Soluble guanylyl cyclase activation by HMR-1766 (ataciguat) in cells exposed to oxidative stress

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Submitted 15 January 2008; accepted in final form 20 August 2008

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Am J Physiol Heart Circ Physiol 295: H1763–H1771, 2008. First published August 29, 2008; doi:10.1152/ajpheart.51.2008.—Many vascular diseases are characterized by increased levels of ROS that destroy the biological activity of nitric oxide and limit cGMP formation. In the present study, we investigated the cGMP-forming ability of HMR-1766 in cells exposed to oxidative stress. Pretreatment of smooth muscle cells with H2O2 reduced cGMP production stimulated by sodium nitroprusside (SNP) or BAY 41-2272. However, pretreatment with H2O2 significantly increased HMR-1766 responses. Similar results were obtained with SIN-1, menadione, and rotenone. In addition, HMR-1766 was more effective in stimulating heme-free sGC compared with the wild-type enzyme. Interestingly, in cells expressing heme-free sGC, H2O2 inhibited instead of potentiated HMR-1766 responses, suggesting that the ROS-induced enhancement of cGMP formation was heme dependent. Moreover, using truncated forms of sGC, we observed that the NH2-terminus of the β1-subunit is required for the action of HMR-1766. Finally, to study tolerance development to HMR-1766, cells were pretreated with this sGC activator and reexposed to HMR-1766 or SNP. Results from these experiments demonstrated lack of tolerance development to HMR-1766 as well as lack of cross-tolerance with SNP. We conclude that HMR-1766 is an improved sGC activator as it has the ability to activate oxidized/heme-free sGC and is resistant to the development of tolerance; these observations make HMR-1766 a promising agent for treating diseases associated with increased vascular tone combined with enhanced ROS production.

Reactive oxygen species; cGMP; nitric oxide; soluble guanylyl cyclase
known about BAY 58-2667. In the present study, we sought to characterize the ability of HMR-1766 to stimulate cGMP production in cells under oxidative stress conditions and in cells expressing heme-deficient sGC. Moreover, we identified the region of sGC that is important for the action of HMR-1766 and evaluated whether tolerance to this agent develops.

MATERIALS AND METHODS

Materials. DMEM, HBSS, and FCS were obtained from GIBCO-BRL (Paisley, UK). Cell culture flasks and plates were obtained from Greiner Labortecnik (Frickenhausen, Germany). Dulbecco’s PBS, penicillin, and streptomycin were from Biochrom (Berlin, Germany). Polyvinylidene difluoride (PVDF) membranes were from Macherey-Nagel (Düren, Germany). Monoclonal anti-V5 antibody, Platinum Pfx DNA polymerase, and the pcDNA3.1 Directional TOPO Expression kit were from Invitrogen (Paisley, UK). The transfection reagent JetPEI was purchased from Polysciences (West Chester, PA). The antibody against the sGC α1-subunit was from Chemicon (Temecula, CA). The antibody against the sGC α1-subunit, monoclonal anti-c-Myc (9E10) antibody, ODQ, rotenone, 3-morpholinosydnonimine (SIN-1), polyvinyl pyrrolidone, and His105 to cysteine (H105C) of the sGC 1-subunit were produced using SuperSignal West Pico chemiluminescent substrate was purchased from Pierce (Rockford, IL). The DC Protein assay kit, Tween 20, and other immunoblot reagents were obtained from Bio-Rad (Munich, Germany). Monoclonal anti-actin antibody was from Chemicon (Temecula, CA). The antibody against the sGC β1-subunit was purchased from Caymen Chemical (Ann Arbor, MI). The antibody against the sGC α1-subunit, monoclonal anti-c-Myc (9E10) antibody, ODQ, rotenone, 3-morpholinosydnonimine (SIN-1), menadione, IBMX, sodium nitroprusside (SNP), carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCFDA), catalase, BSA, PMSF, EGTA, EDTA, aprotinin, leupeptin, pepstatin A, and all other chemical reagents were purchased from Sigma (St. Louis, MO).

Generation of constructs. A NH2-terminal myc-tagged full-length rat sGC α1-subunit, a COOH-terminal V5-tagged full-length rat sGC β1-subunit, and NH2-terminal-deleted mutants of α1- or β1-subunits were constructed by PCR and cloned into the pcDNA3.1/V5-His TOPO vector. Point mutations of His105 to phenylalanine (H105F) and His105 to cysteine (H105C) of the β1-subunit were produced using the QuickChange site-directed mutagenesis kit (Stratagene) following the manufacturer’s instruction. All constructs were sequenced before being used.

Cell culture. Rat aortic smooth muscle cells (RASMCs) were isolated from 12- to 14-wk-old male Wistar rats as previously described (27), according to a protocol approved by our Regional Animal Study Committee. Cells between passages 3 and 5 were used for all experiments. RASMCs and African green monkey kidney cells (COS7 cells) were routinely cultured in DMEM containing 4.5 g/l glucose and supplemented with 10% FBS and antibiotics. For transfection, COS7 cells were plated in 24-well plates at a density of 5 × 10^4 cells/well and allowed to grow overnight. Cells were then transfected using the JetPEI transfection reagent according to the manufacturer’s instruction. All constructs were sequenced before being used.

ROS production. RASMCs were plated in C-24 plates. After cells had reached confluence (~3.6 × 10^5 cells/well), ROS were assayed using the ROS-sensitive fluorescent dye carboxy-DCFDA. Cells were preincubated with carboxy-DCFDA (10 μM) in serum-free DMEM for 1 h. Excess dye was removed by washing the cultures twice with PBS. Cells were then incubated with the indicated agent in serum-free DMEM supplemented with 0.1% BSA for 1 h. At the end of this time, cells were washed with cold PBS and lysed in 100 μl lysis buffer [10 mM Tris-HCl (pH 7.6), 2 mM EDTA, and 1% Triton X-100]. The relative fluorescence was 536 nm after excitation at 485 nm. cGMP enzyme immunoassay. RASMCs were plated in 24-well plates. Confluent RASMCs were serum starved for 4 h and treated with vehicle, H2O2, or other ROS-generating agents for the indicated times. The vehicle for H2O2, menadione, and SIN-1 was PBS, whereas for ODQ and rotenone, DMSO was used. Cells were then washed twice with HBSS and incubated in HBSS in the presence of IBMX (1 mM) for 15 min with or without sGC activators. Media were then aspirated, and 300 μl of 0.1 N HCl were added into each well to extract cGMP. After 30 min, HCl extracts were collected and centrifuged at 600 g for 10 min to remove debris. Supernatants were directly analyzed for cGMP by enzyme immunoassay. After HCl extracts had been removed, 100 μl of 0.5 M NaOH were added to each well, and total protein was determined by the Lowry method. For transiently transfected cells, all treatments were performed 48 h after transfection.

Western blot analysis. Cells were harvested 48 h after transfection and lysed in a buffer containing 1% Triton 100-X, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 0.1 mM EGTA, 1 mM Na2VO4, 0.5% deoxycholic acid, 0.1% SDS, 10 μM aprotinin, 10 μM leupeptin, 10 μM pepstatin A, and 100 μM PMSF. Cellular debris was pelleted (12,000 g, 10 min), supernatants were then collected, and the protein concentration was subsequently determined. Lysates were subjected to SDS-PAGE on 10% polyacrylamide gels and transferred to PVDF membranes. Following transfer, membranes were blocked with 5% dry milk in Tris-buffered saline with 0.2% Tween (TBST) for 1 h at room temperature. Blots were then incubated with the primary antibody diluted in TBST at 4°C. Subsequently, blots were incubated with secondary antibody for 2 h at room temperature. Immunoreactive proteins were detected by chemiluminescence using the SuperSignal reagent kit.

Computational methods. The recently determined three-dimensional crystal structure of the heme-NO-oxygen (H-NOX)-binding domain of a cyanobacterial homolog (from Nostoc sp) of sGC [Protein Data Bank code: 2009 (18)] was used as a template for the homology modelling of the sGC structure. The process was based on the alignment of the 184 residues of the β1-subunit of human sGC and the 182 residues of Ns H-NOX. Twenty structures of sGC were generated using MODELER (4), and the model with the lowest energy was selected and further refined through energy minimization [SANFER routine of AMBER 9 (41) software]. The ribbon representation of the sGC structure and residues defining the heme cavity are shown in Fig. 6.

Docking simulations and analysis of docking results were performed using AutoDock 3.05 and AutoDock Tools, respectively (22). Protein was treated with the united-atom approximation by merging all nonpolar hydrogen atoms. Kollman partial charges were assigned to all protein atoms. HMR-1766 was also treated with the united-atom approximation and was prepared with the OpenEye suite of programs using the AM1-BCC partial charge distribution (14). The grid maps were centered on the ligand’s binding site (heme cavity) with 90 × 90 × 90 grid points of 0.25 Å spacing. The Lamarckian genetic algorithm was employed with the following parameters: a population size of 100 individuals, a maximum number of 2.5 × 10^6 energy evaluations, a maximum number of 27,000 generations, an elitism value of 1, a mutation rate of 0.02, and a crossover rate of 0.80 (22). For all calculations, 100 docking rounds were performed with step sizes of 0.2 Å for translations and 5° for orientations and torsions. Docked conformations were clustered with 0.5-Å tolerance for the root mean square positional deviation.

Data analysis. Data are expressed as means ± SE. Statistical comparisons between groups were performed using ANOVA followed by a post hoc test or Student’s t-test as appropriate. Differences were considered significant when P < 0.05. GraphPad Prism software (version 4.02, GraphPad Software, San Diego, CA) was used for curve fitting and calculation of EC50 values.

RESULTS

To study whether exposure of cells to agents used to oxidize sGC indeed led to increased generation of ROS intracellularly,
RASMCs were loaded with carboxy-DCFDA, a dye that reacts mainly with H$_2$O$_2$ but also detects the presence of hydroxyl radicals. Indeed, cells exposed to H$_2$O$_2$, menadione, or rotenone exhibited increased fluorescence, whereas those exposed to ODQ or the peroxynitrite/NO donor SIN-1 did not (Fig. 1). We next determined the effect of H$_2$O$_2$ on NO donor- and BAY 41-2272-induced sGC stimulation. In these experiments, RASMCs were pretreated with 500 µM H$_2$O$_2$ for 1 h and then exposed to either SNP or the NO-independent/heme-dependent stimulator BAY 41-2272 (Fig. 2A). Responses to both sGC activators were reduced in cells exposed to H$_2$O$_2$. In contrast, exposure of cells to H$_2$O$_2$ enhanced HMR-1766-stimulated cGMP accumulation throughout the concentration range tested (Fig. 2B). In addition, RASMCs treated with ODQ exhibited enhanced responses to HMR-1766; however, the effects of ODQ and H$_2$O$_2$ on HMR-1766-induced cGMP accumulation were not additive (Fig. 2C). Pretreatment of cells with catalase prevented the potentiating effect of H$_2$O$_2$ on HMR-1766-triggered cGMP levels (Fig. 2D). Similarly to what was observed with H$_2$O$_2$, treatment of cells with the peroxynitrite donor SIN-1 (11) led to an increase in cGMP production in response to HMR-1766 (Fig. 3A). Similar results were also obtained with menadione sodium bisulphite, an agent capable of releasing superoxide anions (10) (Fig. 3B). To determine if endogenously produced ROS have a similar effect on HMR-1766-induced cGMP formation, cells were treated with rotenone, a chemical that inhibits the transfer of electrons from Fe-S centers in complex I to ubiquinone, leading to ROS formation (16). In line with the observations made in the presence of exogenously applied ROS, cells treated with rotenone produced greater amounts of cGMP when stimulated with HMR-1766 (Fig. 3C). It should be noted that SNP responses remained unaltered in menadione- and rotenone-treated RASMCs (Fig. 3, B and C).

To determine whether ROS enhance sGC responsiveness to HMR-1766 by oxidizing the heme prosthetic group or through

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**Fig. 1.** Measurement of ROS in rat aortic smooth muscle cells (RASMCs). Confluent RASMCs were loaded with carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-DCFDA) dye (10µM) for 1 h and then washed twice with PBS. Cells were incubated with vehicle (control), H$_2$O$_2$ (500 µM), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 µM), 3-morpholinosydnonimine (SIN-1; 10 µM), menadione (40 µM), or rotenone (10 µM) for another 1 h. Relative fluorescence [in relative light units (RFU)] was measured as described in MATERIALS AND METHODS. Data are means ± SE; n = 4. *P < 0.05 by one-way ANOVA followed by a Newman-Keuls post hoc test.

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**Fig. 2.** Effect of H$_2$O$_2$ on HMR-1766-stimulated sGC activity. RASMCs were incubated with H$_2$O$_2$ (500 µM) for 1 h and then washed twice with HBSS. A and B: cells were then treated with vehicle (basal) or the indicated soluble guanylyl cyclase (sGC) stimulator or activator in the presence of IBMX for 15 min. cGMP was extracted and measured as described in MATERIALS AND METHODS. ODQ was added 30 min prior to cGMP determination (C), whereas catalase was added 2 h prior to H$_2$O$_2$ (D). SNP, sodium nitroprusside. Data are means ± SE; n = 4 wells. *P < 0.05 by unpaired t-test for A and B and by one-way ANOVA followed by a Newman-Keuls test for C and D.
could not be activated by SNP but was modestly stimulated by HMR-1766 (Fig. 4C). Unlike what was observed with SMCs and wild-type α1/β1-subunit-expressing COS cells, pretreatment of cells overexpressing the α1-subunit/H105F β1-subunit mutant or α1-subunit/H105C β1-subunit mutant with H2O2 led to reduced HMR-1766-stimulated cGMP formation (Fig. 5). The differential responses of the wild type and H105 F/C mutants to H2O2 or ODQ cannot be attributed to differences in

A different redox mechanism, COS7 cells were transfected with the heme-free α1-subunit/H105F β1-subunit sGC mutant (43). As expected, this mutant did not respond to SNP but could still be activated by HMR-1766 (Fig. 4B). In addition, although ODQ inhibited SNP-stimulated cGMP formation, it potentiated HMR-1766-stimulated cGMP accumulation in cells expressing wild-type sGC (Fig. 4A). On the other hand, ODQ did not alter HMR-1766 responses of heme-free α1-subunit/H105F β1-subunit sGC (Fig. 4B). In addition to the low-activity α1-subunit/H105F heme-deficient sGC mutant, a mutant with high basal activity was also used (α1-subunit/H105C β1-subunit). In line with what is known in the literature (19), this heme-free mutant

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**Fig. 3.** Effect of SIN-1, menadione, and rotenone on HMR-1766-induced cGMP production. Confluent RASMCs were incubated with SIN-1 (500 μM) for 1 h (A), menadione (40 μM) for 1 h (B), or rotenone (10 μM) for 4 h (C). Cells were washed twice with HBSS and then treated with vehicle (basal), SNP, or HMR-1766 in the presence of IBMX for 15 min. cGMP was extracted and measured as described in MATERIALS AND METHODS. Data are means ± SE; n = 4 wells. *P < 0.05 by unpaired t-test.

**Fig. 4.** Effect of ODQ on cGMP production of wild-type (WT) sGC and heme-free sGC mutants in cells. COS7 cells were cotransfected with myc-tagged α1- subunit + WT β1-subunit (A), the β1-subunit H105F mutant (B), or the β1-subunit H105C mutant (C). Forty-eight hours after transfection, cells were treated with ODQ for 30 min. Cells were then washed with HBSS and incubated with vehicle (basal) or the indicated sGC activator in the presence of IBMX for 15 min. Data are means ± SE; n = 4 wells. *P < 0.05 by unpaired t-test.
left in α1-subunit/H105F β1-subunit versus wild-type sGC in transiently transfected cells (Fig. 6). The EC50 value for HMR-1766 reported herein is similar to that previously published for the purified enzyme (34).

To identify the putative regions through which sGC interacts with HMR-1766, we used a computer modelling approach. To this end, we generated a sGC structure deduced from the H-NOX domain of a cyanobacterial homolog that bears a 33% sequence identity (18). Using this approach, a series of docking calculations were carried out, and the thermodynamically best fits for HMR-1766 in the heme pocket were described. Data analysis revealed that the ligand conformers are clustered in two slightly different orientations. In both orientations, the morpholine ring of HMR-1766 is accommodated into a cavity of hydrophobic residues composed of Leu142, Leu145, Val146, Phe120, Pro118, and Tyr135 (Fig. 7, A and B). This was also the case for the two benzamide rings, which are well positioned to exhibit hydrophobic interactions with the sGC residues Phe4, Val5, Phe74, Leu108, and Tyr83. Stasch and coworkers (35) proposed a model where BAY 58-2667 is in the heme pocket and its carboxylic groups interact with Tyr135 and Arg136 in a similar way that heme propionic groups do in the native enzyme. Based on our docking simulations, it was observed that the sulfonyl group next to the morpholine ring participates in the binding of HMR-1766 through interactions of S=O groups with the guanidinium moiety of Arg139 in a similar way with the carboxylic group of BAY 58-2667.

sGC levels (Suppl. Figs. S1 and S2).1 Taken together, the above-mentioned data suggest that the potentiating effect of H2O2 on HMR-1766-induced cGMP accumulation is dependent on the presence of the heme moiety.

In a different series of experiments using wild-type and α1-subunit/H105F β1-subunit sGC, we determined that the concentration-response curve to HMR-1766 was shifted to the right in α1-subunit/H105F β1-subunit versus wild-type sGC in transiently transfected cells (Fig. 6). The EC50 value for HMR-1766 reported herein is similar to that previously published for the purified enzyme (34).

To identify the putative regions through which sGC interacts with HMR-1766, we used a computer modelling approach. To this end, we generated a sGC structure deduced from the H-NOX domain of a cyanobacterial homolog that bears a 33% sequence identity (18). Using this approach, a series of docking calculations were carried out, and the thermodynamically best fits for HMR-1766 in the heme pocket were described. Data analysis revealed that the ligand conformers are clustered in two slightly different orientations. In both orientations, the morpholine ring of HMR-1766 is accommodated into a cavity of hydrophobic residues composed of Leu142, Leu145, Val146, Phe120, Pro118, and Tyr135 (Fig. 7, A and B). This was also the case for the two benzamide rings, which are well positioned to exhibit hydrophobic interactions with the sGC residues Phe4, Val5, Phe74, Leu108, and Tyr83. Stasch and coworkers (35) proposed a model where BAY 58-2667 is in the heme pocket and its carboxylic groups interact with Tyr135 and Arg136 in a similar way that heme propionic groups do in the native enzyme. Based on our docking simulations, it was observed that the sulfonyl group next to the morpholine ring participates in the binding of HMR-1766 through interactions of S=O groups with the guanidinium moiety of Arg139 in a similar way with the carboxylic group of BAY 58-2667.
The conformation flexibility of HMR-1766 in terms of the benzamide and SO$_2$-thiophene bond rotation seems to allow the placement of the ligand in the same position of the cavity adopting through two conformations and differentiates the docked conformation of this ligand: the two orientations differ in terms of the hydrogen bond between His$^{105}$ and the amide (Fig. 7B, conformer A) or the carbonyl group (Fig. 7C, conformer B) of the benzamide and are similar in terms of van der Waals contacts of the thiophene ring with the sGC residues Met$^1$ and Tyr$^2$. In addition, conformer B displays a hydrogen bond contact between the thiophene sulphur and amide group of Tyr$^2$.

Similar docking calculations were also performed for the His$^{105}$F sGC mutant. Analysis of the contacts between sGC (H105F) and HMR-1766 revealed that the ligand can adopt both orientations described above for the native sGC with the exception that the hydrophobic interactions were more favorable in terms of binding energies in the mutant sGC due to the presence of Phe$^{105}$. However, in addition to these two orientations, a third mode of placement seems to be favorable for the HMR-1766 ligand. In this conformer, the thiophene and chloro-benzamide have different orientations, exchanging their position in the heme cavity, compared with the placement mode described above (Fig. 7D). Specifically, the S=O groups of the sulfonyl group next to morpholine ring interact with Tyr$^{135}$; this is not observed in any other orientation and occurs in a similar way to the carboxylic group of BAY 58-2667.

To determine whether the regulatory domain of the $\beta_1$-subunit and/or the $\alpha_1$-subunit are required for HMR-1766 to exert its stimulatory effect on sGC, we used NH$_2$-terminally truncated mutants of both subunits and compared their responses with the wild-type enzyme. In cells expressing wild-type sGC, HMR-1766 caused a 14-fold increase in cGMP levels. Cells expressing the NH$_2$-terminally deleted form of the $\alpha_1$-subunit along with the wild-type $\beta_1$-subunit exhibited a 38-fold increase in response to HMR-1766; the lower cGMP values observed in cells expressing the heterodimer lacking the $\alpha_1$-subunit NH$_2$-terminus are probably the result of reduced levels of expression of this sGC form (Fig. 8). In contrast, cells expressing a $\beta_1$-subunit form lacking the first 63 residues were unresponsive to HMR-1766, suggesting that the NH$_2$-terminus of the $\beta_1$-subunit is critical for the action of this sGC activator.

Finally, we tested whether tolerance to HMR-1766 and cross-tolerance between HMR-1766 and SNP develops in our system. SMCs were exposed to 10 $\mu$M HMR-1766 for 2 h, and cGMP levels were determined following a second HMR-1766
oxidation anions quenches NO and converts heme to its ferric form, which is unresponsive to NO, thus reducing the ability of NO to stimulate cGMP production (2, 21). In addition, more prolonged exposure of cells to ROS leads to downregulation of sGC expression (8). Although treatment of SMCs with H$_2$O$_2$ reduced the responsiveness of cells to SNP and BAY 41-2272, it promoted HMR-1766-stimulated cGMP accumulation. The effects of H$_2$O$_2$ were of smaller magnitude than those of the selective heme oxidant ODQ; ODQ increased HMR-1766-induced responses by up to fivefold in RASMCs. Similar results to the ones observed with H$_2$O$_2$ and HMR-1766 were obtained with the peroxynitrite donor SIN-1 and the superoxide anion donor menadione. This observation is in line with findings that NS-2028-induced heme oxidation enhances the ability of anthranilic acid derivatives to promote cGMP synthesis by purified sGC (34). Similar results showing that peroxynitrite potentiates the action of the NO/heme-independent sGC activator BAY 58-2667 have been obtained in endothelial cells (38). To determine whether endogenously produced superoxide anions have the capacity to sensitize sGC to the action of HMR-1766, cells were pretreated with rotenone. Results from these experiments extended our observations with exogenously applied ROS and demonstrated that acute endogenous production of ROS enhances the cGMP-elevating ability of NO/heme-independent activators.

Based on experiments performed with the purified enzyme, HMR-1766 has been proposed to preferentially activate the oxidized/heme-depleted form of sGC (34). In the present study, we used two deficient sGC mutants (H105F and H105C) as tools to study the effects of HMR-1766 on heme-deficient sGC in vivo. Consistent with the lack of heme, exposure of cells expressing H105F sGC to SNP did not lead to cGMP accumulation. In contrast, H105F-expressing cells were responsive to HMR-1766; interestingly, HMR-1766 was more effective in stimulating H105F sGC compared with the wild-type enzyme. In agreement with our observations, BAY 58-2667 has been shown to enhance the activity of the H105F mutant (35). The ability of HMR-1766 to enhance the activity of heme-deficient sGC was confirmed using H105C sGC. In contrast to the exposure. “Basal” cGMP levels after the pretreatment and washout of 10 μM HMR-1766 were increased, but the addition of 1 μM HMR-1766 led to a proportionally higher response, demonstrating that tolerance to the action of HMR-1766 does not develop under the conditions studied (Fig. 9). On the other hand, tolerance to SNP could be demonstrated. Pretreatment of cells with SNP and subsequent exposure to HMR-1766 resulted in suprastimulation of sGC, whereas pretreatment with HMR-1766 followed by SNP stimulation yielded additive increases in cGMP levels, demonstrating that no cross-tolerance between the two types of sGC-activating agents develops.

**DISCUSSION**

The major findings of the present study are that 1) exogenously applied or endogenously produced ROS enhance HMR-1766-stimulated cGMP production, 2) the stimulatory effect of ROS on HMR-1766-induced cGMP is heme dependent, 3) HMR-1766 is more effective in stimulating heme-deficient sGC, 4) the NH$_2$-terminus of the β$_1$-subunit is required for the action of HMR-1766, and 5) exposure of cells to HMR-1766 does not lead to the development of tolerance.

Enhanced production of ROS has been implicated in the pathogenesis and maintenance of many cardiovascular diseases (2). Short-term exposure to increased concentrations of super-
results obtained with SNP, HMR-1766 activated H105C sGC, further emphasizing the difference in the mechanism of action between NO donors and the new class of sGC activators.

The greater cGMP accumulation in response to HMR-1766 observed in cells under oxidative stress could be due to redox changes in sGC thiol groups or alterations in the heme oxidation status. The sulphydryl groups of sGC had initially received a lot of attention, leading the redox-sensitive thiol switch theory for sGC activation (40). In addition, many thiol-modifying agents have been shown to alter both basal and stimulated enzyme activity (17, 40). Although the existence of heme-deficient sGC has not been proven to occur in living cells, such mutants overexpressed in cells would allow us to test whether the potentiating action of ROS on HMR-1766-stimulated cGMP generation was heme dependent or thiol related. We found that H2O2 reduced instead of potentiated the effect of HMR-1766 in H105F- and H105C-expressing cells. We attributed this reduction in HMR-1766 responsiveness after H2O2 treatment in sGC mutants to the fact that critical thiol groups of the enzyme are oxidized. A similar reduction probably also occurs in the wild-type enzyme after H2O2 treatment, but it is masked by the effect of H2O2 on heme; one would expect a greater increase by H2O2 in wild-type sGC-expressing cells if there were a way to reduce the thiol groups that become oxidized after H2O2 treatment. Taken together, the data above suggest that the positive effect of ROS on HMR-1766-induced cGMP formation are the result of an alteration in the oxidation state of heme.

sGC is composed of three domains: a NH2-terminal regulatory domain that in the β1-subunit carries the heme-binding region (44), a middle dimerization domain that is broken down into a NH2-terminal and COOH-terminal binding site (45), and a COOH-terminal catalytic domain (42). The NO-independent sGC activator BAY 41-2272 was originally proposed to act by binding to the α1-subunit (36), but later experiments failed to confirm the initial observation (5), whereas BAY 58-2667 binds to both α1- and β1-subunits (37). More recent computational models and site-directed mutagenesis experiments have demonstrated that the NO/heme-independent sGC activator BAY 58-2667 activates sGC as its carboxylic groups compete with H2O2 in the wild-type enzyme after H2O2 treatment, but it is masked by the effect of H2O2 on heme; one would expect a greater increase by H2O2 in wild-type sGC-expressing cells if there were a way to reduce the thiol groups that become oxidized after H2O2 treatment. Taken together, the data above suggest that the positive effect of ROS on HMR-1766-induced cGMP formation are the result of an alteration in the oxidation state of heme.

In summary, we have shown that HMR-1766 is a better stimulus for cGMP production in cells exposed to oxidative stress and that this property of HMR-1766 is dependent on the presence of sGC heme. Moreover, we have shown that HMR-1766 is more potent and efficacious in activating heme-deficient sGC and that the NH2-terminus of the β1-subunit is crucial for the action of this sGC activator. Finally, we have shown that previous exposure of smooth muscle to HMR-1766 does not lead to the development of tolerance, suggesting that this NO/heme-independent sGC activator is devoid of problems associated with NO donors.

GRANTS
This work was supported by the Thorax Foundation (Athens, Greece), the Greek Ministry of Education and the Greek Secretariat of Research and Technology.

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AJP-Heart Circ Physiol • VOL 295 • OCTOBER 2008 • www.ajpheart.org


