Heme Oxygenase-1 Deficiency Leads to Alteration of Soluble Guanylate Cyclase Redox Regulation^S

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ABSTRACT

Heme oxygenase-1 knockout, $H_{\text{mox}}1(-/-)$, mice exhibit exacerbated vascular lesions after ischemia-reperfusion and mechanical injury. Surprisingly, we found no studies that reported contractile responses and sensitivity to vasorelaxants in $H_{\text{mox}}1(-/-)$ mice. The contractile responses [superior mesenteric arteries (SMA), from female $H_{\text{mox}}1(-/-)$ mice] exhibited increased sensitivity to phenylephrine ($p < 0.001$). Cumulative addition of acetylcholine relaxed SMA, with the residual contraction remaining 2 times higher in $H_{\text{mox}}1(-/-)$ mice ($p < 0.001$). Sodium nitroprusside (SNP, an NO donor) and 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole [YC-1; acts directly on soluble guanylate cyclase (sGC)] led to further relaxation, yet the residual contraction remained 2 to 3 times higher in $H_{\text{mox}}1(-/-)$ than $H_{\text{mox}}1(+/+)$ mice ($p < 0.001$). Branches from $H_{\text{mox}}1(-/-)$ mesenteric and renal arteries also showed reduced relaxation ($p < 0.025$). Relaxation of SMA was measured to 4-(((4-carboxybutyl) [2-(5-fluoro-2-[[4'-(trifluoromethyl) biphenyl-4-yl] methoxy]phenyl)ethyl]amino)benzoic

acid (BAY 60-2770), which is a more effective activator of oxidized/heme-free sGC; and to 5-cyclopropyl-2-{1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl}-pyrimidin-4-ylamine (BAY 41-2272), a more effective stimulator of reduced sGC. $H_{\text{mox}}1(-/-)$ arteries were 15 times more sensitive to BAY 60-2770 ($p < 0.025$) than were $H_{\text{mox}}1(+/+)$ arteries. Pretreatment with 1H-[1,2,4]oxadiazolo[3,4-a]quinoxalin-1-one (ODQ), an oxidizer of sGC, predictably shifted the BAY 60-2770 response of $H_{\text{mox}}1(+/+)$ to the left ($p < 0.01$) and BAY 41-2272 response to the right ($p < 0.01$). ODQ had little effect on the responses of $H_{\text{mox}}1(-/-)$ arteries, indicating that much of sGC was oxidized/heme-free. Western analyses of sGC in SMA indicated that both $\alpha 1$ and $\beta 1$ subunit levels were reduced to $< 50\%$ of $H_{\text{mox}}1(+/+)$ level ($p < 0.025$). These findings support the hypothesis that the antioxidant function of $H_{\text{mox}}1$ plays a significant role in the maintenance of sGC in a reduced state, which is resistant to degradation and is sensitive to NO. This function may be especially important in reducing vascular damage during ischemia-reperfusion injury.

Introduction

Heme oxygenase-1 ($H_{\text{mox}}1$) is an inducible cytoprotective enzyme that degrades heme to biliverdin, iron, and CO (Wu and Wang, 2005). It is expressed in vascular tissues and is regarded to play an important role in the production of products that have antioxidant and anti-inflammatory activity (Korthuis and Durante, 2005; Kim et al., 2006). One of the

products, CO, has been the focus of many studies that have linked $H_{\text{mox}}1$ to vascular function. CO was shown to act as a vasodilator and at high concentrations it stimulated soluble guanylate cyclase (sGC) and cGMP formation (Durante et al., 2006; Kim et al., 2006). The resultant activation of protein kinase G (GK) led to effective inhibition of smooth muscle contraction through action on myosin phosphatase, K^+ channels, and cellular calcium. Studies of vascular function have used strategies to stimulate and to inhibit $H_{\text{mox}}1$ directly and to apply its products such as CO (Durante et al., 2006; Kim et al., 2006). For example, hemin injected into 8-week-old spontaneously hypertensive rats increased $H_{\text{mox}}1$ and sGC levels in arteries and lowered blood pressure (Ndisang et al., 2002). Transfection of porcine arteries with $H_{\text{mox}}1$ shifted the phenylephrine-response curves to the right (reduced sensitivity), whereas treatment with the $H_{\text{mox}}1$ inhibitor ZnPPiX elimi-

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ABBREVIATIONS: $H_{\text{mox}}1$, heme oxygenase-1; sGC, soluble guanylate cyclase; GK, protein kinase G; ZnPPiX, zinc protoporphyrin-IX; bp, base pair(s); SMA, superior mesenteric artery(ies); RA, renal artery branch; FA, femoral artery; PS, physiological solution; PE, phenylephrine; SNP, sodium nitroprusside; ACh, acetylcholine; YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole; BAY 41-2272, 5-cyclopropyl-2-{1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl}-pyrimidin-4-ylamine; 5-HT, 5-hydroxytryptamine; Ado, adenosine; ODQ, 1H-[1,2,4]oxadiazolo[3,4-a]quinoxalin-1-one; BAY 60-2770, 4-(((4-carboxybutyl) [2-(5-fluoro-2-[[4'-(trifluoromethyl) biphenyl-4-yl] methoxy]phenyl)ethyl]amino)benzoic acid; eNOS, endothelial nitric-oxide synthase; BAY 58-2667, 4-[[4-carboxybutyl]2-[[4-phenethylbenzyl]oxy]phenethyl]amino)methyl[benzoic] acid.

nated the difference (Duckers et al., 2001). Treatment with lipopolysaccharide induced $H_{\text{mox}}1$ expression in arteries and significantly reduced blood pressure in rats, whereas pretreatment with ZnPPiX prevented the fall in blood pressure (Yet et al., 1997).

Metalloprotoporphyrins have been widely used to study the role of H_{mox} in the regulation of vascular function. These compounds, such as ZnPPiX, tin protoporphyrin-IX, and chromium mesoporphyrin-IX, consistently alter vascular responses in vitro. For example, ZnPPiX increased myogenic tone in mesenteric arteries from rats exposed to chronic hypoxia, a treatment that increased $H_{\text{mox}}1$ activity (Naik and Walker, 2006). A recent study indicated that metalloprotoporphyrins also may have nonspecific constrictor effects on rat cerebral arteries (Andresen et al., 2006). Moreover, these compounds are also effective inhibitors of sGC at concentrations typically used to inhibit H_{mox} (Kim et al., 2006; Stasch et al., 2006). It should be noted that a reduced heme containing Fe^{2+} is necessary for activation of sGC. Inhibition of H_{mox} would remove its antioxidant effect, which would lead to increased levels of oxidized (Fe^{3+}) heme and reduced effect of NO (Wu and Wang, 2005). The interpretation of results derived from the application of an agent that inhibits both H_{mox} and sGC becomes problematic, because these enzymes are closely linked to the signaling pathway operating on smooth muscle contraction.

Another approach to the evaluation of $H_{\text{mox}}1$ has used knockout, $H_{\text{mox}}1(-/-)$, mice (Poss and Tonegawa, 1997). Although these mice exhibited no change in heme oxygenase-2 levels, increased cardiac and renal damage occurred during ischemic conditions (Yet et al., 1999; Wiesel et al., 2001). $H_{\text{mox}}1(-/-)$ mice also exhibited an exacerbation of vascular lesions in response to hyperlipidemia and mechanical and photochemical injury (Duckers et al., 2001; Yet et al., 2003; True et al., 2007). Vascular smooth muscle cells from $H_{\text{mox}}1(-/-)$ mice also exhibited increased proliferation when maintained in cell culture (Duckers et al., 2001). Surprisingly, we have not found studies that used $H_{\text{mox}}1(-/-)$ mice to evaluate arterial contractile responses and their sensitivity to endothelial and pharmacological relaxants.

The present study was undertaken to determine whether arteries from $H_{\text{mox}}1(-/-)$ mice exhibit functional alterations that are similar to those reported during pharmacological manipulation of H_{mox} activity. In addition, we have attempted to determine whether the nitrate tolerance we observed in arteries from $H_{\text{mox}}1(-/-)$ mice resulted from an altered heme redox state, reduced expression of sGC, or both.

Materials and Methods

Animals. Wild-type H129, $H_{\text{mox}}1(+/+)$ breeders were obtained from The Jackson Laboratory (Bar Harbor, ME). $H_{\text{mox}}1(-/-)$ breeders with the H129 background were obtained as a gift from Dr. William Fay (University of Missouri–Columbia, Columbia, MO). All of the mice used in this study were bred at the University of Missouri. The experimental procedures were performed in accordance with institutional guidelines for humane animal care and use. Genotyping was done on lysates from tail samples using standard polymerase chain reaction methods. Primers (HO/e3 and HO/13R, approximately 475 bp for wild type; and NEO1 and HO/e4, approximately 375 bp for HO-1 knockout) were obtained from Invitrogen (Carlsbad, CA). Blood vessels and tissue samples were removed from female mice (9–12 weeks old)

that were first anesthetized with a mixture of ketamine (150 mg/kg body weight i.p.) and xylazine (7.5 mg/kg body weight i.p.).

Vascular Rings. The thoracic aorta, mesenteric arterial bed, renal, and femoral arteries were dissected free of surrounding tissue and placed in ice-cold dissection solution (see composition below). The arteries were trimmed free of fat and cut into rings approximately 1 mm in length. The outside diameter was measured with a calibrated reticle in the dissection microscope (model SMZ-2B; Nikon, Brighton, MI). The range of values is as follows: aorta, 800 to 1000 μm ; superior mesenteric artery (SMA), 440 to 560 μm ; mesenteric artery branch, first- and second-order branches, 200 to 250 μm ; renal artery (RA), first- and second-order branches, 250 to 330 μm ; and femoral artery (FA), 300 to 380 μm . The rings were mounted on force transducers (model FT03; Grass Instruments, West Warwick, RI) that had specially fabricated feet with an open wire (25 or 51 μm in diameter) design. One foot was attached to the cantilever and the other foot to a micrometer mounted on top of the transducer. Rings were gently slipped onto the open end of the wires with the aid of the dissection microscope and stretched 1.25 times their resting length before being placed into physiological solution (PS) (10 ml contained in plastic cups) at 36°C. The rings were tested with high K^+ ($\text{K} = 80$ mM substituted for Na^+ to establish a reference contracture and then returned to PS, relaxed, stretched approximately 5%, and retested with high K^+ and so forth. The rings were regarded to be at or near the length for producing a maximal contraction when the incremental stretch produced less than a 10% increase in contraction. This procedure facilitated the reproducibility of results between vessels and animals.

Drugs were added to the PS in a cumulative manner, with the next level being added either when a steady response occurred or at a predetermined time, e.g., 2 min. The Ca^{2+} chelator EGTA (2.5 mM) was applied at the end of the protocols to determine the basal noncontracted tension.

Western Blots. The thoracic aorta and superior mesenteric artery along with its primary branches were dissected free of fat, placed in vials, frozen in liquid N_2 , and stored at -70°C . For each Western analysis, two aortas and three to four mesenteric arteries were pooled, ground in liquid N_2 , and extracted in buffer (125 mM Tris, pH 6.8, 12.5% glycerol, 2% SDS, 50 mM sodium fluoride, and trace bromophenol blue). The proteins were separated by SDS-polyacrylamide gel electrophoresis. After transfer to a nitrocellulose membrane, the blots were blocked with phosphate-buffered saline and nonfat milk (5%) and then incubated with antibodies directed against sGC $\alpha 1$ (1:1000) and $\beta 1$ (1:750) subunits and β -actin (1:300). The membranes were washed in phosphate-buffered saline, incubated with horseradish peroxidase-conjugated anti-rabbit or anti-goat antibodies, and developed with commercial chemoluminescence reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The expression of sGC $\alpha 1$ and $\beta 1$ protein was quantified by scanning densitometry and normalized with respect to β -actin.

Solutions and Drugs. The PS had the following composition: 138 mM NaCl, 5.0 mM KCl, 1.5 mM CaCl_2 , 1.2 mM MgCl_2 , 1.2 mM NaH_2PO_4 , 10 mM Na-HEPES, pH 7.35, and 11.2 mM glucose. Dissection solution had a similar composition, with Ca^{2+} reduced to 0.15 mM. Phenylephrine (PE), sodium nitroprusside (SNP), acetylcholine (ACh), YC-1, BAY 41-2272, ascorbic acid, and antibodies against sGC $\alpha 1$ and $\beta 1$ were purchased from Sigma-Aldrich (St. Louis, MO). An antibody against β -actin was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). 5-Hydroxytryptamine (5-HT) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Adenosine (Ado) was purchased from Sigma/RBI (Natick, MA). ODQ was purchased from Alexis Biochemicals (San Diego, CA). BAY 60-2770 was a gift from Bayer Schering Pharma AG (Wuppertal, Germany). Stock solutions (10 mM) of PE, 5-HT, ACh, and Ado were prepared in 10 mM ascorbic acid and were frozen (-20°C). SNP (10 mM) was prepared in ascorbic acid and stored at 4°C . YC-1, ODQ, and the BAY compounds were dissolved (10 mM) in dimethyl sulfoxide and stored

at 4°C. Dilutions were made into the appropriate solvent on the day of the experiment.

Data Analyses. Contractile responses were normalized to the maximal response and plotted as log concentration versus percentage of response. The relaxation responses were calculated both as percentage of the remaining maximal contraction and as percentage of the maximal relaxation that was achieved. Only one curve was determined for each animal (n is number of animals), and these were averaged for each group (Franke et al., 2004). The averaged curves for PE, 5-HT, and ACh were fitted with a four-parameter equation (Hill equation: minimal %, maximal %, mid-point concentration [log M for EC_{50} or IC_{50}], and slope h) by means of SigmaPlot software (Systat Software, Inc., San Jose, CA). The software also provided an error analysis for each of the parameters that was used to determine the significance of comparisons between $H_{\text{mox}}1(+/+)$ and $H_{\text{mox}}1(-/-)$ mice. Because the concentration-response curves for the BAY compounds were relatively flat, the IC_{50} value was determined for each ring by linear interpolation between a point just below and just above the 50% response. Student's t test was used to test for differences between group means where one treatment was being evaluated, and $p < 0.05$ was taken to be significant. Data are presented as means $\pm$ S.E.

Results

Contraction. The contractile responses of the FA to 5-HT and the SMA to PE were shifted slightly ($\Delta \log = -0.20 \pm 0.04$) but significantly ($p < 0.001$) to the left in $H_{\text{mox}}1(-/-)$ mice, which showed increased sensitivity to the agonists (Fig. 1). No consistent differences in the maximal contractile response to high K^+ was observed between the two groups of mice, nor did the ratio of the maximal agonist response to the high K^+ response differ.

Relaxation. Cumulative increases in ACh elicited a concentration-dependent relaxation of SMA that was precontracted with PE (Fig. 2A). Significant differences occurred between the two groups. The residual contraction at maximal ACh was more than 2 times higher in $H_{\text{mox}}1(-/-)$ mice ($p < 0.001$), indicating a reduced effectiveness of the endothelial active agent in this group.

Subsequent addition of SNP followed by YC-1 (which acts directly on smooth muscle sGC) caused further relaxation in $H_{\text{mox}}1(-/-)$ mice (Fig. 2B). Still the relaxation remained significantly lower in the $H_{\text{mox}}1(-/-)$ mice ($p < 0.001$). The two groups converged with the final addition of Ado, an

Fig. 1. Contractile responses of SMA (●, ○) to PE (log PE) and FA (▲, △) to 5-HT (log 5-HT). The $H_{\text{mox}}1(+/+)$ mice are indicated by solid symbols, and the $H_{\text{mox}}1(-/-)$ mice are indicated by open symbols. The lines are curves derived from the Hill equation, with the following values for log EC_{50} : SMA = -5.37 ± 0.02 [$n = 14$, $H_{\text{mox}}1(+/+)$] and -5.57 ± 0.02 [$p < 0.001$; $n = 12$, $H_{\text{mox}}1(-/-)$] and FA = -6.35 ± 0.01 [$n = 8$, $H_{\text{mox}}1(+/+)$] and -6.56 ± 0.04 [$p < 0.001$, $n = 12$, $H_{\text{mox}}1(-/-)$]. Data points are means of the percentage of maximal response $\pm$ S.E.

Fig. 2. A, contraction of SMA (PE; 30 μ M) during cumulative addition of ACh (log ACh, M). B, percentage of relaxation of SMA to ACh (10 μ M) with cumulative addition of SNP (10 μ M), YC-1 (30 μ M), and Ado (100 μ M). $H_{\text{mox}}1(+/+)$ mice are noted by solid circles and bars, whereas $H_{\text{mox}}1(-/-)$ mice are noted by open circles and bars. In A, the lines are curves derived from the Hill equation, with the following values for log IC_{50} : $H_{\text{mox}}1(+/+)$ mice = -6.70 ± 0.02 ($n = 14$) and $H_{\text{mox}}1(-/-)$ mice = -6.38 ± 0.08 ($p < 0.005$; $n = 12$). Data points are means $\pm$ S.E. of the percentage of the initial contraction remaining after each addition of ACh. In B, $H_{\text{mox}}1(+/+)$ mice ($n = 14$) are indicated as +/+ and $H_{\text{mox}}1(-/-)$ mice ($n = 12$) as -/-. The bars are means with S.E. shown. The p values are shown above the drug pair being compared.

agent that acts on adenylate cyclase (Franke et al., 2004). The IC_{50} value for ACh (Fig. 2A) was shifted significantly to the right ($\Delta \log = 0.32 \pm 0.08$; $p < 0.005$) in the $H_{\text{mox}}1(-/-)$ mice. This shift is more apparent in Fig. 3 in which the responses were normalized to the maximal response of each group. A similar shift to the right ($\Delta \log = 0.36 \pm 0.11$; $p < 0.01$) was observed in the FA from $H_{\text{mox}}1(-/-)$ mice (Fig. 3). Arteries from five sites were exposed first to a high concentration (10 μ M) of an agonist, followed by a high concentration (3 μ M) of ACh (2–4 min) and then SNP (10 μ M). This protocol was meant to reduce a potential accommodation to ACh and to nitro-compounds that could occur during the extended time (approximately 20 min) used for the cumulative concentration-response curves. The percentage of relaxation to ACh (Fig. 4A) and to SNP (Fig. 4B) is shown. The SMA, mesenteric artery branch, and RA from $H_{\text{mox}}1(-/-)$ mice exhibited significantly less relaxation to ACh and to SNP, whereas the aorta and FA did not. The observation of

Fig. 3. Relaxation of SMA (●, ○) and FA (▲, △) with ACh (log M). The $H_{\text{max}}1(+/+)$ mice are indicated by solid symbols, whereas the $H_{\text{max}}1(-/-)$ mice are indicated by open symbols. The lines are curves derived from the Hill equation, with the following values for IC_{50} : SMA = -6.65 ± 0.04 [$n = 14$, $H_{\text{max}}1(+/+)$] and -6.34 ± 0.04 [$p < 0.001$, $n = 12$, $H_{\text{max}}1(-/-)$] and FA = -7.57 ± 0.08 [$n = 8$, $H_{\text{max}}1(+/+)$] and -7.21 ± 0.07 [$p < 0.01$, $n = 12$, $H_{\text{max}}1(-/-)$]. Data points are means of the percentage of maximal response $\pm$ S.E.

Fig. 4. Percentage of relaxation of arteries to ACh (A) and to SNP (B) plotted as in Fig. 2B. $H_{\text{max}}1(+/+)$ mice are indicated as +/+, and $H_{\text{max}}1(-/-)$ mice are indicated as -/-. The bars are the means with S.E. The number of animals is given in parentheses, and the p values are shown above the arterial pair being compared. MABr, mesenteric artery branch.

reduced relaxation of SMA during the concentration-response measures (Fig. 2B) thus was confirmed.

The effectiveness of ACh and the other dilators on $H_{\text{max}}1(-/-)$ arteries was further evaluated in SMA subjected

to two levels of agonist stimulation. Stimulation with PE alone resulted in a contraction that was 1.2 times greater than that to high K^+ , whereas added 5-HT resulted in a contraction of more than 2.5 times that to high K^+ in both groups (Supplemental Table 1). If the pathways for relaxation have a limited capacity, then one could expect that the percentage of relaxation to a maximal concentration of relaxant would be reduced at the higher level of stimulus. Both groups exhibited reduced responses ($p < 0.01$) to ACh and to SNP at the higher level of stimulation (Supplemental Table 1). The reduction ($p < 0.05$) was greater in $H_{\text{max}}1(-/-)$ mice (3–4-fold) than in $H_{\text{max}}1(+/+)$ mice, which was less than 2-fold.

Nitrate Tolerance. The reduced responses of $H_{\text{max}}1(-/-)$ mice to ACh and to SNP were similar to responses that occur during nitrate tolerance (Bennett et al., 1988; Friebe and Koesling, 2003). Nitrate tolerance can result from multiple causes that include altered redox state of the heme iron in sGC, down-regulation of the amount of sGC expressed in vascular smooth muscle, or both (Friebe and Koesling, 2003; Münzel et al., 2005). We adopted a pharmacological approach to the question of altered sGC redox state. The relaxation responses of SMA to BAY 60-2770, an agent that was reported to be a more effective activator of oxidized/heme-free sGC than reduced/heme-containing sGC, were compared with those to BAY 41-2272, which was a more effective stimulator of reduced sGC (Evgenov et al., 2006; Knorr et al., 2008). We first tested this concept on SMA from $H_{\text{max}}1(+/+)$ mice as shown in Fig. 5. Concentration-dependent relaxations of PE contractures (30 μ M) were conducted in the absence and presence of ODQ, an oxidizer of sGC heme (Evgenov et al., 2006). The relaxation to BAY 60-2770 and to BAY 41-2272 exhibited similar sensitivity under control conditions. In the presence of ODQ (~ 30 -min pretreatment), the response to BAY 60-2770 was shifted to the left ($p < 0.01$), whereas the response to BAY 41-2272 was shifted to the right ($p < 0.01$), which was similar to the effects reported for other preparations (Evgenov et al., 2006; Knorr et al., 2008). The IC_{50} values shown in Fig. 6 indicate that the sensitivity of

Fig. 5. Relaxation of $H_{\text{max}}1(+/+)$ SMA ($n = 8$; PE, 30 μ M) with BAY 60-2770 (log M) without (●) and with (○) ODQ (30 μ M; ~ 30 -min exposure before adding the BAY compounds), and BAY 41-2272 without (▲) and with (△) ODQ. The IC_{50} values are shown at the top of the figure, with the p values for the effects of ODQ. The values used by the software program to compute the curves were not used in the statistical analyses. Data points are means ($n = 8$) of the percentage of maximal response $\pm$ S.E.

Fig. 6. IC₅₀ values for the BAY compounds in the absence and presence of ODQ. H_{mox}1(+/+) mice are indicated as +/+, and H_{mox}1(-/-) mice are indicated as -/-. The bars are the means with 1 S.E. The number of animals is shown in the parentheses, and *p* values are shown above the H_{mox}1 pair being compared.

H_{mox}1(-/-) mice to BAY 60-2770 was approximately 15 times greater (*p* < 0.025) than that of H_{mox}1(+/+) mice, whereas the sensitivity to BAY 41-2272 was reduced but not significantly reduced. In sharp contrast to the ODQ effect on H_{mox}1(+/+) mice (Fig. 5), ODQ had little effect on the responses of H_{mox}1(-/-) mice to the BAY compounds (Fig. 6).

Active sGC is a heterodimer of one α -subunit and one β -subunit to which the heme associates (Pyriochou and Papapetropoulos, 2005). Both the α 1 and β 1 subunit levels in SMA from H_{mox}1(-/-) mice (Fig. 7A) were reduced similarly to 46 and 44% of H_{mox}1(+/+) levels, respectively (*p* < 0.025). The levels of these subunits in the thoracic aorta (Fig. 7B) were also reduced to 88 and 70% of H_{mox}1(+/+) levels (*p* < 0.04). These reductions, however, were significantly less (*p* < 0.001) than the reduction that occurred in the SMA.

Discussion

Our study shows that H_{mox}1 is an important regulator of arterial function. Arteries from H_{mox}1(-/-) mice exhibited increased sensitivity to constrictors, reduced sensitivity to ACh, and greatly reduced relaxation to agents that act via NO and sGC. Reduced relaxation and activity of the GK pathway were reported previously after treatment with metalloprotoporphyrin inhibitors of H_{mox}, but establishing H_{mox}1 as the primary cause was problematic because sGC also could have been inhibited (Stasch et al., 2006). Our study avoided this uncertainty.

Contraction. The increased sensitivity to PE and 5-HT was small and may be secondary to an impaired endothelium. Increased sensitivity to catecholamines occurred after endothelial removal or inhibition of eNOS in normal arteries (Fukuda et al., 1990; Vanhoutte, 1989). Thus, a normally functioning endothelium has an inhibitory effect through basal production of dilators such as NO. Because H_{mox}1(-/-) exhibited normal blood pressure (Wiesel et al., 2001), the effect of increased sensitivity to catecholamines seems to be

A Superior Mesenteric Arteries

B Thoracic Aorta

Fig. 7. Expression of α 1 and β 1 subunits of sGC in SMA (A) and thoracic aorta (B) from H_{mox}1(+/+) and H_{mox}1(-/-) mice. The bars are the means of three to five measures with S.E. shown. The *p* values are shown above the pair being compared.

compensated by regulatory mechanisms. Perturbations (e.g., renal artery clip) that cause hypertension, however, showed an exaggerated response in H_{mox}1(-/-) mice (Wiesel et al., 2001). The enhanced sensitivity of H_{mox}1(-/-) mice to catecholamines indicates that altered receptor signaling could be a factor. This possibility should be explored.

Relaxation. The small decrease in sensitivity to ACh when coupled with reduced capacity to relax (Fig. 2B) also could contribute significantly to exaggerated responses to perturbations that cause hypertension. The reduced responses to ACh occurred in multiple sites, including small arterial branches (Fig. 4A). Thus, the dysfunction may extend throughout the vascular system of $H_{\text{mox}}1(-/-)$ mice.

The dysfunction in $H_{\text{mox}}1(-/-)$ mice extended to the NO donor SNP and to YC-1, a stimulator of sGC (Figs. 2B and 4B). In contrast, Ado, which acts via adenylate cyclase, resulted in equivalent relaxations for both groups. Comparison of the relaxation responses at two levels of contraction (Supplemental Table 1) showed that the dysfunction was more apparent at the greater contraction. This supports the concept that the capacity of sGC is reduced in $H_{\text{mox}}1(-/-)$ mice. At low levels of contraction, sGC had sufficient activity to provide intermediate control, whereas the capacity was insufficient to provide adequate responses at high levels of contraction. This could explain why $H_{\text{mox}}1(-/-)$ mice with normal blood pressure exhibited an exaggerated increase when the system was perturbed with a renal artery clip (Wiesel et al., 2001) and why relatively normal carotid arteries in $H_{\text{mox}}1(-/-)$ mice that had undergone intimal damage exhibited exaggerated remodeling and thrombosis (Duckers et al., 2001; True et al., 2007). The reduced responses we observed to ACh, SNP, and YC-1 indicated that arteries from $H_{\text{mox}}1(-/-)$ mice may have developed nitrate tolerance that involved sGC.

Nitrate Tolerance. Three major factors can contribute to reduced NO-mediated relaxation. First, a reduced ability of ACh to stimulate eNOS could lead to reduced availability of NO. If this were the major factor, then subsequent addition of SNP and YC-1 should have caused relatively normal relaxations. This did not occur (Figs. 2B and 4). Our limited observations do not exclude altered coupling between ACh receptor occupancy and NO production. Elevated levels of reactive oxygen species have been associated with uncoupling of eNOS that resulted in a reduced response to ACh (Münzel et al., 2005). This could occur in $H_{\text{mox}}1(-/-)$ mice because they lack an important antioxidant component (Korthuis and Durante, 2005; Kim et al., 2006). Detailed measures of endothelial function are required to test this hypothesis. Our observations, however, indicate that a major reduction in sGC activation and downstream events is associated with $H_{\text{mox}}1(-/-)$ mice.

A second factor is an altered redox status of sGC. The development of compounds that selectively interact with sGC in the reduced and in the oxidized/heme-free state has provided valuable pharmacological tools to test the redox status of sGC in $H_{\text{mox}}1(-/-)$ mice. YC-1 and BAY41-2272 selectively *stimulate* sGC that contain a reduced heme group, whereas BAY 58-2667 and BAY 60-2770 selectively *activate* heme-free sGC (Evgenov et al., 2006; Knorr et al., 2008; Roy et al., 2008). When heme Fe^{2+} is oxidized, the heme pocket is vacated and available for the binding of the sGC activators (Stasch et al., 2006; Hoffmann et al., 2009). Brief exposure (~30 min) to ODQ can accomplish this oxidation and subsequent removal of heme without reducing sGC levels (Hoffmann et al., 2009) and can serve as a test of these compounds.

Exposure to ODQ predictably reduced the sensitivity to BAY 41-2272 and increased the sensitivity to BAY 60-2770 in

$H_{\text{mox}}1(+/+)$ mice (Fig. 5). It is reasonable to assume that arteries from $H_{\text{mox}}1(+/+)$ mice had a high percentage of sGC in the reduced/heme-containing form before treatment with ODQ. In sharp contrast, treatment with ODQ had no significant effect on the sensitivity of $H_{\text{mox}}1(-/-)$ mice to the BAY compounds (Fig. 6). This is consistent with the conclusion that a high percentage of sGC in $H_{\text{mox}}1(-/-)$ mice was oxidized/heme-free before treatment with ODQ. The observation that the untreated SMA from $H_{\text{mox}}1(-/-)$ mice was 15-fold more sensitive to BAY 60-2770 also supports this conclusion. This difference was negated by ODQ pretreatment of $H_{\text{mox}}1(+/+)$ mice.

The $H_{\text{mox}}1(-/-)$ mice did retain an ability to relax to BAY 41-2272 in the absence and presence of ODQ. This is consistent with the observation that BAY 41-2272 (10 μM) stimulated sGC approximately 4-fold in the presence of ODQ (Stasch et al., 2001). Although this was 10 times less than the stimulation in the absence of ODQ, it could be sufficient for a biological effect. For example, a 3-fold increase in cGMP induced by SNP was associated with 80% relaxation of rat aorta (Magliola and Jones, 1990). As little as 6% of the sGC content was sufficient to support NO relaxation in aorta from $\alpha 1$ -sGC-deficient mice, and the concept was proposed that spare sGC receptors ensure high NO sensitivity (Mergia et al., 2006). BAY 41-2272 also may have an inhibitory action on phosphodiesterase type 5, yielding a sildenafil-like effect (Mullershausen et al., 2004). Our protocol for the BAY compounds used PE contractures that developed half the force achieved with PE plus 5-HT (Supplemental Table 1). Under these conditions, sufficient reduced sGC may be available for BAY 41-2272 to relax SMA from $H_{\text{mox}}1(-/-)$ mice.

A third factor is an increased degradation of oxidized/heme-free sGC. Both $\alpha 1$ and $\beta 1$ subunit levels in vascular tissues were reduced by 20- to 24-h (but not 30-min) treatment with ODQ (Hoffmann et al., 2009; Meurer et al., 2009). Cultured endothelial cells exhibited ~50% reduction in both subunits, and the subunit levels in rat aorta fell to ~30% of controls. Simultaneous treatment of the preparations with BAY 58-2667 prevented the loss of sGC subunits (Hoffmann et al., 2009; Meurer et al., 2009). It was further shown that oxidized/heme-free sGC was selectively targeted for ubiquitination and proteasomal degradation, a process that was inhibited by BAY 58-2667 (Meurer et al., 2009). It seems that oxidation of the sGC heme reduces the responses to NO by acute removal of its receptor heme, and by a slower removal of the subunits that contain the catalytic sites. Our observation of a >50% reduction of $\alpha 1$ - and $\beta 1$ -sGC levels in SMA from $H_{\text{mox}}1(-/-)$ mice (Fig. 7A) is in the range noted above (Hoffmann et al., 2009; Meurer et al., 2009). This finding supports the concept that sGC in $H_{\text{mox}}1(-/-)$ mice has undergone significant oxidation, heme loss, and degradation. At ~50% loss of sGC, we observed significant loss of relaxation in SMA. A lesser reduction in sGC occurred in the aorta (Fig. 7B) that was not associated with a significant change in relaxation (Fig. 4). Vascular tissues seem to contain spare sGC, and a partial loss (12–30%) may not affect relaxation during submaximal contraction and exposure to high concentrations of NO. Our observations on SMA from $H_{\text{mox}}1(-/-)$ mice indicate that the loss of antioxidant function resulted in an ODQ-like effect on sGC. This supports the proposal that

H_{mox}-1 is important for the maintenance of sufficient reduced sGC to meet challenges such as ischemia reperfusion injury.

Antioxidant Function of H_{mox}-1. H_{mox}-1 is recognized to be part of the cellular antioxidant system. Our study identified by means of an H_{mox}-1 knockout model a specific target, sGC, that is maintained in a reduced state as a result of H_{mox}-1. The antioxidant properties of H_{mox}-1 products may be particularly important in preventing oxidation of the vulnerable Fe²⁺ in the weakly bonded heme group on sGC. Maintenance of sensitivity to NO, production of cGMP, and activation of GK has been shown to be especially important for the reduction of ischemia reperfusion injury (Ahluwalia et al., 2004). The sGC pathway is also important for the maintenance of normal cardiovascular function (Münzel et al., 2007; Wolin, 2009). During the initial stages of reperfusion, the cascade of oxidants that are produced may oxidize the sGC heme, causing it to become refractory to the eNOS product NO but not to such compounds as BAY 60-2770 and BAY 58-2667. BAY 58-2667, when given 5 min before cardiac reperfusion, significantly reduced the infarct size (Krieg et al., 2009). Therefore, strategies that recognize the importance of H_{mox}-1 in maintaining reduced sGC could lead to more effective prevention of tissue injury.

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References

- Ahluwalia A, Foster P, Scotland RS, McLean PG, Mathur A, Perretti M, Moncada S, and Hobbs AJ (2004) Antiinflammatory activity of soluble guanylate cyclase: cGMP-dependent down-regulation of P-selectin expression and leukocyte recruitment. *Proc Natl Acad Sci USA* **101**:1386–1391.
- Andresen JJ, Shafi NI, Durante W, and Bryan RM Jr (2006) Effects of carbon monoxide and heme oxygenase inhibitors in cerebral vessels of rats and mice. *Am J Physiol Heart Circ Physiol* **291**:H223–H230.
- Bennett BM, Schröder H, Hayward LD, Waldman SA, and Murad F (1988) Effect of in vitro organic nitrate tolerance on relaxation, cyclic GMP accumulation, and guanylate cyclase activation by glyceryl trinitrate and the enantiomers of isoside dinitrate. *Circ Res* **63**:693–701.
- Duckers HJ, Boehm M, True AL, Yet SF, San H, Park JL, Clinton Webb R, Lee ME, Nabel GJ, and Nabel EG (2001) Heme oxygenase-1 protects against vascular constriction and proliferation. *Nat Med* **7**:693–698.
- Durante W, Johnson FK, and Johnson RA (2006) Role of carbon monoxide in cardiovascular function. *J Cell Mol Med* **10**:672–686.
- Evgenov OV, Pacher P, Schmidt PM, Haskó G, Schmidt HH, and Stasch JP (2006) NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential. *Nat Rev Drug Discov* **5**:755–768.
- Franke R, Yang Y, Rubin LJ, Magliola L, and Jones AW (2004) High-fat diet alters K⁺ currents in porcine coronary arteries and adenosine sensitivity during metabolic inhibition. *J Cardiovasc Pharmacol* **43**:495–503.
- Friebe A and Koesling D (2003) Regulation of nitric oxide-sensitive guanylyl cyclase. *Circ Res* **93**:96–105.
- Fukuda S, Matsumoto M, Nishimura N, Fujiwara N, Shimoji K, Takeshita H, and Lee TJ (1990) Endothelial modulation of norepinephrine-induced constriction of rat aorta at normal and high CO₂ tensions. *Am J Physiol* **258**:H1049–H1054.
- Hoffmann LS, Schmidt PM, Keim Y, Schaefer S, Schmidt HH, and Stasch JP (2009) Distinct molecular requirements for activation or stabilization of soluble guanylyl cyclase upon haem oxidation-induced degradation. *Br J Pharmacol* **157**:781–795.
- Kim HP, Ryter SW, and Choi AM (2006) CO as a cellular signaling molecule. *Annu Rev Pharmacol Toxicol* **46**:411–449.
- Knorr A, Hirth-Dietrich C, Alonso-Alija C, Härter M, Hahn M, Keim Y, Wunder F, and Stasch J-P (2008) Nitric oxide-independent activation of soluble guanylate cyclase by BAY 60-2770 in experimental liver fibrosis. *Arzneimittelforschung* **58**:71–80.
- Korthuis RJ and Durante W (2005) Heme oxygenase-1: a pluripotent sentinel limiting the systemic inflammatory response to extremity ischemia and reperfusion. *Crit Care Med* **33**:2701–2703.
- Krieg T, Liu Y, Rütz T, Methner C, Yang XM, Dost T, Felix SB, Stasch JP, Cohen MV, and Downey JM (2009) BAY 58-2667, a nitric oxide-independent guanylyl cyclase activator, pharmacologically post-conditions rabbit and rat hearts. *Eur Heart J* **30**:1607–1613.
- Magliola L and Jones AW (1990) Sodium nitroprusside alters Ca²⁺ flux components and Ca²⁺-dependent fluxes of K⁺ and Cl[−] in rat aorta. *J Physiol* **421**:411–424.
- Mergia E, Friebe A, Dangel O, Russwurm M, and Koesling D (2006) Spare guanylyl cyclase NO receptors ensure high NO sensitivity in the vascular system. *J Clin Invest* **116**:1731–1737.
- Meurer S, Ploch S, Pabst T, Opitz N, Schmidt PM, Beckhaus T, Wagner K, Matt S, Gegenbauer K, Geschka S, et al. (2009) Nitric oxide-independent vasodilator rescues heme-oxidized soluble guanylate cyclase from proteasomal degradation. *Circ Res* **105**:33–41.
- Müllershausen F, Russwurm M, Friebe A, and Koesling D (2004) Inhibition of phosphodiesterase type 5 by the activator of nitric oxide-sensitive guanylyl cyclase BAY 41-2272. *Circulation* **109**:1711–1713.
- Münzel T, Daiber A, and Mülsch A (2005) Explaining the phenomenon of nitrate tolerance. *Circ Res* **97**:618–628.
- Münzel T, Daiber A, Ullrich V, and Mülsch A (2005) Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cGMP-dependent protein kinase. *Arterioscler Thromb Vasc Biol* **25**:1551–1557.
- Münzel T, Gentz-Zotz S, and Hink U (2007) Targeting heme-oxidized soluble guanylate cyclase: solution for all cardiorenal problems in heart failure? *Hypertension* **49**:974–976.
- Naik JS and Walker BR (2006) Role of vascular heme oxygenase in reduced myogenic reactivity following chronic hypoxia. *Microcirculation* **13**:81–88.
- Ndisang JF, Zhao W, and Wang R (2002) Selective regulation of blood pressure by heme oxygenase-1 in hypertension. *Hypertension* **40**:315–321.
- Poss KD and Toneyawa S (1997) Heme oxygenase 1 is required for mammalian iron reutilization. *Proc Natl Acad Sci USA* **94**:10919–10924.
- Pyriochou A and Papapetropoulos A (2005) Soluble guanylyl cyclase: more secrets revealed. *Cell Signal* **17**:407–413.
- Roy B, Mo E, Vernon J, and Garthwaite J (2008) Probing the presence of the ligand-binding haem in cellular nitric oxide receptors. *Br J Pharmacol* **153**:1495–1504.
- Stasch JP, Becker EM, Alonso-Alija C, Apeler H, Dembowsky K, Feurer A, Gerzer R, Minuth T, Perzborn E, Pleiss U, et al. (2001) NO-independent regulatory site on soluble guanylate cyclase. *Nature* **410**:212–215.
- Stasch JP, Schmidt PM, Nedvetsky PI, Nedvetskaya TY, H S AK, Meurer S, Deile M, Taye A, Knorr A, Lapp H, et al. (2006) Targeting the heme-oxidized nitric oxide receptor for selective vasodilation of diseased blood vessels. *J Clin Invest* **116**:2552–2561.
- True AL, Olive M, Boehm M, San H, Westrick RJ, Raghavachari N, Xu X, Lynn EG, Sack MN, Munson PJ, et al. (2007) Heme oxygenase-1 deficiency accelerates formation of arterial thrombosis through oxidative damage to the endothelium, which is rescued by inhaled carbon monoxide. *Circ Res* **101**:893–901.
- Vanhoutte PM (1989) Endothelium and control of vascular function. State of the Art lecture. *Hypertension* **13**:658–667.
- Wiesel P, Patel AP, Carvajal IM, Wang ZY, Pellacani A, Maemura K, DiFonzo N, Rennke HG, Layne MD, Yet SF, et al. (2001) Exacerbation of chronic renovascular hypertension and acute renal failure in heme oxygenase-1-deficient mice. *Circ Res* **88**:1088–1094.
- Wolin MS (2009) Reactive oxygen species and the control of vascular function. *Am J Physiol Heart Circ Physiol* **296**:H539–H549.
- Wu L and Wang R (2005) Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. *Pharmacol Rev* **57**:585–630.
- Yet SF, Layne MD, Liu X, Chen YH, Ith B, Sibling NE, and Perrella MA (2003) Absence of heme oxygenase-1 exacerbates atherosclerotic lesion formation and vascular remodeling. *FASEB J* **17**:1759–1761.
- Yet SF, Pellacani A, Patterson C, Tan L, Polta SC, Foster L, Lee WS, Hsieh CM, and Perrella MA (1997) Induction of heme oxygenase-1 expression in vascular smooth muscle cells. A link to endotoxic shock. *J Biol Chem* **272**:4295–4301.
- Yet SF, Perrella MA, Layne MD, Hsieh CM, Maemura K, Kobzik L, Wiesel P, Christou H, Kourembanas S, and Lee ME (1999) Hypoxia induces severe right ventricular dilation and infarction in heme oxygenase-1 null mice. *J Clin Invest* **103**:R23–R29.

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