Effects of hydrogen peroxide on relaxation through the NO/sGC/cGMP pathway in isolated rat iliac arteries

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ABSTRACT
The production of reactive oxygen species, including hydrogen peroxide (H$_2$O$_2$), is increased in diseased blood vessels. Although H$_2$O$_2$ leads to impairment of the nitric oxide (NO)/soluble guanylate cyclase (sGC)/cGMP signaling pathway, it is not clear whether this reactive molecule affects the redox state of sGC, a key determinant of NO bioavailability. To clarify this issue, mechanical responses of endothelium-denuded rat external iliac arteries to BAY 41-2272 (sGC stimulator), BAY 60-2770 (sGC activator), nitroglycerin (NO donor), acidified NaNO$_2$ (exogenous NO) and 8-Br-cGMP (cGMP analog) were studied under exposure to H$_2$O$_2$. The relaxant response to BAY 41-2272 ($pD_{2}$: 6.79 ± 0.10 and 6.62 ± 0.17), BAY 60-2770 ($pD_{2}$: 9.57 ± 0.06 and 9.34 ± 0.15) or 8-Br-cGMP ($pD_{2}$: 5.19 ± 0.06 and 5.24 ± 0.08) was not apparently affected by exposure to H$_2$O$_2$. In addition, vascular cGMP production stimulated with BAY 41-2272 or BAY 60-2770 in the presence of H$_2$O$_2$ was identical to that in its absence. On the other hand, nitroglycerin-induced relaxation was markedly attenuated by exposing the arteries to H$_2$O$_2$ ($pD_{2}$: 8.73 ± 0.05 and 8.30 ± 0.05), which was normalized in the presence of catalase ($pD_{2}$: 8.59 ± 0.05). Likewise, H$_2$O$_2$ exposure impaired the relaxant response to acidified NaNO$_2$ ($pD_{2}$: 6.52 ± 0.17 and 6.09 ± 0.16). These findings suggest that H$_2$O$_2$ interferes with the NO-mediated action, but the sGC redox equilibrium and the downstream target(s) of cGMP are unlikely to be affected in the vasculature.

Introduction
Soluble guanylate cyclase (sGC), an intracellular receptor for nitric oxide (NO), facilitates the conversion of GTP to cGMP, leading to vasorelaxation through the activation of cGMP-dependent kinase (PKG) [1]. Although the NO/sGC/cGMP signaling is thus crucial for the regulation of vascular tone, this pathway is disrupted in pathological conditions. In the first place, NO activates sGC by binding to its reduced ferrous Fe$_2^+$ heme moiety, but oxidation to ferric Fe$_3^+$ state or loss of heme renders sGC insensitive to NO [2,3]. This alteration of the heme iron redox state has attracted attention as an important mechanism responsible for dysfunction of the NO/sGC/cGMP pathway. In fact, it has been reported that the sGC redox equilibrium is shifted toward the NO-insensitive form in a certain diseased vasculature [4–6].

Although NO cannot activate the heme-oxidized/heme-free sGC, these NO-insensitive forms can still catalyze cGMP formation in the same manner as the NO-sensitive form [7,8]. In addition, there are also compounds that stimulate sGC in an NO-independent manner [9]. Like NO, sGC stimulators can increase sGC activity only when it contains a prosthetic heme group with reduced iron [10]. In contrast, sGC activators preferentially and effectively activate the NO-unresponsive, heme-oxidized or heme-free enzyme [11]. There is no way to quantify the relative expression ratio of the reduced, oxidized and heme-free sGC, but sGC stimulators and sGC activators are emerging as valuable tools for evaluating the redox state of sGC.

Reactive oxygen and nitrogen species (ROS/RNS), such as superoxide and peroxynitrite, respectively, interfere with sGC activity via multiple mechanisms [12,13]. In this regard, accumulating evidence suggests that both superoxide and peroxynitrite have a potential to oxidize the sGC heme moiety in the vasculature [5,14,15]. Hydrogen peroxide (H$_2$O$_2$), a ROS generated by a range of oxidase enzymes and through the dismutation of superoxide, has been implicated in the etiology of various diseases while functioning as an endothelium-derived hyperpolarizing factor (EDHF) under certain conditions [16]. Indeed, it has been demonstrated that the vascular and/or circulating H$_2$O$_2$ levels are markedly increased in various pathological conditions [17–21].
Mechanical responses

Isometric contractions and relaxations were displayed on an ink-writing oscillograph. The tissues were contracted twice with 60 mM KCl followed each time by repeated washout with modified Ringer-Locke’s solution. The second response was taken as an index of the contractile ability. At this time, endothelial denudation was confirmed by the lack of acetylcholine-induced relaxation. After equilibration, the preparations were exposed for about 60 min to \( \text{H}_2\text{O}_2 \) (100 \( \mu \text{M} \)) or its vehicle (referred to as “control”) in the bathing solution. This concentration was determined based on our preliminary study, in which exposure to \( \text{H}_2\text{O}_2 \) up to 100 \( \mu \text{M} \) had no obvious influence on the contractile ability (Supplementary Figure 1). The strips were then partially contracted with phenylephrine (10\( \text{E}^{-7} \)–10\( \text{E}^{-5} \) M) and/or prostaglandin (PG) \( \text{F}_2\text{\alpha} \) (10\( \text{E}^{-7} \)–10\( \text{E}^{-5} \) M) in a range between roughly 20 and 60% of the contraction induced by 60 mM KCl. The reason why two types of vasoconstrictors were used is to obtain above precontraction level, which was not statistically different between the groups or the agonists (Supplementary Table 1). After the contraction reached a plateau, concentration-response curves for BAY 41-2272 (sGC stimulator), BAY 60-2770 (sGC activator), nitroglycerin (NO donor), acidified NaNO\(_2\) (exogenous NO) and 8-Br-cGMP (cGMP analog) were obtained by adding the drug directly to the bathing media in cumulative concentrations. A major difference between nitroglycerin and acidified NaNO\(_2\) is whether metabolism within the cell is needed in producing NO [14]. At the end of each experiment, papaverine (100 \( \mu \text{M} \)) was added to induce the maximal relaxation, which was taken as 100% for relaxations induced by the agonists. The effect of pretreatment with SOD (200 U/mL) or catalase (1200 U/mL) on the responses under exposure to \( \text{H}_2\text{O}_2 \) was also evaluated. Briefly, these drugs were added to the bathing solution just before exposure to \( \text{H}_2\text{O}_2 \). And experiments with endothelium-intact preparations were performed in the presence of the NO synthase inhibitor \( \text{N}^\text{G}\)-nitro-L-arginine (L-NA, 10 \( \mu \text{M} \)) to exclude the influence of endothelium-derived NO. The concentration of these drugs was determined based on previous works [14,22,23].

cGMP measurements

The content of cGMP in endothelium-denuded rat iliac artery strips was measured according to the method described previously [15]. Briefly, the strips were incubated without (referred to as “basal”) and with BAY 41-2272 (10\( \text{E}^{-7} \) M) or BAY 60-2770 (10\( \text{E}^{-9} \) M) for about 20 min after exposing to each condition and were then...
immediately plunged into liquid nitrogen. The tissues were homogenized in 0.5 mL of 5% trichloroacetic acid at 0 °C with a glass homogenizer. After centrifugation at 3000 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 the determination of cGMP, using a commercial enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI). 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: H_{2}O_{2} (Santoku Chemical Industries Co., Ltd., Tokyo, Japan); 8-Br-cGMP, L-NA, phenylephrine and SOD (Sigma Chemical Co., St. Louis, MO); nitroglycerin (Nihonkayaku Co., Tokyo, Japan); 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); acetylcholine (Daiichi-Sankyo Co., Tokyo, Japan); PGF_{2α} (Pharmacia-Upjohn, Tokyo, Japan); papaverine hydrochloride (Dainippon-Sumitomo Pharma Co., Osaka, Japan); sodium pentobarbital (Kyoritsu Seiyaku Co., Tokyo, Japan); sodium bicarbonate buffer (pH 9.2) was used as a solvent for BAY compounds and PGF_{2α}, respectively. Acidified NaNO_{2} was prepared by dissolving NaN0_{2} in 0.1 N HCl titrated to pH 2.0. In this solution, NaNO_{2} forms nitrous acid, which decomposes to NO. 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 ± SEM. Concentration–response curves were analyzed by nonlinear curve fitting using Graph Pad Prism 6.0 software (Graph Pad Software Inc., San Diego, CA). The negative logarithm of the dilator concentration that caused half of the maximal response (pD_{2}) was obtained. Statistical analysis was done for responses to individual concentrations of vasodilator drugs, pD_{2} values and cGMP levels. Comparisons between two groups were performed with an unpaired two-tailed Student’s t-test. Comparisons among four groups were performed with one-way measures analysis of variance followed by the Tukey–Kramer post-test. Differences were considered significant at p < 0.05.

**Results**

**Effects of H_{2}O_{2} on BAY compound-induced vasorelaxation**

As shown in Figure 1A, in endothelium-denuded rat iliac arteries, exposure to H_{2}O_{2} had no obvious effect on the concentration–response curve for the sGC stimulator BAY 41-2272 when compared with the curve obtained in the control. pD_{2} values in the presence of H_{2}O_{2} were not also significantly different from those in its absence (Table I). Preincubation with SOD or catalase, of course, made little impact on the BAY 41-2272-induced relaxation under H_{2}O_{2} stress (Figure 1A; Table I).

As is the case with the response to BAY 41-2272, the relaxation induced by the sGC activator BAY 60-2770 was
unaffected by exposure to H$_2$O$_2$ regardless of whether or not SOD or catalase is present (Figure 1B; Table I).

**Effects of H$_2$O$_2$ on BAY compound-induced cGMP formation**

Figure 2 demonstrates that exposure to H$_2$O$_2$ had no obvious influence on basal cGMP level in endothelium-denuded rat iliac arteries: 0.59 ± 0.14 pmol/mg protein (control) versus 0.45 ± 0.09 pmol/mg protein (H$_2$O$_2$). Additionally, in parallel with the vascular response, cGMP formation stimulated with either BAY 41-2272 (10$^{-7}$ M) or BAY 60-2770 (10$^{-9}$ M) was not affected by exposure to H$_2$O$_2$: BAY 41-2272: 2.75 ± 0.30 pmol/mg protein (control) versus 2.61 ± 0.25 pmol/mg protein (H$_2$O$_2$); BAY 60-2770: 4.57 ± 0.26 pmol/mg protein (control) versus 5.29 ± 0.56 pmol/mg protein (H$_2$O$_2$).

**Effects of H$_2$O$_2$ on NO-donating drug-induced vasorelaxation**

In contrast to the relaxant responses to BAY compound, exposure to H$_2$O$_2$ produced a rightward displacement of the concentration-response curve for nitroglycerin (Figure 3A) with a decrease in the $pD_2$ values (Table I). These undesirable effects of H$_2$O$_2$ on the relaxant response to nitroglycerin were also observed in the presence of SOD. On the other hand, the impaired

| Compound          | Control $pD_2$ | H$_2$O$_2$ $pD_2$ | + SOD $pD_2$ | + Catalase $pD_2$ |
|-------------------|---------------|-------------------|--------------|------------------|
| BAY 41-2272       | 6.79 ± 0.10   | 6.62 ± 0.17       | 6.63 ± 0.26  | 6.79 ± 0.16      |
| BAY 60-2770       | 9.57 ± 0.06   | 9.34 ± 0.15       | 9.45 ± 0.10  | 9.54 ± 0.04      |
| Nitroglycerin     | 8.73 ± 0.05   | 8.30 ± 0.05**     | 8.32 ± 0.06**| 8.59 ± 0.05†     |
| Acidified NaNO$_2$| 6.52 ± 0.17   | 6.09 ± 0.16       | 6.19 ± 0.18  | 4.69 ± 0.13**††  |
| 8-Br-cGMP         | 5.19 ± 0.06   | 5.24 ± 0.08       | ND           | ND               |

ND, no data. Values are the mean ± SEM of eight experiments.

* $p < 0.01$, compared with the control.
†† $p < 0.01$, compared with H$_2$O$_2$.  

Figure 3. Effects of H$_2$O$_2$ on nitroglycerin (A)- and acidified NaNO$_2$ (B)-induced relaxation of endothelium-denuded rat iliac arteries in the absence or presence of the antioxidant enzyme. Circle = control; Square = H$_2$O$_2$; Triangle = + SOD; Diamond = + catalase. Each point and bar represents the mean ± SEM of eight experiments.
relaxation by exposure to H₂O₂ was normalized by preincubation with catalase (Figure 3A; Table I).

Similar results as nitroglycerin were also obtained for the response to exogenous NO. That is, the relaxant response to acidified NaNO₂ was impaired by exposure to H₂O₂, which was also observed in the presence of SOD. However, contrary to the case with nitroglycerin, the impairment by H₂O₂ exposure of acidified NaNO₂-induced vasorelaxation was further deteriorated by treatment with catalase (Figure 3B; Table I).

**Effect of H₂O₂ on cGMP analog-induced vasorelaxation**

As shown in Figure 4, H₂O₂ exposure did not affect the concentration-response curve for the cGMP analog 8-Br-cGMP. In addition, there was no significant difference in pD₂ values between exposure to H₂O₂ and its vehicle (Table I).

**Influence of endothelium on the action of H₂O₂**

In endothelium-intact rat iliac arteries treated with L-NA, the relaxation induced by neither BAY 41-2272 nor BAY 60-2770 was affected by exposure to H₂O₂ (Figure 5): pD₂ to BAY 41-2272: 6.60 ± 0.10 (control) versus 6.54 ± 0.09 (H₂O₂); pD₂ to BAY 60-2770: 9.15 ± 0.11 (control) versus 9.12 ± 0.10 (H₂O₂).

**Discussion**

Oxidative stress is a major factor leading to dysfunction of the NO/sGC/cGMP pathway in the vasculature. In the previous study, we have shown that exposure to superoxide or peroxynitrite, both of which are produced under oxidative stress conditions, impairs the relaxation of endothelium-denuded rat iliac arteries by an sGC stimulator, but enhances that by an sGC activator [14,15]. As opposed to these results, the present study showed that exposure of endothelium-denuded rat iliac arteries to H₂O₂ has little influence on the relaxations induced by the sGC stimulator BAY 41-2272 and the sGC activator BAY 60-2770. Besides, vascular cGMP formation stimulated by these drugs in the presence of H₂O₂ was identical to that in its absence. It is of course natural that each of ROS/RNS has different biological activity [24,25], and H₂O₂ may not affect the sGC redox equilibrium in the vasculature. However, this speculation is not in line with previous reports showing inhibitory and stimulatory effects of H₂O₂ exposure on the cGMP production.
induced by an sGC stimulator and by an sGC activator, respectively, in vascular smooth muscle cells [4,26]. Although we cannot find out the reason for this discrepancy, one possibility is that H$_2$O$_2$ exposure conditions (e.g. concentration and duration) in these studies are not consistent with those in the present study. Furthermore, it is not uncommon that findings in cell-culture experiments differ from those in isolated tissue experiments [27,28]. In fact, previous reports have demonstrated different effects of H$_2$O$_2$ on isolated arteries and cultured smooth muscle cells [29,30]. Anyway, the present study, for the first time, revealed the influence of H$_2$O$_2$ exposure on the sGC redox state in blood vessels.

Although results of the present study suggest that the heme moiety of sGC was not modified by H$_2$O$_2$, there is no guarantee that the NO/sGC/cGMP pathway functions normally. Actually, several studies reported an inhibitory effect of H$_2$O$_2$ on vascular response to NO [4,26,31,32]. Among others, Mian and Martin show that exposure of endothelium-denuded rat aorta to H$_2$O$_2$ impairs the relaxant response to the NO donor nitroglycerin [31]. In accordance with this finding, nitroglycerin-evoked relaxation of endothelium-denuded rat iliac arteries was attenuated by H$_2$O$_2$ exposure in the present study. Daiber et al. demonstrated that the enzyme that catalyzes nitroglycerin biotransformation is inhibited by H$_2$O$_2$ [33]. However, the relaxant response to acidified NaNO$_2$ which does not need such metabolism was also aggravated in the presence of H$_2$O$_2$, suggesting that H$_2$O$_2$ exposure impairs NO-mediated vascular signaling. On the other hand, it is true that there is a conflicting report showing the potentiating effect of H$_2$O$_2$ on NO-induced vasorelaxation [34]. To definitely answer what makes this discrepancy is difficult under present circumstances.

To the best of our knowledge, there is no evidence to suggest that H$_2$O$_2$ directly interacts with cGMP, while H$_2$O$_2$ exposure is well known to induce thiol oxidation-mediated subunit dimerization of PKG [35,36]. According to a report written by Müller et al., this structural modification by H$_2$O$_2$ is possible to slightly inhibit cGMP-stimulated PKG activity [36]. Unfortunately, the present study did not confirm whether or not PKG is oxidized by exposure to H$_2$O$_2$. But the cGMP analog 8-Br-cGMP-induced relaxation of endothelium-denuded rat iliac arteries in the presence of H$_2$O$_2$ was identical to that in its absence, suggesting that the upstream signaling pathway of cGMP seems to be influenced under H$_2$O$_2$ stress conditions. The point is that the underlying mechanism is at least not through an alteration of the sGC redox equilibrium toward the NO-insensitive oxidized/heme-free form.

Catalase is an enzyme that rapidly breaks down H$_2$O$_2$ into oxygen and water. Preincubation with catalase almost completely eliminated the inhibitory effect of H$_2$O$_2$ on the relaxation induced by nitroglycerin, supporting the crucial role of H$_2$O$_2$ itself. Incidentally, although catalase failed to normalize the attenuated relaxant response to acidified NaNO$_2$ under exposure to H$_2$O$_2$, this is probably because catalase is a heme-containing and a membrane-impermeable enzyme. NO has a high affinity for heme, and a couple of studies have shown that NO can bind to heme iron in catalase [37,38]. It is reasonable that if NO is consumed by the reaction with heme proteins other than sGC, biological effects of NO via activation of this enzyme are limited. In fact, the relaxation of endothelium-denuded rat iliac arteries by acidified NaNO$_2$ was markedly attenuated in the presence of catalase, as is the case with coincubation with H$_2$O$_2$ and catalase (data not shown).

As shown in the present study, SOD can eliminate only extracellular superoxide because its membrane permeability, but we have shown that intracellular superoxide not only scavenges NO but also causes a shift of sGC redox equilibrium [14], which does not correlate well with the effects of H$_2$O$_2$ in the present study. Consequently, it is likely that H$_2$O$_2$ impairs NO-induced vascular relaxation in a superoxide-independent manner.

There are some possible mechanisms by which H$_2$O$_2$ limits the biological activity of NO. For example, the chemical interaction of H$_2$O$_2$ with NO has been demonstrated in in vitro studies [41,42]. That is, H$_2$O$_2$ might react with and scavenge NO in the same way as superoxide, resulting in a decrease in its vascular bioavailability. Another possibility is that exposure to H$_2$O$_2$ leads to redox modification of cysteine residues in proteins. Accumulating evidence has shown that sGC is desensitized to NO by the oxidation of cysteine residues [32,43,44]. Indeed, Maron et al. confirmed an oxidizing action of H$_2$O$_2$ on a cysteine residue in sGC in mammalian-cultured cells [32]. Furthermore, Fernhoff et al.
reported that the sGC stimulator YC-1, structurally related to BAY 41-2272, can activate sGC under the conditions in which NO-stimulated its activity is inhibited by cysteine oxidation [43]. In this regard, exposure of endothelium-denuded rat iliac arteries to the thiol oxidant diamide resulted in an impairment of the relaxation induced by nitroglycerin or acidified NaNO3, while it did not affect the relaxant response to BAY 41-2272 nor BAY 60-2770 (authors’ unpublished data). Consequently, the impairment by H2O2 of NO-mediated relaxation in the present study might be due to oxidation of sGC cysteine(s). Anyway, further studies are needed to clarify how H2O2 interferes with NO-dependent relaxation in the vasculature.

The present study paid attention to an oxidizing property of H2O2, but this molecule also acts as an EDHF and evokes a relaxation in some vascular beds [45]. Indeed, H2O2 had a strong relaxing effect in endothelium-denuded rat iliac arteries precontracted with phenylephrine and PGF2α (Supplementary Figure 2). However, this EDHF-like response is less likely to mask the effect of NO by competing for the same pathway. If H2O2-induced relaxation is mediated by the NO/sGC/cGMP pathway, the response to an sGC stimulator, an sGC activator and/or a cGMP analog is expected to be also affected.

The upper limit of physiological H2O2 concentration has been estimated to be in the range of 1 to 15 μM [46]. From this perspective, the concentration of H2O2 used in the present study is considered to be a pathophysiological level. However, it is not exactly clear how high levels of H2O2 are in the diseased vasculature. Nonetheless, an impairment of vascular relaxations by NO-donating drugs [47,48] has been shown in spontaneously hypertensive rats in which vascular H2O2 production is elevated [49]. Therefore, it is at least possible that a similar phenomenon as observed in the present study is occurring in vivo. This is also a future research issue.

Main focus of the present study was to investigate the effect of H2O2 on the sGC redox equilibrium in the vasculature. As described previously, this was determined by using two types of drugs, an sGC stimulator and an sGC activator, in the present study. What matters is that effects of sGC stimulators and sGC activators are synergistic and additive to NO, respectively [9–11]. That is, the presence of endothelium-derived NO makes it difficult to judge whether or not the influence of H2O2 is only due to an alteration of the sGC redox state. However, there is a possibility that the influence of H2O2 is not identical between endothelium-intact and endothelium-denuded arteries. In this regard, the exposure of endothelium-intact rat iliac arteries in the presence of L-NA to H2O2 did not affect the relaxant response to either an sGC stimulator or an sGC activator, again suggesting no influence of H2O2 on vascular sGC redox equilibrium.

In conclusion, the present study demonstrated that H2O2 exerts no substantial effects on the redox state of sGC in rat iliac arteries, although it interferes with the relaxation through the NO/sGC/cGMP pathway probably by reacting with NO or by modifying cysteine residue(s) in sGC. These findings will provide important insights into how the NO/sGC/cGMP pathway is regulated in the diseased blood vessel associated with increased H2O2 level.

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Supplementary material available online
Supplementary Figures 1 and 2 and Table 1.