROS, to judge from the results of DHE staining. In vivo treatment with apocynin, a potent antioxidant (31) that was introduced as a positive control, decreased ROS production to a similar level as hemin. The combined treatment with hemin plus apocynin did not have an additive effect in reducing ROS production. These results confirm the potent antioxidant effect of HO-1. The antioxidant effect of HO-1 induction is mainly due to the degradation of free heme and the production of bilirubin. The breakdown of cytotoxic heme prevents the formation of ROS (48). Additionally, degradation of cellular heme reduces the catalysis of NADPH oxidase and cyclooxygenase, which are two main ROS-generating enzymes in the vascular wall (1, 38, 59, 61). In addition, the HO product bilirubin itself is a potent antioxidant (57).

The reduced ROS level resulting from HO-1 induction must contribute to the improvement of EDH-type relaxations. Indeed, the effect of hemin is comparable to that of apocynin as regards acetylcholine-induced EDH-type responses, K+−induced relaxations of vascular smooth muscle, and protein presence of Na+−K+−ATPase. Moreover, the combined treatment with hemin and apocynin did not cause a greater potentiation than either apocynin or hemin given alone. These observations suggest that upregulation of HO-1 causes a similar improvement in EDH-type relaxations as apocynin because of a reduction in oxidative stress.

It remains unclear how these antioxidant properties help to improve EDH-mediated responses, in particular the expression and activity of Na+−K+−ATPase. One explanation is that HO-1 (like apocynin) protects Na+−K+−ATPase from damage by superoxide anions. Indeed, superoxide anions can inhibit Na+−K+−ATPase (20, 27, 35, 55). The ability of HO-1 induction to diminish ROS generation probably helps to preserve the activity of the sodium pump.

The present study attempted to determine whether or not the hemin-improved EDH-type response could be attributed to the HO products, CO and bilirubin. CO may cause vasodilatation by stimulating BKCa and is regarded as a potential EDH factor (63, 64). However, BKCa-activated relaxations did not appear to participate to the hemin-potentiated relaxations. Furthermore, in vitro incubation with a CO donor did not alter acetylcholine-induced EDH-type relaxations in SHR mesenteric arteries in the presence of l-NNAME and indomethacin, indicating that CO per se is not likely to contribute to the improved response following hemin-treatment. By contrast, in vitro incubation with bilirubin potentiated EDH-mediated relaxations to acetylcholine, indicating that the HO product may contribute to the hemin-improved EDH-type relaxations. Likewise, bilirubin potentiated K+−induced, ouabain-sensitive relaxations in preparations without endothelium, suggesting improved activity of Na+−K+−ATPase in vascular smooth muscle. Bilirubin acts as an antioxidant (57). The observation that bilirubin did not further potentiate acetylcholine-induced relaxations in mesenteric arteries of apocynin-treated SHR supports the view that the effect of the HO product on EDH-type relaxations is due to its antioxidant properties. Although these in vitro experiments suggest that bilirubin improves EDH-type relaxations, the serum bilirubin level was not changed after HO-1 induction, while the concentration of bilirubin in the mesenteric arteries was below detection level. Thus a definitive conclusion cannot yet be reached as regards the possible contribution of bilirubin to the improved endothelial function following HO-1 induction.

In summary, the present study reveals that upregulation of HO-1 by hemin potentiates EDH-type relaxations in the mesenteric artery of the SHR. The improvement in EDH-type responses caused by HO-1 induction can be attributed to an increased expression of Na+−K+−ATPase, facilitating relaxations involving the IKCa-Na+−K+−ATPase pathway. This beneficial effect can be attributed to the antioxidant properties of HO-1 and may be due in part to the production of bilirubin. This improvement of EDH-type relaxations, together with the previous findings of an impairment of endothelium-dependent contractions, suggests that upregulation of HO-1 improves endothelial function and thus may be a therapeutic strategy for treating endothelial dysfunction in hypertension. However, in view of the reported toxic effects of generalized HO-1 induction (45, 46, 49), such therapeutic approach should be vascular specific.

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