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Soluble Guanylate Cyclase Redox State Under Hypoxia or Hypoxia/Reoxygenation in Isolated Monkey Coronary Arteries

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Abstract. Hypoxia or hypoxia/reoxygenation impairs nitric oxide (NO)-mediated relaxation through the increase in superoxide generation in monkey coronary arteries. Soluble guanylate cyclase (sGC), the target enzyme of NO, has been shown to change from the NO-sensitive reduced form to the NO-insensitive oxidized/heme-free form under substantial oxidative stress, so the present study investigated whether hypoxia or hypoxia/reoxygenation influences sGC redox equilibrium. In isolated monkey coronary arteries without endothelium, the relaxation caused by the sGC stimulator BAY 41-2272 (E_{max}: 93.3% ± 2.2%) was somewhat impaired under hypoxia (E_{max}: 86.3% ± 2.6%) or hypoxia/reoxygenation (E_{max}: 86.1% ± 3.2%), whereas that by the sGC activator BAY 60-2770 (E_{max}: 86.0% ± 3.2%) was significantly augmented under hypoxia (E_{max}: 94.4% ± 1.3%) or hypoxia/reoxygenation (E_{max}: 95.5% ± 1.1%). In addition, cGMP formation in response to BAY 41-2272 and BAY 60-2770 was inhibited and stimulated, respectively, under hypoxia or hypoxia/reoxygenation. The effects of hypoxia or hypoxia/reoxygenation on BAY 41-2272– and BAY 60-2770–induced vasorelaxation were completely canceled by the treatment with the superoxide dismutase mimetic tempol. These findings suggest that sGC redox equilibrium in the coronary artery is shifted towards the NO-insensitive form under hypoxia or hypoxia/reoxygenation and that superoxide seems to play an important role in this shift.
[Supplementary Figures: available only at http://dx.doi.org/10.1254/jphs.14046FP]

Keywords: soluble guanylate cyclase, cGMP, hypoxia, reoxygenation, coronary artery

Introduction

The coronary artery plays an important role in supplying fresh nutrients and oxygen to myocardium and in maintaining the cardiac function (1). Accordingly, the performance of coronary artery during hypoxia or hypoxia/reoxygenation is of great importance in clinical conditions such as angina pectoris and atherosclerosis. Although the tone of the coronary artery is modulated by several endogenous and exogenous substances acting on smooth muscle, this regulation has been reported to be affected in varying degrees in the process of hypoxia/reoxygenation injury in several animal species, including rat (2, 3), cattle (4, 5), swine (3, 5 – 8), monkey (9), and human (3). There is a need to better understand the influence of hypoxia or hypoxia/reoxygenation on coronary function in order to establish an effective treatment strategy.

Soluble guanylate cyclase (sGC) is a primary receptor for the gaseous messenger nitric oxide (NO) and mediates vasorelaxation through elevation of intracellular cGMP levels and activation of cGMP-dependent kinase (PKG) (10). NO increases sGC activity by binding to the prosthetic heme moiety of the enzyme, but this requires the presence of reduced ferrous Fe^{2+} heme moiety and oxidation to its ferric Fe^{3+} state or loss of heme renders sGC insensitive to NO (11, 12). This sGC redox equilibrium can be shifted to the NO-insensitive heme-oxidized/heme-free form by reactive oxygen and nitrogen species such as superoxide and peroxynitrite, which are generated under conditions of oxidative stress (13, 14). Actually, an altered redox state of sGC under substantial oxidative stress contributes to impairing the relaxation mediated by

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its natural ligand NO in diseased vasculatures (12, 15).

Two different types of compounds that act directly on sGC (sGC stimulators and sGC activators) have been recently developed (13, 16). sGC stimulators, such as BAY 41-2272 {3-(4-amino-5-cyclopropylpyrimidine-2-yl)-1-(2-fluorobenzyl)-1H-pyrazolo[3,4-*b*]pyridine}, enhance the sensitivity of the reduced heme of sGC to low levels of bioavailable NO and can also stimulate this form of the enzyme in the absence of NO (17). In contrast, sGC activators, such as BAY 60-2770 (4-({(4-carboxybutyl)[2-(5-fluoro-2-{[4′-(trifluoromethyl) biphenyl-4-yl]methoxy}phenyl)ethyl]amino}methyl) benzoic acid), preferentially and effectively activate sGC when it is in the NO-unresponsive, heme-oxidized or heme-free state (18). These compounds therefore are emerging as valuable tools for elucidating the physiology and pathophysiology of the NO–sGC–cGMP pathway in more detail.

In the previous study, we have reported that hypoxia or hypoxia/reoxygenation impairs the relaxation by NO donors, but not that by a cGMP analog, through the increase in intracellular superoxide generation in isolated endothelium-denuded monkey coronary arteries (9). However, it was still unclear whether or not this impairment of NO signaling is mediated via a shift of the sGC redox equilibrium towards the NO-insensitive oxidized/heme-free state. Therefore, the present study was undertaken to investigate this point by using the sGC stimulator BAY 41-2272 and the sGC activator BAY 60-2770.

Materials and Methods

Animals

Eleven Japanese monkeys (*Macaca fuscata*) of either sex, weighing 3 – 10 kg, were used for the present study. The Animal Care and Use Committee at Shiga University of Medical Science and The Institutional Animal Care and Use Committee at Primate Research Institute of Kyoto University approved the use of monkey materials along with the experimental protocols in this study. These guidelines are in accordance with the recommendations of the Weatherall Report on "The Use of Non-Human Primates in Research". The monkeys were group-housed or housed individually in cages under controlled conditions of humidity, temperature, and light; and they were monitored daily by the researchers and the animal care staff to check the conditions of health and welfare. A commercial primate diet and fresh fruit/vegetable were provided daily, and water was provided ad libitum. The environmental enrichment consisted of various toys. Every effort was made to alleviate animal discomfort and pain by appropriate and routine use of anesthetic and/or analgesic agents.

Preparation

Under deep general anesthesia with ketamine (10 mg/kg, i.m.) and sodium pentobarbital (40 mg/kg, i.v.), each monkey was sacrificed by bleeding. The heart was rapidly removed. Coronary arteries (1.0 – 2.5 mm outside diameter) were isolated, the surrounding tissues cleaned off of them, and then the arteries were cut helically into strips. The endothelium of the strips was intentionally removed by gently rubbing the intimal surface with a ball of cotton wool. The strips were then fixed vertically between hooks in a muscle bath (10 mL capacity) containing the modified Ringer-Locke solution with the following composition: 120 mM NaCl, 5.4 mM KCl, 2.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25.0 mM $NaHCO_3$, and 5.6 mM dextrose. The solution was bubbled with a gas mixture of 95% O_2 and 5% CO_2 (pH 7.4), and the temperature was maintained at 37°C ± 0.3°C. The hook anchoring the upper end of the strip was connected to the lever of a force-displacement transducer (Nihon Kohden Kogyo Co., Tokyo). The resting tension was adjusted to 1.0 g, which is optimal for inducing the maximum contraction. Before starting the experiments, all of the preparations were allowed to equilibrate in the bathing medium for 60 – 90 min, during which time the solution was replaced every 10 – 15 min.

Mechanical responses

Isometric contractions and relaxations were displayed on an ink-writing oscillograph. The contractile response to 30 mM KCl was first obtained, and the arterial strips were repeatedly washed and equilibrated. The preparations were then separately exposed to any of the following conditions: i) Aerobic conditions, exposure to the bathing media continuously with 95% O_2 and 5% CO_2; ii) Hypoxic conditions, exposure for about 60 min to the bathing media with 95% N_2 and 5% CO_2 instead of 95% O_2 and 5% CO_2; iii) Reoxygenated conditions, reexposure for about 30 min to the bathing media with 95% O_2 and 5% CO_2 after hypoxia. After exposing to each condition, the strips were partially contracted with prostaglandin (PG) $F_{2\alpha}$ in a concentration range of 0.1 – 10 μM. Concentration–response curves for BAY 41-2272 and BAY 60-2770 were obtained by adding the drug directly to the bathing media in cumulative concentrations. The concentration ranges for these drugs were determined based on our preliminary research. At the end of each experiment, 100 μM papaverine was added to induce the maximal relaxation, which was taken as 100% for relaxations induced by the agonists. The partial O_2 pressure in the solution exposed to aerobic, hypoxic, and reoxygenated conditions were in a range of 431 – 524, 58 – 71, and 476 – 532 mmHg, respectively. The effects of tempol (3 mM), a membrane-permeable superoxide

dismutase mimetic, on the response of the strips treated for 10 – 30 min were also evaluated. The concentration of tempol was determined based on the previous work (9).

cGMP measurements

The content of cGMP in endothelium-denuded monkey coronary artery strips was measured according to the method described previously with minor modifications (19). Briefly, the strips were incubated for about 20 min with BAY 41-2272 (10^{-5} M) or BAY 60-2770 (10^{-7} M) after exposing to each condition and were then immediately plunged into liquid nitrogen. The tissues were homogenized in 1.0 mL of 5% trichloroacetic acid at 0°C with a Polytron-type homogenizer. After centrifugation at 3,000 rpm for 10 min, the supernatant was extracted with water-saturated ether. The residual ether was removed from the aqueous layer by heating the supernatant for 5 min to 70°C. An aliquot of the extract was then used for determination of cGMP, using a commercial enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI, USA). The cGMP level in the tissue was expressed as the relative value divided by the protein content measured in the same extract.

Drugs

The following drugs were used: BAY 41-2272 and BAY 60-2770 (kindly provided by Dr. Johannes-Peter Stasch of the Institute of Cardiovascular Research, Pharma Research Centre, Bayer AG, Wuppertal, Germany); tempol (MP Biomedicals, Aurora, OH, USA); $PGF_{2\alpha}$ (Pharmacia-Upjohn, Tokyo); ketamine (Sankyo, Tokyo); sodium pentobarbital and papaverine hydrochloride (Dainippon-Sumitomo Pharma Co., Osaka). Dimethyl sulfoxide was used as a solvent for BAY 41-2272 and BAY 60-2770. $PGF_{2\alpha}$ was dissolved in sodium bicarbonate buffer (pH 9.2). These solvents at the concentrations used in the present study did not show significant influence on the vascular response. Distilled water was used to dissolve all other drugs and to prepare serial dilutions, as required, from stocks on the day of the experiment.

Statistics

All values are expressed as the mean ± S.E.M. Concentration–response curves were analyzed by nonlinear curve fitting using Graph Pad Prism 6.0 software (Graph Pad Software Inc., San Diego, CA, USA). The maximal response (E_{max}) and the negative logarithm of the dilator concentration that caused half of the E_{max} (pD_2) were obtained. Concentration–response curves were assessed by two-way repeated measures analysis of variance (ANOVA) and Tukey-Kramer test. E_{max} and pD_2 values were compared with one-way ANOVA followed by the Tukey-Kramer posthoc test. Comparison for the cGMP level was performed using the Tukey-Kramer multiple comparisons test after one-way ANOVA. Differences were considered significant at $P < 0.05$.

Results

Influence of hypoxia or hypoxia/reoxygenation on BAY compounds–induced vasorelaxation

The addition of either BAY 41-2272 at concentrations of 10^{-9} – 10^{-5} M or BAY 60-2770 at 10^{-11} – 10^{-7} M produced a dose-dependent relaxation in coronary arteries without endothelium in which ACh did not produce any relaxation (data not shown). The dose–response curve to BAY 41-2272 showed the tendency to be shifted to the right under hypoxic or under reoxygenated conditions as compared with those under aerobic conditions (Fig. 1A). E_{max} and pD_2 values were also somewhat decreased by hypoxia or by hypoxia/reoxygenation (Table 1). On the other hand, the concentration–response curve to BAY 60-2770 was shifted to the left under hypoxic or under reoxygenated conditions (Fig. 1B). In addition, E_{max} and pD_2 values were increased significantly and slightly, respectively, by hypoxia or by hypoxia/reoxygenation (Table 1). The potencies of attenuation for BAY 41-2272 and augmentation for BAY 60-2770 were not obviously different between hypoxic and reoxygenated conditions.

Influence of hypoxia or hypoxia/reoxygenation on BAY compounds–induced cGMP production

As shown in Fig. 2, cGMP levels in endothelium-denuded coronary arteries stimulated with BAY 41-2272 (10^{-5} M) were lower under hypoxic (15.74 ± 2.01 pmol/mg protein) or under reoxygenated conditions (14.80 ± 2.39 pmol/mg protein) than that under aerobic conditions (23.31 ± 2.84 pmol/mg protein). On the other hand, BAY 60-2770 (10^{-7} M)-induced cGMP formation was enhanced by hypoxia or by hypoxia/reoxygenation (aerobic conditions, 11.43 ± 1.12 pmol/mg protein; hypoxic conditions, 23.34 ± 4.63 pmol/mg protein; reoxygenated conditions, 26.41 ± 4.54 pmol/mg protein).

Influence of hypoxia or hypoxia/reoxygenation on BAY compounds–induced vasorelaxation in the presence of tempol

In coronary arteries without endothelium that were preincubated with tempol, the magnitudes of relaxation by BAY 41-2272 were not significantly different between aerobic, hypoxic, and reoxygenated conditions (Fig. 3A). Similar results were also obtained in the case of BAY 60-2770 (Fig. 3B).

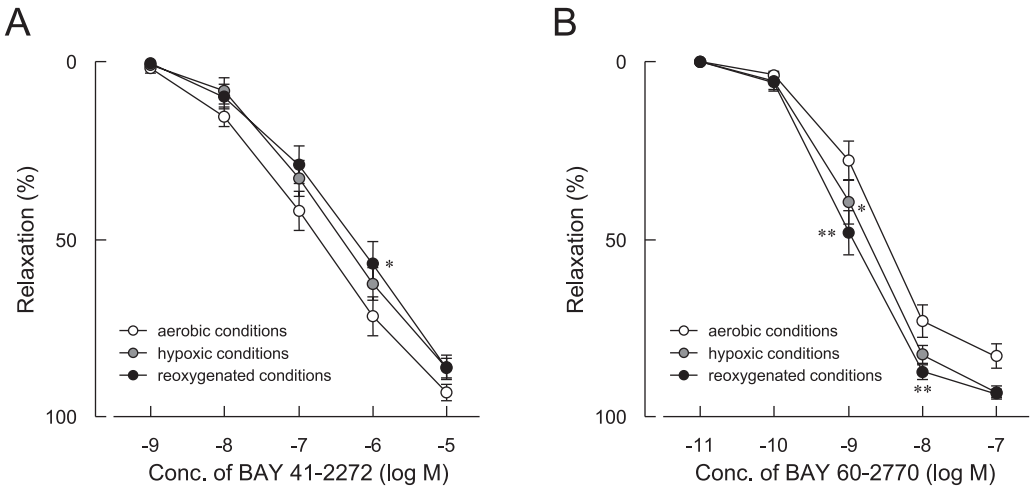

Fig. 1. BAY 41-2272 (panel A)- and BAY 60-2770 (panel B)-induced relaxation of endothelium-denuded monkey coronary arteries under aerobic (white circle), hypoxic (gray circle), and reoxygenated conditions (black circle). Each point and bar represents the mean ± S.E.M. (n = 8 from 6 separate hearts). *$P < 0.05$ and **$P < 0.01$, compared with aerobic conditions.

Table 1. pD_2 and E_{max} to BAY 41-2272 and BAY 60-2770

	Aerobic conditions	Hypoxic conditions	Reoxygenated conditions
BAY 41-2272			
pD_2	6.73 ± 0.17	6.64 ± 0.16	6.35 ± 0.19
E_{max} (%)	93.3 ± 2.2	86.3 ± 2.6	86.1 ± 3.2
BAY 60-2770			
pD_2	8.71 ± 0.10	8.87 ± 0.10	9.04 ± 0.09
E_{max} (%)	86.0 ± 3.2	94.4 ± 1.3*	95.5 ± 1.1*

Values are the mean ± S.E.M. (n = 8 from 6 separate hearts). *$P < 0.05$, compared with aerobic conditions. E_{max}, the maximal response; pD_2, the negative logarithm of the dilator concentration that caused half of the E_{max}.

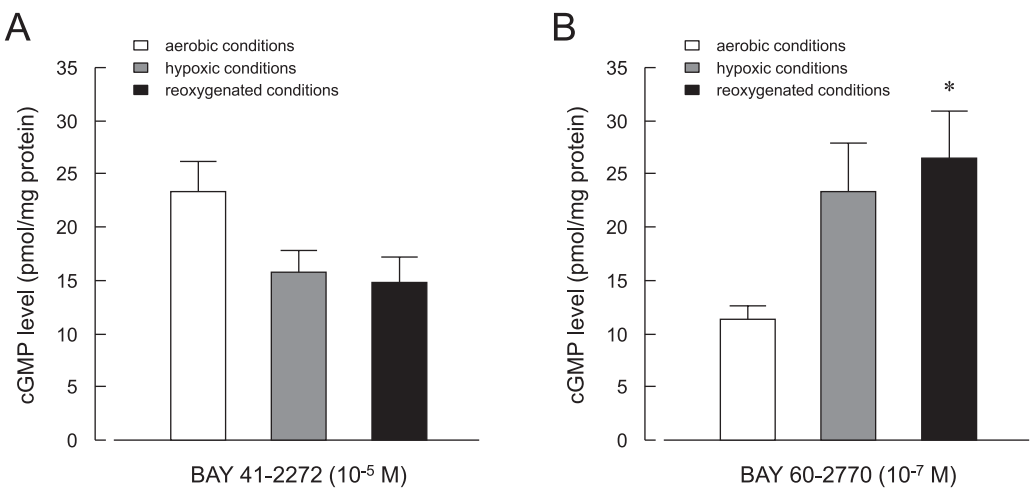

Fig. 2. BAY 41-2272 (10^{-5} M) (panel A)- and BAY 60-2770 (10^{-7} M) (panel B)-induced cGMP formation in endothelium-denuded monkey coronary arteries under aerobic (white column), hypoxic (gray column), and reoxygenated conditions (black column). Each column and bar represents the mean ± S.E.M. (n = 5 from 3 separate hearts). *$P < 0.05$, compared with aerobic conditions.

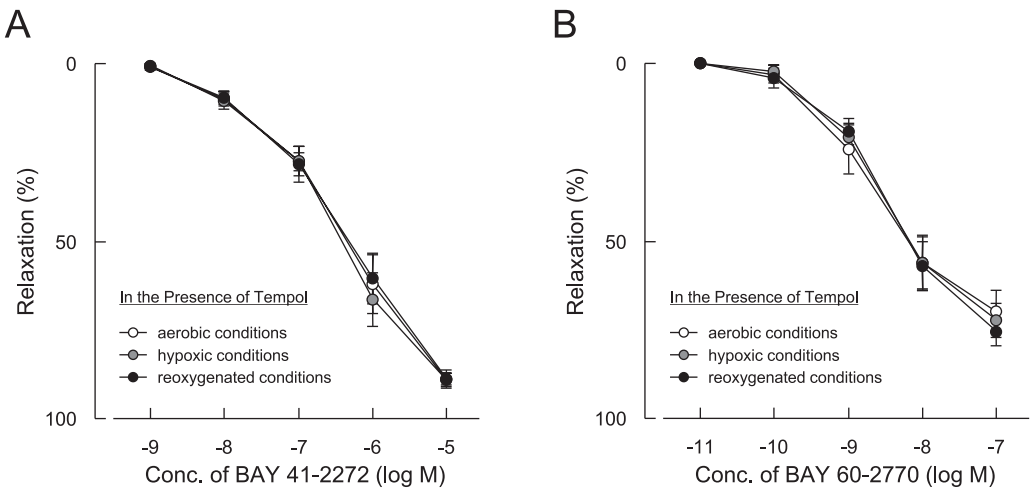

Fig. 3. BAY 41-2272 (panel A)- and BAY 60-2770 (panel B)-induced relaxation of endothelium-denuded monkey coronary arteries in the presence of tempol under aerobic (white circle), hypoxic (gray circle), and reoxygenated conditions (black circle). Each point and bar represents the mean ± S.E.M. (n = 6 from 5 separate hearts).

Discussion

The present study revealed that responses of endothelium-denuded monkey coronary arteries to BAY 41-2272, an NO-independent but heme-dependent sGC stimulator, were attenuated by hypoxia or by hypoxia/reoxygenation, whereas those to BAY 60-2770, an NO- and heme-independent sGC activator, were augmented. In addition, cGMP formation induced by high concentrations of BAY 41-2272 and BAY 60-2770 was inhibited and stimulated, respectively, under hypoxia or under hypoxia/reoxygenation, reflected in the vascular responses. Considering that sGC stimulators and sGC activators preferentially act on the reduced form and the oxidized/heme-free form of sGC, respectively (17, 18), these findings suggest that the sGC redox equilibrium is shifted to the NO-insensitive heme-oxidized/heme-free form under hypoxic or reoxygenated conditions in monkey coronary arteries. To the best of our knowledge, this is a first report showing that hypoxia or hypoxia/reoxygenation affects the sGC redox state in the coronary artery. Although this outcome provides important information in view of the fact that monkeys are primates, as are humans, further studies using several different animal species are hoped to be performed. This is because coronary vascular dynamics in monkeys is not exactly the same as that in humans (20).

Although sGC is recognized to exist mainly as the reduced (NO-sensitive) form under physiological conditions, the ratio of oxidized and/or heme-free forms has been proposed to rise in some cardiovascular diseases (12, 15). It has been already revealed that a crucial factor for the maintenance of this equilibrium is reactive oxygen and nitrogen species such as superoxide and peroxynitrite, respectively (13, 14). That is, these highly reactive species oxidize the sGC heme moiety from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state and subsequently remove the heme from the enzyme, resulting in the increase in the ratio of the oxidized/heme-free form to the reduced form. In the previous study, we have shown that the tissue level of superoxide is increased by hypoxia or by hypoxia/reoxygenation in isolated endothelium-denuded monkey coronary arteries (9). Consequently, the shift of sGC redox state under hypoxia or under hypoxia/reoxygenation observed in the present study was considered to be due to superoxide and/or its derivatives. In fact, pretreatment with the superoxide scavenger tempol before exposing to hypoxia or to hypoxia/reoxygenation completely eliminated the influences on BAY 41-2272– and BAY 60-2770–induced vasorelaxation under these two conditions, suggesting again that superoxide generated under hypoxia or subsequent reoxygenation contributes to a conversion of ferrous heme in sGC to the ferric state or dissociation of the heme from the enzyme.

Unfortunately, it is still unclear whether superoxide itself has potential to alter the sGC redox state since this radical easily reacts with other molecules or radicals (21). Zhou et al. have demonstrated that not only superoxide-generating agents but also hydrogen peroxide or peroxynitrite-generating agent limit the cGMP formation stimulated by the NO donor or the sGC stimulator, whereas they enhance that by the sGC activator in rat aortic smooth muscle cells (14). This means that there is a possibility that secondary oxidants such as hydrogen peroxide and peroxynitrite, but not superoxide itself, convert the ferrous heme of sGC to the ferric state or

remove the heme from the enzyme. Anyway, it is certain that the increase in superoxide level results in a shift of the sGC redox equilibrium towards the NO-insensitive heme-oxidized/heme-free form because it is the primary radical among reactive oxygen and nitrogen species.

From the results of Figs. 1 and 3, the treatment with tempol seems to have a certain effect on both responses to BAY 41-2272 and BAY 60-2770 under aerobic conditions. However, the dose–response curves in Fig. 1 should not be simply compared with those in Fig. 3. This is because monkeys used in the present study have different genetic backgrounds and vascular reactivity varies slightly among individuals. Basically, the comparison should be made between preparations from individuals used for the experiment of both Figs. 1 and 3. In fact, if only those data are compared, BAY compounds–induced relaxation was not affected by the absence or presence of tempol (see Supplementary Fig. 1: available in the online version only). Although the number of samples is insufficient to make a conclusion, the treatment with tempol under aerobic conditions is likely to have no effect on the relaxation of endothelium-denuded coronary arteries evoked by BAY compounds. This idea is supported by our observation that the same concentration of tempol does not affect the relaxant responses to BAY 41-2272 and BAY 60-2770 in isolated endothelium-denuded rat iliac arteries (unpublished data).

The impairment by hypoxia or by hypoxia/reoxygenation of BAY 41-2272–evoked relaxation in endothelium-denuded monkey coronary arteries observed in the present study was not so severe as that of NO donors–induced relaxation observed in our previous study (9). Incidentally, this impairment of vasorelaxation in response to NO donors was also prevented by treatment with tempol (9), indicating that the crucial factor underlying the impairment is also superoxide. In this regard, it is common sense that superoxide reacts with NO to form peroxynitrite and other nitrogen species, thereby causing an effective reduction in NO availability (22). Consequently, the increased superoxide is considered to quench NO and decrease the reduced form of sGC, thus limiting the ability of NO to relax vascular smooth muscle. However, it is also important to remember that more prolonged exposure to reactive oxygen species leads to downregulation of sGC expression. Gerassimou et al. have confirmed that sGC expression is reduced in freshly isolated rat aortic strips by exposure to superoxide-generating agents or hydrogen peroxide for a long period (23). In any case, taken together, the response to agents activating the reduced sGC would be diminished sooner or later if the generation of reactive oxygen and nitrogen species was stimulated.

Growing evidence highlights the advantage of the sGC activator in disease states associated with oxidative stress (12). In that respect, it has been demonstrated that superoxide production is increased in coronary arteries from patients with coronary artery disease (24, 25). Putting this fact together with our present findings, the sGC activator probably provides some advantages as a vasodilator in coronary artery disease. However, we have to remember that the coronary artery relaxation induced by BAY 60-2770 was not as acute as that by the organic nitrate nitroglycerin (see Supplementary Fig. 2: available in the online version only). Since either form of sGC is present in the cytosol, this may be due to membrane permeability of the drug used. Although we do not know if other sGC activators possess the same vasorelaxant kinetics as BAY 60-2770, this type of drug, unlike the organic nitrate, might be unsuitable for the treatment of acute attacks. At this point, further studies are needed to fully assess the potential clinical benefit of the sGC activator in coronary artery disease.

The present study has a few limitations. First, the basal cGMP levels in endothelium-denuded coronary arteries were not measured. However, a number of previous studies have shown that the basal cGMP levels in endothelium-denuded arteries are extremely low as compared with the levels stimulated with sufficient dosages of stimulants (26 – 29). Therefore, our conclusion that cGMP formation by BAY 41-2272 and BAY 60-2770 was inhibited and stimulated, respectively, under hypoxia or under hypoxia/reoxygenation is probably not influenced. Next, it is well acknowledged that the influence of hypoxia or hypoxia/reoxygenation is not identical between coronary arteries with and without endothelium (30, 31). Unfortunately, our previous (9) and present studies have not addressed the question of whether the presence of endothelium makes a difference in outcomes. Consequently, this point must be inevitably found out in order to better understand functional changes of coronary arteries under hypoxic or reoxygenated conditions.

In summary, the present study showed that hypoxia or hypoxia/reoxygenation induces a shift of the sGC redox equilibrium towards the NO-insensitive oxidized/heme-free form in endothelium-denuded monkey coronary arteries. Additionally, the increased superoxide by hypoxia or by hypoxia/reoxygenation seems to contribute to this process.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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