Preconditioning with soluble guanylate cyclase activation prevents postischemic inflammation and reduces nitrate tolerance in heme oxygenase-1 knockout mice

Walter Z. Wang,1 Allan W. Jones,1,2 Meifang Wang,1 William Durante,1 and Ronald J. Korthuis1,2

1Department of Medical Pharmacology and Physiology, University of Missouri, Columbia, Missouri; and 2The Dalton Cardiovascular Research Center, University of Missouri, Columbia, Missouri

Submitted 29 October 2012; accepted in final form 12 June 2013

Preconditioning with soluble guanylate cyclase activation prevents postischemic inflammation and reduces nitrate tolerance in heme oxygenase-1 knockout mice. Am J Physiol Heart Circ Physiol 305: H521–H532, 2013. First published June 14, 2013; doi:10.1152/ajpheart.00810.2012—Previously we have shown that, unlike wild-type mice (WT), heme oxygenase-1 knockout (HO-1−/−) mice developed nitrate tolerance and were not protected from inflammation caused by ischemia-reperfusion (IR) when preconditioned with a H2S donor. We hypothesized that stimulation (with BAY 41-2272) or activation (with BAY 60-2770) of soluble guanylate cyclase (sGC) would precondition HO-1−/− mice against an inflammatory effect of IR and increase arterial nitrate responses. Intravital fluorescence microscopy was used to visualize leukocyte rolling and adhesion to postcapillary venules of the small intestine in anesthetized mice. Relaxation to ACh and BAY compounds was measured on superior mesenteric arteries isolated after IR protocols. Preconditioning with either BAY compound 10 min (early phase) or 24 h (late phase) before IR reduced postischemic leukocyte rolling and adhesion to sham control levels and increased superior mesenteric artery responses to ACh, sodium nitroprusside, and BAY 41-2272 in WT and HO-1−/− mice. Late-phase preconditioning with BAY 60-2770 was maintained in HO-1−/− and endothelial nitric oxide synthase knockout mice pretreated with an inhibitor (ts-propargylglycine) of enzymatically produced H2S. Pretreatment with BAY compounds also prevented the IR increase in small intestinal TNF-α. We speculate that increasing sGC activity and related PKG acts downstream to H2S and disrupts signaling processes triggered by IR in part by maintaining low cellular Ca2+. In addition, BAY preconditioning did not increase sGC levels, yet increased the response to agents that act on reduced heme-containing sGC. Collectively these actions would contribute to increased nitrate sensitivity and vascular function.

BAY 41-2272; BAY 60-2770; protein kinase G; endothelial nitric oxide synthase; ischemia-reperfusion; leukocyte rolling and adhesion; vascular smooth muscle

PREVIOUS STUDIES have shown that heme oxygenase (HO)-1 knockout (HO-1−/−) mice do not exhibit delayed (late phase) protection from ischemia-reperfusion (IR)-induced inflammation afforded by preconditioning with H2S or ethanol, whereas wild-type (WT) mice do (48, 49). Moreover, HO-1−/− mice have been reported to develop nitrate tolerance, which was associated with reduced levels of soluble guanylate cyclase (sGC) (20). HO-1 is an inducible enzyme that conveys cytoprotection by producing biliverdin along with the products iron and carbon monoxide (45). It has been proposed to play an important role in the antioxidant and anti-inflammatory activity associated with delayed preconditioning in WT mice (21, 22). Indeed, a number of preconditioning strategies have been associated with increased levels of HO-1 protein during delayed or late-phase preconditioning (21, 48). We proposed that an important component of that protection was related to HO-1 maintaining the heme iron of sGC in a reduced state (Fe2+) that would retain sensitivity to nitric oxide (NO) (20). In that study, we made use of two drugs: one that was a stimulator (BAY 41-2272) of reduced sGC and one that was a more effective activator (BAY 60-2770) of oxidized (heme-free) sGC (10, 24). Superior mesenteric arteries (SMAs) from HO-1−/− mice were more responsive to the sGC activator than those from WT mice (20). This indicated that HO-1−/− had more of sGC in an oxidized heme-free state, which would not be responsive to NO. It has been recognized that a number of agents that convey preconditioning protection to IR injury act via the NO-cGMP-PKG pathway, with potassium channels [e.g., large-conductance Ca2+-activated K+ (BKCa) and ATP-sensitive K+ (KATP) channels] being downstream targets (8, 32, 38). Therefore, one strategy to convey protection to IR injury under conditions where sGC is compromised by oxidative stress (e.g., in HO-1−/− mice) would be to precondition with an agent that would act preferentially on oxidized heme-free sGC.

This strategy has been tested in several studies that evaluated the effectiveness of cinaciguat as a cardioprotective agent during oxidative stress and IR injury. Chronic treatment provided cardiopulmonary protection to fetal sheep exposed to intrauterine pulmonary hypertension (6). Acute preconditioning before cardiopulmonary bypass in dogs led to improved recovery of cardiac contractility and coronary artery responses to ACh (35). Acute preconditioning before coronary artery occlusion in rabbits and mice also significantly reduced infarct size after reperfusion (37). In addition, cinaciguat reduced infarct size when given just before reperfusion (8, 37). At that time, flow of oxygenated blood into ischemic tissue can lead to the production of ROS and damage including oxidation of heme in sGC (21). Therefore, a sGC activator would be a rational choice to promote tissue protection under such conditions.

The above studies with sGC activators did not use conditions to determine whether delayed or late-phase preconditioning (e.g., 24 h before ischemia) can be induced by a sGC activator or a stimulator such as BAY 41-2272 and whether these compounds will inhibit inflammatory responses associated with IR injury. It was our objective to determine whether preconditioning with BAY compounds 10 min (early phase) or 24 h (late phase) before IR would convey protection from...
inflammation and retain responses to ACh in mesenteric vessels. We also evaluated the effectiveness of these compounds in HO-1−/− mice that were susceptible to ROS damage, where sGC levels are reduced and nitrate tolerance develops with an associated reduction in responses to ACh (20).

**MATERIALS AND METHODS**

**Animals**

WT H129 breeders and endothelial NO synthase (eNOS) knockout (eNOS−/−) mice (C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME), whereas HO-1−/− breeders (H129 background) were a gift from Dr. William Fay (University of Missouri, Columbia, MO). All of the female HO-1−/− mice used in this study were bred in University of Missouri facilities and were used at 8–12 wk of age. Genotyping was done on lysates from tail samples according to previously used methods (20). Experimental procedures were performed in compliance with institutional guidelines for humane animal care and use and were approved by the Institutional Animal Care and Use Committee.

**Surgical Procedures and Induction of I/R**

The procedures were similar to those previously used in our laboratory to maintain continuity (16, 48, 49). Mice were anesthetized with a mixture of ketamine (150 mg/kg body wt) and xylazine (7.5 mg/kg) followed by a midline abdominal incision. The SMA was identified and later occluded for 45 min [0 min for sham-operated (sham) mice]. The left jugular vein was cannulated for the administration of carboxyfluorescein diacetate succinimidyl ester (CFDAse; Molecular Probes, Eugene, OR) to label leukocytes after ischemia. CFDAse stock (5 mg/ml) was prepared before adding DMSO; the resulting solution was then aliquoted and stored in light-tight containers at −70°C until use. A 5-min infusion (20-fold dilution in saline) of the fluorescent probe allowed leukocyte-endothelial interactions to be observed via intravital fluorescence microscopy over minutes 30–40 and 60–70 of reperfusion.

**Intravital Fluorescence Microscopy**

Mice were placed on a Plexiglas board, and a section of the small intestine was exteriorized over a glass coverslip and superfused with a bicarbonate-buffered saline (37°C, pH 7.4). Body temperature was maintained between 36.5 and 37.5°C by means of a thermostatically controlled heat lamp. The Plexiglas board was mounted on the stage of an inverted microscope (Eclipse TE2000, Nikon), and the intestinal microcirculation was observed through a ×20 objective lens. Fluorescent images (excitation: 420–490 nm and emission: 520 nm) were detected with a charge-coupled device camera (Photometrics COOLSNAP ES). Images were projected onto a television monitor (PVM-1953MD, Sony) and recorded on a DVD recorder (DMR-E50, Panasonic), a time-date generator (WJ810, Panasonic) displayed this information onto an inverter (SNAP ES). Images were projected onto a television monitor (PVM-1953MD, Sony) and recorded on a DVD recorder (DMR-E50, Panasonic). A 5-min infusion (20-fold dilution in saline) of the fluorescent probe allowed leukocyte-endothelial interactions to be observed via intravital fluorescence microscopy over minutes 30–40 and 60–70 of reperfusion.

**In Situ Experimental Protocols**

The experimental design for each group is shown in Fig. 1 and is described below. Identical protocols were used for WT, HO−1−/−, and eNOS−/− mice, with 5–8 mice/group.

Group 1: sham. The surgical procedures used in group 1 were identical to the other groups. Pretreatment was with vehicle only (no drugs), and the exposed SMA was not occluded.

Group 2: I/R. Group 2 was similar to group 1 but with added occlusion of the SMA for 45 min.

Group 3: acute pretreatment with BAY 41-2272 or BAY 60-2770 + I/R. Mice received BAY 41-2272 (3 mg/kg ip) or BAY 60-2770 (3, 10, or 30 μg/kg ip) 10 min before I/R.

Group 4: delayed pretreatment with BAY 41-2272 or BAY 60-2770 + I/R. Mice received BAY 41-2272 (3 mg/kg ip) or BAY 60-2770 (30 or 300 μg/kg ip) 24 h before I/R.

Group 5: paxilline + BAY 60-2770 + I/R. Mice received paxilline (2.5 mg/kg ip) 10 min before BAY 60-2770 (300 μg/kg ip) 24 h before I/R. Paxilline was used to inhibit BKCa channel activity, which has been associated with delayed protection to H2S (49).

Group 6: DL-propargylglycine + BAY 60-2770 + I/R. Mice received DL-propargylglycine (PAG; 50 mg/kg ip) 10 min before BAY 60-2770 (300 μg/kg ip) 24 h before I/R and again 30 min before I/R. PAG was used to inhibit H2S production by cystathionine-γ-lyase (CSE), which has been associated with BAY 58-2667 preconditioning (37).

**Vascular Rings**

After the in situ protocol, SMAs were removed, dissected free of surrounding tissue, and placed in ice-cold low-Ca2+ (0.15 mM) physiological solution (PS). Arteries were trimmed free of fat and cut into rings ~0.5 mm long. The outside diameter (range: 400–500 μm) was measured with a calibrated reticle in the dissection microscope (SMZ-2B, Nikon). Rings were mounted on force transducers (FT03, Grass Instruments) that had specially fabricated feet with an open wire (diameter: 51 μm) design. Rings were gently slipped onto the open end with the aid of the microscope and stretched 1.2 times their resting diameter before being placed into PS (10 ml in plastic cups) at 36°C. Rings were tested with high K+ (K+: 80 mM substituted for Na+) to establish a reference contracture and then returned to PS, relaxed, stretched 5%, and retested with high K+ and so forth. Rings were considered to be at or near the optimum length for force production when 5% stretch produced a <10% increase in contraction. This procedure facilitated the reproducibility of results between vessels and animals. Drugs were added to PS in a cumulative manner, with the next concentration being added when a steady-state response had occurred or at a predetermined time, e.g., 5 min. The Ca2+ chelator EGTA (5.0 mM) was applied at the end of the protocols to determine the basal noncontracted tension.

**Circulating Neutrophil Counts**

Whole blood was obtained via cardiac puncture after the reperfusion recordings. Samples were diluted 1:20 with 1% gentian violet solution, and total leukocytes were counted by means of a hemocytometer. To obtain the values for neutrophils, a differential count was done on samples stained with Wright Giesma stain, and the product of the total leukocyte count and the percent neutrophils yielded the number of cells per microliter of whole blood.
TNF-α Assay

Segments of the jejunum from sham mice or those subjected to the I/R protocols were ground in liquid N₂, homogenized in 1 ml lysate buffer [10 mM Tris·HCl (pH 7.4), 250 mM sucrose, 20 mM HEPES, 1 mM EDTA, 1 mM PMSF, and 10 μl/ml protease inhibitor cocktail], and then sonicated for 20 s. The homogenate was centrifuged at 12,000 g for 20 min at 4°C, and aliquots of the supernatant were stored at -70°C. TNF-α was measured in duplicate by ELISA (KMC3012, Invitrogen) (27). The minimum detectable level of TNF-α was ≤4 pg/ml, and levels were expressed as picograms per milligram of protein.

Western Blot Analysis

The SMA was dissected free of fat, placed in vials, frozen in liquid N₂, and stored at -70°C. For each Western blot analysis, three to four SMAs were pooled, ground in liquid N₂, and extracted in buffer [125 mM Tris (pH 6.8), 12.5% glycerol, 2% SDS, 50 mM NaF, and trace bromphenol blue]. Proteins were separated by SDS-PAGE. After transfer to a nitrocellulose membrane, blots were blocked with PBS and nonfat milk (5%) and then incubated with antibodies directed against sGC α₁- and sGC β₁-subunits along with β-actin in one set of experiments and against eNOS and ERK1 in a second set of experiments. Membranes were washed in PBS, 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 proteins was quantified by scanning densitometry and normalized with respect to β-actin or ERK1.

Solutions and Drugs

PS used for the isolated ring experiments had the following composition (in mM): 138 NaCl, 5.0 KCl, 1.5 CaCl₂, 1.2 MgCl₂, 1.2 NaHPO₄, 10 Na-HEPES (pH 7.35), and 11.2 glucose. PE, 5-hydroxytryptamine, SNP, ACh, BAY 41-2272, 5-hydroxydecanoic acid (5-HD), and PAG were purchased from Sigma-Aldrich. Paxilline was purchased from Enzo Life Sciences. Antibodies against eNOS and ERK1 were from BD Transduction Laboratories and Santa Cruz Biotechnology, respectively. Bay 60-2770 was a gift from Bayer Schering Pharma (Wuppertal, Germany). Stock solutions (10 mM) of PE and ACh were prepared in 10 mM ascorbic acid and frozen (-20°C). SNP (10 mM) was prepared in ascorbic acid and stored at 4°C. BAY compounds (10 mM) and paxilline (5 mg/ml) were dissolved in DMSO and stored at 4 and 20°C, respectively. PAG (5 mg/ml) was dissolved in DMSO/saline on the day of the experiment, whereas 5-HD was dissolved in saline only. Dilutions of the various drugs were made into the appropriate solvent on the day of the experiment.

Data Analyses

Data are presented as means ± SE. Student’s t-test was used to test for differences between group means where one treatment was being evaluated. ANOVA and the Newman-Keuls method applied post hoc
were used to evaluate multiple comparisons. P values of <0.05 were deemed to be significant.

RESULTS

Inflammatory Responses

Figure 2, A and B, shows mean values for the numbers of rolling and adherent leukocytes for experimental groups 1–4. After 30 min of reperfusion, significant increases occurred for both measures in WT mice as well as HO-1—/— mice that had not received any BAY compounds. Similar results were also observed at 60 min of reperfusion (data not shown). ANOVA indicated that the rolling leukocyte response to I/R was greater in HO-1—/— mice (P < 0.02). No significant increases in leukocyte rolling and adhesion occurred in WT and HO-1—/— mice that had been pretreated with BAY compounds at either 10 min or 24 h before the ischemic episode (Fig. 2). A similar result was observed at 60 min of reperfusion (data not shown). The protective effects of BAY 60-2770 and BAY 41-2272 compounds were similar in both WT and HO-1—/— mice and could be induced by early-phase as well as late-phase preconditioning.

The dose-dependent effects of Bay 60-2770 were measured during early-phase preconditioning to determine whether an activator of oxidized (heme-free) sGC would be more effective in HO-1—/— mice, a model with reduced antioxidant reserve. As shown in Fig. 3, A and B, leukocyte rolling in WT mice remained significantly greater than in sham mice after pretreatment with 3 or 10 μg/kg BAY 60-2770, whereas leukocyte adhesion remained elevated after pretreatment with 3 μg/kg. In sharp contrast, at these doses, leukocyte rolling and adhesion in HO-1—/— mice did not significantly differ from sham levels. Similar results were also observed at 60 min of reperfusion (data not shown). Therefore, the reduced doses of BAY 60-2770 had a greater effect on early-phase preconditioning in HO-1—/— mice than in WT mice.

Experimental protocols 5 and 6 (Fig. 1) were conducted on HO-1—/— mice to evaluate K+ channel activation and H2S production during late-phase preconditioning with BAY 60-2770. The 10× dose (300 μg/kg) was used to insure maximal late-phase preconditioning in this and subsequent experiments. H2S production was reported to be an important factor in the protection conveyed by cinaciquat (BAY 58-2667) on I/R injury in the heart (37), whereas K+ channel activation has been proposed to be an important downstream effector for various preconditioning treatments (8, 49). We used two PAG treatments for the 24-h protocol to insure inhibition of CSE before BAY 60-2770 was administered as well as during I/R and subsequent measures of leukocyte-venule interactions. As shown in Fig. 4, A and B, in the presence of PAG, the numbers of rolling and adherent leukocytes remained at or below sham levels during 30 min of reperfusion. Similar results were found with paxilline pretreatment (Fig. 4, A and B). The protocol used for paxilline was identical to that used previously, which has been shown to block late-phase preconditioning with NaHS (49). Likewise, an inhibitor of mitochondrial KATP channels, 5-HD (15, 33), did not significantly alter the late-phase protection conveyed by BAY 60-2770 (Fig. 4, A and B).

Previous measures of sGC in SMAs showed lower levels in HO-1—/— mice than in WT mice (20). In a separate series, we repeated these measures on WT and HO-1—/— mice pretreated 24 h with BAY 60-2770 (300 μg/kg) to determine whether its interaction with sGC might increase levels. As shown in Fig. 5A, the 24-h pretreatment did not increase sGC. Instead, a significant reduction occurred in sGC β1-subunits for both groups. The HO-1—/— group also exhibited reduced sGC β1-subunits compared with the WT group, consistent with our earlier report (20).

It has been reported that eNOS may be essential for the late-phase preconditioning induced by ethanol (46) and for the cardioprotection associated with cinaciquat (BAY 58-2667).
Although sGC levels in SMAs were significantly reduced in HO-1\(^{-/-}\) mice compared with WT mice (20), measures of eNOS levels were equivalent, as shown in Fig. 5B; hence, eNOS could be a factor in BAY 60-2770-induced preconditioning in WT and HO-1\(^{-/-}\) mice. Protocols 1–4 and 6 were repeated on eNOS\(^{-/-}\) mice to determine whether its expression was required for preconditioning with BAY 60-2770. As shown in Fig. 6, A and B, leukocyte rolling and adhesion remained near sham levels after early- and late-phase preconditioning. Moreover, treatment of eNOS\(^{-/-}\) mice with PAG did not alter the late-phase preconditioning response to BAY 60-2770.

Measures of jejunum TNF-\(\alpha\), as shown in Fig. 7, were done to determine whether late-phase preconditioning with BAY compounds had an anti-inflammatory effect upstream to the venules. A significant three- to fourfold increase in TNF-\(\alpha\) occurred after I/R in WT and HO-1\(^{-/-}\) mice, whereas the increase in eNOS\(^{-/-}\) mice was twofold. Pretreatment with both BAY compounds prevented the increase in WT and

Fig. 3. Effects of preconditioning with BAY 60-2770 at various doses on responses to I/R. Leukocyte rolling is shown in A and adhesion in B for WT mice (solid bars) and HO-1\(^{-/-}\) mice (cross-hatched bars). BAY 60-2770 was applied 10 min before the ischemic period at three different concentrations. Data for sham, IR, and 30 \(\mu\)g/kg BAY 60-2770 groups are also shown in Fig. 2. Bars indicate means \(\pm\) SE; \(n = 6\). *\(P < 0.05\) compared with the sham group within each set of mice.

Fig. 4. Effects of PAG, paxiline (Pax), and 5-hydroxydecanoic acid (5-HD) on 24-h preconditioning with BAY 60-2770. Leukocyte rolling is shown in A and adhesion in B for HO-1\(^{-/-}\) mice. 5-HD (10 mg/kg) was given intraperitoneally 10 min before the ischemic episode, and the protocols for PAG and Pax are shown in Fig. 1. Data for the sham and IR groups are also shown in Fig. 2. Measures at 30 min postischemia are shown as means \(\pm\) SE; \(n = 6\). *\(P < 0.05\) compared with the sham group.
of circulating neutrophil changes in response to BAY compounds differed from that for leukocyte-venule interactions. This indicated that mechanisms in addition to a reduction in circulating neutrophils were operative during preconditioning with BAY compounds.

**Contralve Responses**

The SMA was studied because it is the feeder vessel to the intestine that was subjected to I/R. Measures were made of PE

![Graph A](image1)

**Graph A**

**SMA sGC**

![Graph B](image2)

**Graph B**

**SMA eNOS**

Fig. 5. Expression of soluble guanylate cyclase (sGC) and endothelial nitric oxide (NO) synthase (eNOS) in superior mesenteric arteries (SMAs) from HO-1−/− and HO-1+/+ mice. Bars are the means of 3–4 measures ± SE. As shown in A, two-way ANOVA indicated that the expression of the sGC β1-subunit was negatively affected by both HO-1−/− mouse type (P < 0.01) and BAY 60-2770 pretreatment (300 μg/kg 24 h, P < 0.03) with no significant interaction. The expression of the sGC α1-subunit exhibited a greater variance, and no significant effects were observed. As shown in B, the expression of eNOS from untreated mice was similar in both groups.

HO-1−/− mice and Bay 60-2770 in eNOS−/− mice, similar to leukocyte-endothelium interactions. Also, there was a tendency for HO-1−/− mice to exhibit higher content of TNF-α than WT and eNOS−/− mice under equivalent conditions.

I/R was also associated with increased circulating levels of neutrophils in WT, HO-1−/−, and eNOS−/− mice, as shown in Fig. 8. The response was greatest in HO-1−/− mice. In contrast to the effects on leukocyte-venule interactions and tissue TNF-α, late-phase preconditioning of WT mice with BAY 41-2272 did not prevent the increase in neutrophils, BAY 60-2770 prevented the increase in both WT and HO-1−/− mice but not in eNOS−/− mice. Unlike WT mice, BAY 41-2272 appeared to be effective in HO-1−/− mice. Overall, the pattern

![Graph C](image3)

**Graph C**

**eNOS−/− Leukocyte Adhesion - 30 min**

Fig. 6. Effects of preconditioning with BAY 60-2770 on I/R responses in eNOS knockout (eNOS−/−) mice. Leukocyte rolling is shown in A and adhesion in B. The protocols were the same as those shown in Fig 2. Data for measures at 30 min postischemia are shown as means ± SE; n = 6. *P < 0.05 compared with the sham group.
sensitivity to ACh compared with HO-1 
should be noted that sham WT mice exhibited 5-fold greater (cross-hatched bars), and eNOS 
Fig. 8. Effects of 24-h preconditioning with BAY 60-2770 and BAY 41-2272 on TNF-α content of the jejunum from WT (solid bars), HO-1 
pretreatment (both early and late) with BAY compounds had 
concentration-dependent contractures and subsequent relaxation to agents that directly or indirectly act on sGC. SMAs from all three mouse groups exhibited significant responses to the agents. The IC50 for ACh was shifted to a higher concentration (lower sensitivity) by I/R in HO-1 
from all three mouse groups exhibited significant responses to BAY 41-2272 in both WT and HO-1 
Fig. 7. Effects of 24-h preconditioning with BAY 60-2770 and BAY 41-2272 on intestinal TNF-α. Data are shown as means ± SE; n = 6. *P < 0.05 compared with the sham group within each set of mice.

**Intestinal TNF-α**

**WT**

**HO-1** 

**eNOS**

**Blood Neutrophils**

**WT**

**HO-1** 

**eNOS**

Fig. 8. Effects of 24-h preconditioning with BAY 60-2770 and BAY 41-2272 on neutrophil concentration in whole blood from WT (solid bars), HO-1 
**Drug Sensitivity**

**A**

**PE**

**Ach**

**BAY60**

**BAY41**

**Change in sensitivity**

**PE**

**Ach**

**BAY60**

**BAY41**

Fig. 9. Effects of I/R and preconditioning with BAY compounds on drug sensitivity of SMAs from WT, HO-1 
significant effects on sensitivity, as shown by the changes in log M50 (Fig. 9B). The sensitivity to PE was reduced (positive change) in WT mice, whereas it was unchanged in HO-1 
and eNOS 
Fig. 9A) was nullified by pretreatment with BAY compounds, eNOS 
AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00810.2012 • www.ajpheart.org

Downloaded from http://ajpheart.physiology.org/ by on July 5, 2017
Pretreatment with BAY compounds also affected the percent relaxation of the SMA, as shown in Fig. 10. The relaxation was significantly increased in HO-1^{-/-} mice to all sGC active compounds and to three of four sGC active compounds in WT mice. BAY pretreatments had significantly greater effects on HO-1^{-/-} mice for the three compounds that work via stimulation of reduced sGC. eNOS^{-/-} mice exhibited significantly greater (P < 0.05) relaxation to ACh (68 ± 7%, n = 6) compared with WT mice (40 ± 9%, n = 12). The relaxation of eNOS^{-/-} mice to the various agents (Fig. 10) was not significantly affected by pretreatment with BAY compounds.

**DISCUSSION**

The principle findings of these experiments show that pretreatment with an activator or a stimulator of sGC will convey protection in HO-1^{-/-} mice. BAY pretreatments had significantly greater effects on HO-1^{-/-} mice compared with WT mice (40 ± 9%, n = 12). The relaxation of eNOS^{-/-} mice to the various agents (Fig. 10) was not significantly affected by pretreatment with BAY compounds.

**Leukocyte-Endothelial Interactions**

As previously reported, ischemia of the small intestine in WT and HO-1^{-/-} mice was associated with increased leukocyte rolling and adhesion to the venular endothelium within 30 min of reperfusion (48, 49). Pretreatment with relatively high doses of BAY compounds conveyed protection to both groups, whether given 10 min or 24 h before ischemia (Fig. 2). Although HO-1^{-/-} mice would be expected to have less sGC in the reduced form and less total content (Fig. 5A) than WT mice, there appears to be sufficient sGC in reduced and oxidized states (i.e., spare NO receptors) to allow responses to both BAY compounds (20). When pretreated at 10 min with doses of BAY 60-2770 of ≤10 µg/kg (Fig. 3), HO-1^{-/-} mice maintained near sham levels of rolling and adhesion after I/R, whereas WT mice exhibited significant increases. This is consistent with the presence of a higher level of oxidized heme-free sGC in HO-1^{-/-} mice than in WT mice. (See Refs. 10, 24, 28, and 30 for background concerning the highly specific interaction between BAY 58-2667 and BAY 60-2770 with the vacated heme pocket of sGC that occurs after oxidation of the heme Fe^{2+} to Fe^{3+}.) This difference in sGC may have persisted after 24 h preconditioning, since treatment with BAY 60-2770 was associated with a reduction in sGC levels in SMAs as well as reduced levels in HO-1^{-/-} mice compared with WT mice (Fig. 5A). This finding was unexpected in light of an in vitro study (30) showing that BAY 58-2667 prevented an oxidation-induced downregulation of sGC. In that study (30), rat aortas were maintained in tissue culture for 20 h with 10 µM BAY 58-2667 and the oxidant 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ); in contrast, our in vivo protocol used one dose of BAY 60-2770, which would be expected to result in a lower concentration (≤1 µM) that would fall progressively during 24 h. Also, another in vitro study (23) indicated that simulation of rat aortic sGC with YC-1 for 24 h reduced sGC levels, possibly through an effect of the cGMP pathway on the stability of sGC mRNA. Under our conditions, it appears that early-phase as well as late-phase preconditioning conveyed protection by BAY 60-2770 occurs through targets of the sGC-cGMP-PKG pathway rather than a major increase in sGC levels. The possibility also exists that the relative balance between reduced and oxidized sGC may be shifted toward the reduced state. Several of these concepts are shown in Fig. 11 as a diagram. Because altered vascular redox regulation is associated with many pathological states (44), it is anticipated that the use of an activator of oxidized heme-free sGC, such as BAY 58-2667 or BAY 60-2770, would provide the more efficacious approach to activating the sGC-cGMP-PKG pathway.

We also studied the effects of several interventions that have been reported to be effective in blocking preconditioning pathways induced by other agents. As noted above, the BAY compounds were effective in providing early- and late-phase protection in HO-1^{-/-} mice, whereas NaHS did not (48). These observations support the concept that HO-1 expression and activity in the endothelium is an important mediator of the pretreatment with an H_{2}S donor. In another study (47), NaHS-induced preconditioning was found to depend on eNOS, with subsequent production of NO. In the present study (Fig. 4), the anti-inflammatory effects of sGC-cGMP-PKG signaling occurred downstream and could be initiated by NO as well as other sGC active agents without changes in HO-1 expression. It has been proposed, however, that the increased production of H_{2}S in response to BAY 58-2667 (acting similarly to BAY 60-2770) is a necessary step in postischemic myocardial protection (37). The administration of BAY 58-2667 to mice 30 min before ischemia was associated with an 80% reduction in infarct size 24 h after reperfusion. The reduction in infarct size

---

**Fig. 10.** Effects of preconditioning with BAY compounds on relaxation of SMAs from WT, HO-1^{-/-}, and eNOS^{-/-} mice. Changes in percent relaxation associated with the treatments of BAY 60-2770 and BAY 41-2272 and the sham group are presented as averages ± SE for the five protocols, as shown in Fig. 9B. The P values at the top indicate the significance of the changes associated with the BAY treatments. The brackets and associated P values represent comparisons between WT and HO-1^{-/-} or eNOS^{-/-} mice.
was blocked entirely when PAG, a CSE inhibitor, was given 30 min before BAY 58-2667. We tested the H2S dependence of late-phase protection in HO-1−/− mice, which exhibit a compromised antioxidant capacity. Two PAG treatments (30 min before BAY 60-2770 and 30 min before ischemia) were used with the 24-h preconditioning protocol to ensure that CSE (and additional H2S production) would be blocked before the acute effects of BAY 60-2770 and ischemia occurred. Under these conditions, the venules were fully protected from leukocyte rolling and adhesion (Fig. 4). It appears that increased H2S production may not be required for late-phase preconditioning protection against an early inflammatory response to I/R. This observation does not rule out later consequences of BAY 58-2667 treatment, e.g., tissue infarct size after 24 h, that may be affected by increased CSE activity and the resultant H2S as well as other factors, such as shifts in eNOS activity.

It has been reported that the cardioprotection conveyed by BAY 58-2667 (applied during the last 5 min of ischemia) can be blocked by simultaneous treatment with N-nitro-L-arginine methyl ester (8). Unlike preconditioning with agents that increase PKG activity, they proposed that PKG was active upstream to eNOS and NO production when the conditioning agent was applied during ischemia. We tested the dependence of early- and late-phase preconditioning on eNOS by means of a knockout model. Under these conditions, BAY 60-2770 conveyed protection against the venular inflammatory response (Fig. 6). It is of further interest that treatment of eNOS−/− mice with PAG had no effect on late-phase protection with BAY 60-2770. Future experiments where BAY compounds are given during the last 5–10 min of ischemia should be conducted to determine whether a similar or different series of signals are involved in protection against this inflammatory response. It is important to resolve this issue, since a potential clinical application would involve giving such agents before reestablishing blood flow to an ischemic organ, where the option of giving it before the ischemic episode (as was done in our study) is nonexistent.

**Intestinal TNF-α**

In addition to vascular effects, pretreatment with BAY compounds prevented the increase in small intestinal TNF-α
associated with I/R (Fig. 7). Since the venules receive blood and extracellular fluid draining from this tissue, reduced TNF-\(\alpha\) perfusion and extracellular diffusion may have contributed to the reduced leukocyte-endothelial interactions (Fig. 11). TNF-\(\alpha\) is synthesized and released from macrophages and parenchymal cells via highly regulated processes that are sensitive to conditions existing during I/R (29). The role of PKG activation in the regulatory process is not well understood, and comments based on our data would be highly speculative. The presence of TNF-\(\alpha\) after I/R, however, could be a major factor. For instance, I/R-induced vascular dysfunction in hearts was absent in TNF-\(\alpha\)-deficient mice (14).

**Linkage Between BAY Pretreatments and Reduced Leukocyte-Endothelial Interactions**

Extensive literature indicates that the primary effect of BAY compounds is on sGC leading to the production of cGMP (30, 40). This relation is shown in Fig. 11. The question arises as to how this translates to the inhibition of leukocyte rolling and adhesion in venules that we observed after I/R in three mouse models. Insights and potential experiments can be derived from the current literature. The process of leukocyte-endothelial interaction after I/R is regarded to occur in several stages (5, 18). Endothelial cells become “activated” in response to various factors (e.g., ROS, TNF-\(\alpha\), and \(\alpha\)-thrombin) released during reperfusion. Part of this process involves receptor-G protein-coupled events, whereas others, e.g., ROS, may have intracellular actions leading to significant elevation of cytosolic Ca\(^{2+}\) (7). Ca\(^{2+}\) is regarded to be an important cofactor for the fusion of P-selectin storage vesicles with the surface membrane of endothelial cells. Fusion results in the extracellular exposure of the elongated arm of P-selectin, which acts as a receptor for the tethering of leukocytes (5). This results in leukocyte rolling and primes them for subsequent adhesive arrest. The percentage of cultured endothelial cells that tested positive for P-selectin after an inflammatory stimulus was reduced by half in the presence of BAY 41-2772 (1). It was also shown that inhibition of the \(\alpha\)-thrombin-induced Ca\(^{2+}\) signal with a chelator reduced the amount of P-selectin incorporated into the membranes of endothelial cells (7). Thus, the entry and release of cellular Ca\(^{2+}\) is an important component of P-selectin signaling and may be influenced by cGMP signaling (Fig. 11).

Extensive literature has established linkages between increases in cGMP, PKG activity, and altered cellular Ca\(^{2+}\) regulation (12). Phosphorylation of several PKG substrates directly reduces cytosolic Ca\(^{2+}\) during exposure to receptor active agents. The targeted substrates include 1) inositol 1,4,5-trisphosphate receptor-associated proteins, which, in turn, lead to inhibition of Ca\(^{2+}\) release from the endoplasmic reticulum (ER); 2) phospholamban, leading to increased Ca\(^{2+}\) uptake into the ER; and 3) phospholipase C and regulators of G protein signaling, resulting in reduced inositol 1,4,5-trisphosphate production and subsequent Ca\(^{2+}\) release from the ER. The entry of Ca\(^{2+}\) is also inhibited via PKG phosphorylation of transient receptor potential channels, which are particularly important for Ca\(^{2+}\) influx into endothelial cells during activation with agonists (25, 39). One study (11) demonstrated that an NO donor caused 90% inhibition of the Ca\(^{2+}\) transient induced by \(\alpha\)-thrombin in endothelial cells, indicating the extent to which activated PKG can maintain Ca\(^{2+}\) at basal levels in the presence of agonists. These effects on Ca\(^{2+}\) are shown in Fig. 11.

The inhibitory effects of PKG activation on cytosolic Ca\(^{2+}\) may have important consequences in preventing the endothelial dysfunction caused by TNF-\(\alpha\) released from tissues undergoing I/R (14). TNF-\(\alpha\) is regarded to be a major factor during the early inflammatory response in the endothelium and is associated with increased membrane expression of P-selectin (18, 19). Since this process is Ca\(^{2+}\) dependent (from the discussion above), it would be subject to inhibition by BAY compounds. TNF-\(\alpha\) is also associated with increased cytosolic phospholipase A\(_2\) activity (cPLA\(_2\)) and the release of arachidonic acid and its metabolites (2). cPLA\(_2\) contains multiple regulatory sites for Ca\(^{2+}\) as well as phosphorylation that are required for maximal activation (3, 17, 34). It would be expected that a PKG effect that reduces Ca\(^{2+}\) would prevent full activation of cPLA\(_2\) (Fig. 11). The importance of TNF-\(\alpha\)-mediated signaling through type 1 receptors and cPLA\(_2\) is underscored by the attenuation of I/R injury in cPLA\(_{2\alpha}\)-deficient mice and in TNF-\(\alpha\) receptor type 1-deficient mice (9, 36). Therefore, agents such as the BAY compounds used in this study may be particularly useful in preventing early inflammatory responses.

Additional targets for PKG may also be involved in the protection conveyed by BAY compounds. The activity of K\(_{ATP}\) and Ca\(^{2+}\)-sensitive K\(^+\) channels can be increased, whereas that of L-type Ca\(^{2+}\) channels is inhibited (12). Since K\(_{ATP}\) channels are expressed on mitochondria, activation can be involved in protecting against the damaging effects of I/R (8, 21). These actions may be of additional importance to the dilator responses of arterial smooth muscle, which exhibit high expression of the various K\(^+\) and Ca\(^{2+}\) channels. Pretreatment with paxilline as well as 5-HD, however, failed to block late-phase preconditioning with BAY 60-2770 (Fig. 4). Hence, alternate PKG targets (discussed above) may be more relevant to the prevention of I/R induced leukocyte-endothelial interactions in HO-1\(^{-/-}\) venules.

**Vascular Smooth Muscle Relaxation**

The SMA from HO-1\(^{-/-}\) exhibited nitrate tolerance of the type previously reported (20); moreover, I/R was associated with decreased sensitivity to ACh (Fig. 9A) consistent with a reduced sensitivity of sGC to NO. It should be noted that eNOS expression, a source of NO during ACh stimulation, was similar in WT and HO-1\(^{-/-}\) mice (Fig. 5B). The SMA from eNOS\(^{-/-}\) mice had a greater relaxation response to ACh than that from WT mice. This was expected from previous reports (4, 42) that showed ACh relaxation of mesenteric arteries was shifted to a greater dependence on EDHF and/or cyclooxygenase products. This shift in regulation may underlie the lack of effect of changes in drug sensitivity and relaxation in SMAs from eNOS\(^{-/-}\) mice pretreated with BAY compounds (Figs. 9B and 10).

The heme Fe\(^{2+}\) on sGC is prone to oxidation to Fe\(^{3+}\) under conditions of increased ROS with subsequent loss of sensitivity to NO (20, 31, 44). Although other antioxidant systems are operative, HO-1 appears to be particularly important for the maintenance of sGC in a reduced NO-sensitive state (Fig. 11). Caveolin-1 has been shown to act as a platform for the colocalization of sGC, HO-1, and eNOS in endothelial cells...
It has been hypothesized that the close proximity of a source of NO and carbon monoxide to sGC would convey a functional advantage. Moreover, the tethering of sGC near a source of antioxidant activity would convey protection from Fe²⁺ oxidation. Such protection was compromised in pressure-overloaded hearts, which exhibited reduced NO-stimulated sGC activity (41). The reduction was associated with translocation of sGC from caveolin-3 to cytosolic fractions where oxidation of sGC occurred more readily. As expected, the cGMP response to BAY 60-2770 in the cytosolic fractions remained at near control levels. The sGC heme can be subject to immediate oxidation by micromolar peroxynitrite (a product of NO and O₂⁻) even in the presence of 5 mM reduced glutathione (13). BAY 58-2667 and BAY 60-2770 are structurally similar and have been shown to fit into the subsequently vacated heme pocket (28). In vitro treatment of the rat aorta with BAY 58-2667 under oxidized conditions led to (1) activation of heme-free sGC with subsequent production of cGMP and 2) protection of sGC from ubiquitination and proteasomal degradation (30). Under our in vivo conditions, it appears that sGC levels were not increased by 24-h pretreatment with a single dose of BAY 60-2770 (Fig. 5A); hence, sGC activation with a possible increase in the relative amount of reduced sGC may be the main contributor to the increased sensitivity and relaxation to ACh, BAY 41-2272, and SNP after preconditioning (Figs. 9B and 10).

The leukocyte-endothelium section emphasized PKG sensitive Ca²⁺ linkages that also apply to smooth muscle and have been previously reviewed (12, 38). The increase in SMA sensitivity to ACh and BAY 41-2272 exhibited by both WT and HO-1⁻/⁻ mice (Fig. 9B) could reflect an increased availability of reduced heme-containing sGC. This would be expected to support the increased relaxation that was observed for NO stimulation (ACh and SNP) as well as stimulation by BAY 41-2272 (Fig. 10). It is uncertain what mechanisms underlie these increased responses under conditions where sGC levels did not increase. Rather than increase sensitivity, persistent cGMP signaling has been documented to desensitize smooth muscle to NO and other sGC active agents (12). Much of the focus has been on the role of phosphodiesterases, especially phosphodiesterase 5, in this process (12), and this is shown in Fig. 11. “Feedforward” mechanisms have also been suggested to explain some observations of increased sensitivity, but these have not been defined in much detail. H₂S may also have an important role in preserving cGMP through its action as an antioxidant and as a phosphodiesterase inhibitor (43), as shown in Fig. 11. This area of sGC regulation will require further study to determine how preconditioning with BAY compounds can insure a responsive vascular system to an organ affected by an episode of I/R.

Conclusions

A growing body of evidence has indicated that stimulation of the sGC-cGMP-PKG pathway provides significant cardiovascular protection against inflammatory events involving ROS. This pathway has targets that apparently act downstream (or parallel) to eNOS, H₂S, and some K⁺ channels, which in themselves can also support preconditioning. Among the agents available, activators of sGC (e.g., BAY 58-2667 and BAY 60-2770) are of particular interest in that they preferentially act on oxidized heme-free sGC, which can be elevated under pathological conditions where antioxidant systems are stressed, as in HO-1⁻/⁻ mice. sGC activation appears to be important for the preservation of nitrate sensitivity in the vascular system and normal vascular responsiveness to endothelial control. Connections need to be established more definitively between the actions of sGC activators and altered immune responses, such as TNF-α expression and release. There is much more happening than can be explained by cellular Ca²⁺ regulation alone. This is an important area to be explored and is highly relevant to developing strategies to minimize the damage done by and during treatment of occlusive vascular disease.

Acknowledgments

The authors thank Kelly J. Peyton, Derek Wang, Alaina Boyett, and Christine Korthuis for excellent technical assistance and Bayer Schering Pharma for the gift of BAY 60-2770.

Grants

This work was supported by National Institutes of Health Grants AA-014945, HL-59976, and HL-095486.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

Author Contributions


References


