Heme oxygenase-1 deficiency leads to alteration of soluble guanylate cyclase redox regulation

Allan W. Jones, William Durante, and Ronald J. Korthuis.

Department of Medical Pharmacology and Physiology (AWJ, WD, RJK)
and the Dalton Cardiovascular Research Center (AWJ, RJK).

University of Missouri-Columbia, Columbia, Missouri
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Address correspondence to Allan W. Jones, Department of Medical Pharmacology and Physiology, MA 415 Medical Science building, University of Missouri-Columbia, Columbia, MO 65212

(Tel: 573-882-8059; Fax 573-884-4276; e-mail: jonesa@health.missouri.edu)

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**ABBREVIATIONS:** Ado, adenosine; Ach, acetylcholine; CO, carbon monoxide; NO, nitric oxide; ROS, reactive oxygen species; 5-HT, 5-hydroxytryptamine; PE, phenylephrine; SNP, sodium nitroprusside; BAY 41-2272, (5-cyclopropyl-2-{1-(2-fluoro-benzyl) -1H-pyrazolo [3,4-b]pyridin-3-yl} -pyrimidin-4-ylamine); BAY 60-2770 (4-{{(4-carboxybutyl) [2-(5-fluoro-2-[[4'- (trifluoromethyl) biphenyl-4-yl] methoxy] phenyl] ethyl} amino} benzoic acid); ODQ, (1H-[1,2,4] oxadiazolo [3,4-a] quinoxalin-1-one); YC-1, (3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole); eNOS, endothelial NO synthase; GK, protein kinase G; sGC, soluble guanylate cyclase; CrMPIX, chromium mesoporphyrin-IX; SnPPIX, tin protoporphyrin-IX; ZnPPIX, zinc protoporphyrin IX; H\textsubscript{max}\textsuperscript{1}, heme oxygenase-1; H\textsubscript{max}\textsuperscript{2}, heme oxygenase-2; H\textsubscript{max} \textsuperscript{1\\/+}, wild type; H\textsubscript{max} \textsuperscript{1\\/-}, knock-out; FA, femoral artery; RA, renal artery branch; MABr, mesenteric artery branch; SMA, superior mesenteric artery; PS, physiological solution; DS, dissection solution.
ABSTRACT

Heme oxygenase-1 knock-out mice, $\text{Hmox1}^{1/-}$, exhibit exacerbated vascular lesions after ischemia-reperfusion and mechanical injury. Surprisingly we found no studies that reported contractile responses and sensitivity to vaso-relaxants in $\text{Hmox1}^{1/-}$. The contractile responses (superior mesenteric arteries, SMA, from female $\text{Hmox1}^{1/-}$) exhibited increased sensitivity to phenylephrine ($p<0.001$). Cumulative addition of Ach relaxed the SMA with the residual contraction remaining 2 times higher in $\text{Hmox1}^{1/-}$ ($p<0.001$). SNP (NO donor) and YC-1 (acts directly on sGC) led to further relaxation, yet the residual contraction remained 2-3 times higher in $\text{Hmox1}^{1/-}$ than $\text{Hmox1}^{1^{+/+}}$ ($p<0.001$). Branches from $\text{Hmox1}^{1/-}$ mesenteric and renal arteries also showed reduced relaxation ($p<0.025$). Relaxation of SMA was measured to BAY 60-2770, which is a more effective activator of oxidized/heme-free sGC; and to BAY 41-2272, a more effective stimulator of reduced sGC. $\text{Hmox1}^{1/-}$ arteries were 15 times more sensitive to BAY 60-2770 ($p<0.025$) than were $\text{Hmox1}^{1^{+/+}}$. Pretreatment with ODQ, an oxidizer of sGC, predictably shifted the BAY 60-2770 response of $\text{Hmox1}^{1^{+/+}}$ to the left ($p<0.01$) and BAY 41-2272 to the right ($p<0.01$). ODQ had little effect on the responses of $\text{Hmox1}^{1/-}$ 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 $\text{Hmox1}^{1^{+/+}}$ ($p<0.025$). These findings support the hypothesis that the anti-oxidant function of $\text{Hmox1}$ 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, \( \text{H}_{\text{max}} \text{ox} \text{1} \), is an inducible cytoprotective enzyme that degrades heme to biliverdin, iron and carbon monoxide, 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 (Kim et al., 2006; Korthuis and Durante, 2005). One of the products, CO, has been the focus of many studies that have linked \( \text{H}_{\text{max}} \text{ox} \text{1} \) to vascular function. CO was shown to act as a vasodilator and at high concentrations it stimulated soluble guanylate cyclase (sGC) and cGMP formation (Kim et al., 2006; Durante 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 \( \text{H}_{\text{max}} \text{ox} \text{1} \) directly and to apply its products such as CO (Kim et al., 2006; Durante et al., 2006). For instance hemin injected into 8 week old spontaneously hypertensive rats increased \( \text{H}_{\text{max}} \text{ox} \text{1} \) and sGC levels in arteries and lowered blood pressure (Ndisang et al., 2002). Transfection of porcine arteries with \( \text{H}_{\text{max}} \text{ox} \text{1} \) shifted the phenylephrine – response curves to the right (reduced sensitivity) while treatment with the \( \text{H}_{\text{max}} \text{ inhibitor, ZnPPX} \), eliminated the difference (Duckers et al., 2001). Treatment with lipopolysaccharide induced \( \text{H}_{\text{max}} \text{ox} \text{1} \) expression in arteries and significantly reduced blood pressure in rats, while pretreatment with ZnPPIX prevented the fall in blood pressure (Yet et al., 1997).

Metalloprotoporphyrins have been widely used to study the role of \( \text{H}_{\text{max}} \text{ox} \text{1} \) in the regulation of vascular function. These compounds, such as ZnPPIX, SnPPIX, and CrMPIX, consistently alter vascular responses in-vitro. For instance ZnPPIX increased myogenic tone in mesenteric arteries from rats exposed to chronic hypoxia, a treatment that increased \( \text{H}_{\text{max}} \text{ox} \text{1} \) activity (Naik and Walker, 2006). A recent study indicated that metalloprotoporphyrins may also have non-specific
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 Hmox (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 Hmox would remove its anti-oxidant 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 Hmox and sGC becomes problematic, since these enzymes are closely linked to the signaling pathway operating on smooth muscle contraction.

Another approach to the evaluation of Hmox1 has used knock-out mice, Hmox1$^{-/-}$ (Poss and Tonegawa, 1997). Although these mice exhibited no change in Hmox2 levels, increased cardiac and renal damage occurred during ischemic conditions (Yet et al., 1999; Wiesel et al., 2001). Hmox1$^{-/-}$ also exhibited an exacerbation of vascular lesions in response to hyperlipidemia, mechanical and photochemical injury (Duckers et al., 2001; True et al., 2007; Yet et al., 2003). Vascular smooth muscle cells from Hmox1$^{-/-}$ also exhibited increased proliferation when maintained in cell culture (Duckers et al., 2001). Surprisingly, we have not found studies that used Hmox1$^{-/-}$ to evaluate arterial contractile responses and their sensitivity to endothelial and pharmacological relaxants.

The present study was undertaken to determine whether arteries from Hmox1$^{-/-}$ exhibit functional alterations that are similar to those reported during pharmacological manipulation of Hmox activity. In addition, we have attempted to determine whether the nitrate tolerance we observed in arteries from Hmox1$^{-/-}$ resulted from an altered heme redox-state and/or reduced expression of sGC.

**METHODS**

**Animals.** Wild type H129, Hmox1$^{+/+}$ breeders were obtained from the Jackson Laboratories.
(Bar Harbor, ME). Hmo1−/− breeders with the H129 background were obtained as a gift from Dr. William Fay (University of Missouri Columbia). All 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 PCR methods. Primers (HO/e3 and HO/13R, ~475bp, for wild type; and NEO1 and HO/e4, ~375bp, for HO-1 knock-out) were obtained from Invitrogen (Carlsbad, CA). Blood vessels and tissue samples were removed from female mice (9-12 weeks old) which were first anesthetized with a mixture of ketamine (150mg/kg body weight, i.p.) and xylazine (7.5mg/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 cold dissection solution (DS, composition below). The arteries were trimmed free of fat and cut into rings about 1 mm long. The outside diameter was measured with a calibrated reticle in the dissection microscope (Nikon model SMZ-2B, Brighton, MI). The range of values (in µm) is: aorta, 800-1,000; superior mesenteric artery (SMA), 440-560; mesenteric artery branch (MABr), 1st and 2nd order branches, 200-250; renal artery (RA), 1st and 2nd order branches, 250-330; femoral artery (FA), 300-380. The rings were mounted on force transducers (Grass model FT03, West Warwick, RI) which had specially fabricated feet with an open wire (25 or 51 µm diameter) design. One foot was attached to the cantilever and the other 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 1.25x resting length before being placed into physiological solution (PS) (10ml contained in plastic cups) at 36°C. The rings were tested with K80 (K=80mM substituted for Na) to establish a reference contracture then returned to PS, relaxed, stretched about 5%, and retested with
K80 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 minutes. The Ca chelator, EGTA=2.5mM, was applied at the end of the protocols to determine the basal non-contracted 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 3-4 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 $\alpha_1$ (1:1,000) and $\beta_1$ (1:750) subunits and $\beta$–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 chemo-luminescence reagents (GE Healthcare, Waukesha, WI). The expression of sGC $\alpha_1$ and $\beta_1$ protein was quantified by scanning densitometry and normalized with respect to $\beta$–actin.

**Solutions and drugs.** The PS had the following composition (in mM): 138 NaCl, 5.0 KCl, 1.5 CaCl$_2$, 1.2 MgCl$_2$, 1.2 NaH$_2$PO$_4$, 10 NaHEPES (pH 7.35), and 11.2 glucose. DS had a similar composition with Ca$^{2+}$ reduced to 0.15 mM. Phenylephrine (PE), sodium nitroprusside (SNP), acetylcholine (Ach), YC-1 (see ABBREVIATIONS for names of complex chemicals), BAY 41-2272, ascorbic acid and antibodies against sGC $\alpha_1$ and $\beta_1$ were purchased from Sigma Chemical.
Company (St.Louis, MO). An antibody against β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 5-hydroxytryptamine (5-HT) was purchased from Aldrich Chemical Company (St. Louis, MO). Adenosine (Ado) was purchased from Research Biochemical International (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 (10mM) of PE, 5-HT, Ach, and Ado were prepared in 10mM ascorbic acid and were frozen (-20°C). SNP (10mM) was prepared in ascorbic acid and stored at 4°C. YC-1, ODQ and the BAY compounds were dissolved (10mM) in dimethylsulfoxide (DMSO) 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 verses percent response. The relaxation responses were calculated both as percent of the remaining maximal contraction and as percent of the maximal relaxation that was achieved. Only one curve was determined for each animal (n=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: minimum %, maximum %, midpoint concentration [log M for EC50 or IC50], and slope h) by means of Sigma Plot software. The software also provided an error analysis for each of the parameters which was used to determine the significance of comparisons between Hmox1+/+ and Hmox1−/−. Because the concentration-response curves for the BAY compounds were relatively flat, the IC50 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 ± SE.
RESULTS

Contraction. The contractile responses of the femoral artery (FA) to 5-HT and the superior mesenteric artery (SMA) to PE were shifted slightly (Δlog = -0.20±0.04), but significantly (p<0.001) to the left in Hmox1-/- which showed increased sensitivity to the agonists (Fig. 1). No consistent differences in the maximal contractile response to K80 was observed between the two groups of mice, nor did the ratio of the maximal agonist response to the high K response differ (Table 1, Supplemental data).

Relaxation. Cumulative increases in Ach elicited a concentration-dependent relaxation of SMA that was pre-contracted with PE (Fig. 2A). Significant differences occurred between the two groups. The residual contraction at maximal Ach was over 2 times higher in Hmox1-/- (p<0.001) which indicated 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 Hmox1-/- (Fig. 2B). Still the relaxation remained significantly lower in the Hmox1-/- (p<0.001). The two groups converged with the final addition of Ado, an agent that acts on adenylate cyclase (Franke et al., 2004).

The IC50 for Ach in Fig. 2A was shifted significantly to the right (Δlog = 0.32±0.08, p<0.005) in the Hmox1-/- This shift is more apparent in Fig. 3 where the responses were normalized to the maximal response of each group. A similar shift to the right (Δlog = 0.36 ± 0.11, p<0.01) was also observed in the FA from Hmox1-/- (Fig. 3).

Arteries from 5 sites were exposed first to a high concentration (10µM) of an agonist, followed by a high concentration (3µM) of Ach (2-4 minutes) and then SNP (10µM). This protocol was meant to reduce a potential accommodation to Ach and to nitro-compounds which could occur during the extended time (about 20 minutes) used for the cumulative concentration-response curves. The percent relaxation to Ach appears in Fig. 4A and to SNP in Fig. 4B. The
SMA, MABr, and RA from Hmox1⁻/⁻ exhibited significantly less relaxation to Ach and to SNP, while the aorta and FA did not. The observation of reduced relaxation of SMA during the concentration-response measures (Fig. 2B) was thus confirmed.

The effectiveness of Ach and the other dilators on Hmox1⁻/⁻ 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 K80, whereas added 5-HT resulted in a contraction of over 2.5 times that to K80 in both groups (Table 1, Supplemental data). If the pathways for relaxation have a limited capacity, then one could expect that the percent 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 (Table 1, Supplemental data). The reduction (p<0.05) was greater in Hmox1⁻/⁻ (3-4 fold) than in Hmox1⁺/⁺, which was less than 2 fold.

**Nitrate tolerance.** The reduced responses of Hmox1⁻/⁻ 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 which include altered redox-state of the heme Fe in sGC and/or down regulation of the amount of sGC expressed in vascular smooth muscle (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 an agent (BAY 60-2770) that was reported to be a more effective activator of oxidized/heme-free sGC than reduced/heme-containing sGC were compared to BAY 41-2272 which was a more effective stimulator of reduced sGC (Knorr et al., 2008; Evgenov et al., 2006). We first tested this concept on SMA from Hmox1⁺/⁺ as shown in Fig. 5. Concentration-dependent relaxations of PE contractures (30µM) 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 (Knorr et al, 2008; Evgenov et al., 2006). The IC50s shown in Fig. 6 indicate that the sensitivity of Hmox1-/- to BAY 60-2770 was about 15 times greater (p<0.025) than Hmox1+/+, whereas, the sensitivity to BAY 41-2272 was reduced, but not significantly. In sharp contrast to the ODQ effect on Hmox1+/+ (Fig. 5), ODQ had little effect on the responses of Hmox1-/- to the BAY compounds (Fig. 6).

Active sGC is a hetero-dimer of one α-subunit and one β-subunit to which the heme associates (Pyriochou and Papapetropoulos, 2005). Both the α1 and β1 subunit levels in SMA from Hmox1-/- (Fig 7A) were reduced similarly to 46% and 44% of Hmox1+/+ (p<0.025). The levels of these subunits in the thoracic aorta (Fig. 7B) were also reduced to 88% and 70% of Hmox1+/+ (p<0.04). These reductions, however, were significantly less (p<0.001) than the reduction which occurred in the SMA.

**DISCUSSION**

Our study shows that Hmox1 is an important regulator of arterial function. Arteries from Hmox1-/- 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 previously reported after treatment with metalloprotoporphyrin inhibitors of Hmox, but establishing Hmox1 as the primary cause was problematic since sGC could also 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. Since $H_{\text{max1}}^{-/-}$ exhibited normal blood pressure (Wiesel et al., 2001), the effect of increased sensitivity to catecholamines appears to be compensated by regulatory mechanisms. Perturbations (e.g. renal artery clip) that cause hypertension, however, showed an exaggerated response in $H_{\text{max1}}^{-/-}$ (Wiesel et al., 2001). The enhanced sensitivity of $H_{\text{max1}}^{-/-}$ 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) could also 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 $H_{\text{max1}}^{-/-}$.

The dysfunction in $H_{\text{max1}}^{-/-}$ 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 (Table 1, Supplemental data) showed that the dysfunction was more apparent at the greater contraction. This supports the concept that the capacity of sGC is reduced in $H_{\text{max1}}^{-/-}$. 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 $H_{\text{max1}}^{-/-}$ 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 $H_{\text{max1}}^{-/-}$ which 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 Hmox1-/- may have developed nitrate tolerance which 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 which resulted in a reduced response to Ach (Münzel et al., 2005). This could occur in Hmox1-/- since they lack an important anti-oxidant component (Kim et al., 2006; Korthuis and Durante, 2005). Detailed measures of endothelial function will be required to test this. Our observations, however, indicate that a major reduction in sGC activation and downstream events is associated with Hmox1-/-.

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 Hmox1-/. YC-1 and BAY41-2272 selectively stimulate sGC that contain a reduced heme group, while 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 (Hoffman et al., 2009, Stasch et al., 2006). Brief exposure (~30 min) to ODQ can accomplish this oxidation and subsequent removal of heme without reducing sGC levels (Hoffman et al., 2009) and can serve as a test of these compounds.

Exposure to ODQ predictably reduced the sensitivity to BAY-42272 and increased the
sensitivity to BAY 60-2770 in Hmox1+/+ (Fig. 5). It is reasonable to assume that arteries from Hmox1+/+ 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 Hmox1−/− to the BAY compounds (Fig. 6). This is consistent with the conclusion that a high percentage of sGC in Hmox1−/− was oxidized/heme-free before treatment with ODQ. The observation that the untreated SMA from Hmox1−/− was 15 fold more sensitive to BAY 60-2770 also supports this conclusion. This difference was negated by ODQ pretreatment of Hmox1+/+.

The Hmox1−/− 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 (10µM) stimulated sGC about 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 instance 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 α1-sGC deficient mice and the concept was proposed that spare sGC receptors ensure high NO sensitivity (Mergia et al., 2006). BAY 41-2272 may also have an inhibitory action on phosphodiesterase type 5 (PDE5) yielding a sidenafil-like effect (Mullershausen et al., 2004). Our protocol for the BAY compounds employed PE contractures which developed half the force achieved with PE plus 5-HT (Table 1, Supplemental data). Under these conditions sufficient reduced sGC may be available for BAY 41-2272 to relax SMA from Hmox1−/−.

A third factor is an increased degradation of oxidized/heme-free sGC. Both α1 and β1 subunit levels in vascular tissues were reduced by 20-24 hr (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 appears 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 which contain the catalytic sites. Our observation of a >50% reduction of \( \alpha_1 \)- and \( \beta_1 \)-sGC levels in SMA from Hmox1-/- (Fig. 7A) is in the range noted above (Hoffmann et al., 2009, Meurer et al., 2009). This finding supports the concept that sGC in Hmox1-/- 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) which was not associated with a significant change in relaxation (Fig. 4). Vascular tissues appear to contain spare sGC and a partial loss (12% to 30%) may not affect relaxation during sub-maximal contraction and exposure to high concentrations of NO. Our observations on SMA from Hmox1-/- indicate that the loss of anti-oxidant function resulted in an ODQ-like effect on sGC. This supports the proposal that Hmox1 is important for the maintenance of sufficient reduced sGC to meet challenges such as ischemia reperfusion injury.

**Anti-oxidant function of Hmox1.** Hmox1 is recognized to be part of the cellular anti-oxidant system. Our study identified by means of a Hmox1 knockout model a specific target, sGC, that is maintained in a reduced state as a result of Hmox1. The antioxidant properties of Hmox1 products may be particularly important in preventing oxidation of the vulnerable Fe\(^{2+}\) 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 minutes before cardiac reperfusion, significantly reduced the infarct size (Krieg et al., 2009). Therefore strategies which recognize the importance of Hmox1 in maintaining reduced sGC could lead to more effective prevention of tissue injury.

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Footnotes

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Legends for figures.

**Fig. 1.** Contractile responses of SMA (●,○) to PE (Log PE) and FA (▲,Δ) to 5-HT (Log 5-HT). The $H_{\text{max}}^{1+/+}$ are solid symbols while the $H_{\text{max}}^{1-/-}$ are 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, $H_{\text{max}}^{1+/+}$) and -5.57±0.02 (p<0.001, n=12, $H_{\text{max}}^{1-/-}$); and FA = -6.35±0.01 (n=8, $H_{\text{max}}^{1+/+}$) and -6.56±0.04 (p<0.001, n=12, $H_{\text{max}}^{1-/-}$). Data points are means of the percent maximal response ± SE.

**Fig. 2.** Contraction of SMA (PE, 30µM) during cumulative addition of Ach (Log Ach,M) appear in Panel A. Percent relaxation of SMA to Ach (10µM) with cumulative addition of SNP (10µM), YC-1 (30µM) and Ado (100µM) appear in Panel B. $H_{\text{max}}^{1+/+}$ are noted by solid circles and bars, while $H_{\text{max}}^{1-/-}$ are noted by open circles and bars. In Panel A the lines are curves derived from the Hill equation, with the following values for log IC$_{50}$: $H_{\text{max}}^{1+/+}$ = -6.70±0.02 (n=14) and $H_{\text{max}}^{1-/-}$ = -6.38±0.08 (p<0.005, n=12). Data points are means ± SE of the percent of the initial contraction remaining after each addition of Ach. In Panel B, $H_{\text{max}}^{1+/+}$ (n=14) are indicated as +/+ and $H_{\text{max}}^{1-/-}$ (n=12) as -/- . The bars are means with SE shown. The p values appear above the drug pair being compared.

**Fig. 3.** Relaxation of SMA (●,○) and FA (▲,Δ) with Ach (Log M). The $H_{\text{max}}^{1+/+}$ are solid symbols while the $H_{\text{max}}^{1-/-}$ are 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 percent maximal response ± SE.
Fig. 4. Percent relaxation of arteries to Ach (Panel A) and to SNP (Panel B) plotted as in Fig. 2B. Hmox1+/+ are indicated as +/- and Hmox 1-/- as -/-: The bars are the means with SE shown. The number of animals appears in the parentheses and the p values appear above the arterial pair being compared.

Fig. 5. Relaxation of Hmox1+/+ SMA (n=8, PE, 30µM) with BAY 60-2770 (Log M) without (●) and with (○) ODQ (30µM, ~30 minute exposure before adding the BAY compounds), and BAY 41-2272 without (▲) and with (Δ) ODQ. The IC50 appear 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 percent maximal response ± SE.

Fig. 6. IC50 for the BAY compounds in the absence and presence of ODQ. Hmox1+/+ are indicated as +/- and Hmox 1-/- as -/-: The bars are the means with one SE shown. The number of animals appears in the parentheses and p values appear above the Hmox1 pair being compared.

Fig. 7. Expression of α1 and β1 subunits of sGC in SMA (Panel A) and thoracic aorta (Panel B) from Hmox1+/+ and Hmox 1-/-: The bars are the means of the 3-5 measures with SE shown. The p values appear above the pair being compared.
Fig. 1

Δ FA +/+, EC50 -6.35±0.01
Δ FA -/-, EC50 -6.56±0.04
P<0.001

○ SMA +/+, EC50 -5.37±0.02
○ SMA -/-, EC50 -5.57±0.02
P<0.001

% Contraction

PE, 5-HT, Log M

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Fig. 2

Ach, Log M

% PE Contraction

H_{max}^{1+/-}

IC50 - 6.38±0.08

P< 0.005

H_{max}^{1+/-}

IC50 - 6.70±0.02

7.00 ± 0.02
Fig. 2

B

<table>
<thead>
<tr>
<th>Drug</th>
<th>Ach+/-</th>
<th>SNP+/-</th>
<th>YC-1+/-</th>
<th>Ado+/-</th>
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<tbody>
<tr>
<td>Relaxation (%)</td>
<td>80 ± 5</td>
<td>60 ± 4</td>
<td>80 ± 3</td>
<td>60 ± 3</td>
</tr>
</tbody>
</table>

P < 0.001  0.001  0.001  n.s.
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 3

% Relaxation

Ach, Log M

Δ FA +/+, IC50 -7.57±0.08
Δ FA -/-, IC50 -7.21±0.07

● SMA +/+, IC50 -6.65±0.04
● SMA -/-, IC50 -6.34±0.04

P<0.01

P<0.001
Fig. 4

SNP % Relaxation

Artery

RA +/+  (8)  
RA -/-  (12)  
MABr +/+  (12)  
MABr -/-  (12)  
SMA +/+  (14)  
SMA -/-  (14)  
FA +/+  (8)  
FA -/-  (12)  
Aorta +/+  (12)  
Aorta -/-  (12)

P < 0.025  0.025  0.005  n.s.

B

n.s.

n.s.
• BAY 60-2770, IC50 -6.33 ± 0.22  △ BAY 41-2272, IC50 -6.53 ± 0.30
○ BAY 60 + ODQ, IC50 -7.79 ± 0.53  △ BAY 41 + ODQ, IC50 -5.60 ± 0.03

P<0.01
IC50, Log M

Drug

BAY60 +/+  
BAY60 -/-  
BAY60 +ODQ +/+  
BAY60 +ODQ -/-  
BAY41 +/+  
BAY41 -/-  
BAY41 +ODQ +/+  
BAY41 +ODQ -/-  

P < 0.025  
n.s.  
n.s.  
n.s.  
n.s.  
n.s.  

n.s.
Fig. 7

A Superior Mesenteric Arteries

<table>
<thead>
<tr>
<th>Protein (relative to wild type)</th>
<th>Hmox1+/+</th>
<th>Hmox1−/−</th>
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<tbody>
<tr>
<td>sGC α1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sGC β1</td>
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<tr>
<td>β-actin</td>
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</table>

P < 0.025
Fig. 7

B  Thoracic Aorta

Hmox1+/+  Hmox1−/−

sGC α1  sGC β1

β-actin

Protein (relative to wild type)

P< 0.04  0.04

Hmox1+/+  Hmox1−/−

sGC α1  sGC β1
Supplemental data

The Journal of Pharmacology and Experimental Therapeutics

Heme oxygenase-1 deficiency leads to alteration of soluble guanylate cyclase redox regulation

Allan W. Jones, William Durante, and Ronald J. Korthuis

**TABLE 1**

Percent relaxation of superior mesenteric artery at two levels of contractile stimulation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Ratio: PE/K80†</th>
<th>p&lt;</th>
<th>PE+5-HT/K80</th>
<th>p&lt;</th>
<th>PE+5-HT/PE</th>
<th>p&lt;</th>
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</thead>
<tbody>
<tr>
<td>Hmox1+/+</td>
<td>14</td>
<td>1.2±0.1</td>
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<td>2.6±0.2</td>
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<td>2.4±0.2</td>
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<tr>
<td>Hmox1−/−</td>
<td>12</td>
<td>1.2±0.1</td>
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<td>2.9±0.1</td>
<td>ns</td>
<td>2.7±0.3</td>
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</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Relaxation</th>
<th>% Relaxation</th>
<th>Ratio: PE+5-HT/PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ach</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Hmox1+/+</td>
<td>14</td>
<td>84±3</td>
<td>47±9</td>
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<tr>
<td>Hmox1−/−</td>
<td>12</td>
<td>47±10</td>
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<tr>
<td>SNP</td>
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</tr>
<tr>
<td>Hmox1+/+</td>
<td>14</td>
<td>85±5</td>
<td>53±7</td>
</tr>
<tr>
<td>Hmox1−/−</td>
<td>12</td>
<td>55±9</td>
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<td>YC-1</td>
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<td>84±6</td>
<td>72±6</td>
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<tr>
<td>Hmox1−/−</td>
<td>11</td>
<td>71±7</td>
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<td>Ado</td>
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<tr>
<td>Hmox1+/+</td>
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<td>90±2</td>
<td>89±2</td>
</tr>
<tr>
<td>Hmox1−/−</td>
<td>12</td>
<td>82±5</td>
<td>ns</td>
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</tbody>
</table>

Values are means ± SE and p is for Hmox1+/+ verses Hmox1−/−. PE, 10µM; 5-HT, 10µM; Ach, 3µM; SNP, 10µM; YC-1, 30µM; Ado, 100µM.

†Contractile responses to K80 were 101±10 and 78±7 mg for Hmox1+/+ and Hmox1−/− respectively (ns).