

Full Paper

Impairment by Hypoxia or Hypoxia/Reoxygenation of Nitric Oxide–Mediated Relaxation in Isolated Monkey Coronary Artery: the Role of Intracellular SuperoxideMasashi Tawa¹, Kohei Yamamizu², Ayman Geddawy¹, Takashi Shimosato¹, Takeshi Imamura¹, Kazuhide Ayajiki³, and Tomio Okamura^{1,*}¹Department of Pharmacology, Shiga University of Medical Science, Otsu, Shiga 520-2192, Japan²Laboratory of Stem Cell Differentiation, Stem Cell Research Center, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Kyoto 606-8507, Japan³Department of Pharmacy, School of Pharmacy, Hyogo University of Health Sciences, Kobe, Hyogo 650-8530, Japan

Received February 16, 2011; Accepted April 5, 2011

Abstract. To investigate the effect of hypoxia or hypoxia/reoxygenation on vascular smooth muscle function, mechanical response of monkey coronary artery without endothelium was studied under normoxia, hypoxia, and hypoxia/reoxygenation. Hypoxia or hypoxia/reoxygenation impaired the relaxation by nitroglycerin or isosorbide dinitrate but not that by 8-bromoguanosine-3',5'-cyclic monophosphate or isoproterenol. Tempol restored the impaired relaxation by nitroglycerin or isosorbide dinitrate, but superoxide dismutase had no effect. Apocynin, an NADPH oxidase inhibitor, improved the nitroglycerin-induced relaxation under hypoxia, but not under reoxygenation. Under combined treatment of apocynin with oxypurinol (xanthine oxidase inhibitor), rotenone (mitochondria electron transport inhibitor), or both, hypoxic impairment of vasorelaxation was restored more effectively. Similarly, impairment of the nitroglycerin-induced vasorelaxation under hypoxia/reoxygenation was restored by combined treatment with three inhibitors, apocynin, oxypurinol, and rotenone. Increase in superoxide production under hypoxia tended to be inhibited by apocynin and that under hypoxia/reoxygenation was abolished by combined treatment with three inhibitors. These findings suggest that increased intracellular superoxide production under hypoxia or hypoxia/reoxygenation attenuates vasodilation mediated with a nitric oxide/soluble guanylyl cyclase, but not adenylyl cyclase, signaling pathway. The main source of superoxide production under hypoxia seems to be different from that under reoxygenation: superoxide is produced by NADPH oxidase during hypoxia, whereas it is produced by xanthine oxidase, mitochondria, or both during reoxygenation.

[Supplementary Figure: available only at <http://dx.doi.org/10.1254/jphs.11031FP>]**Keywords:** hypoxia, reoxygenation, coronary artery, nitric oxide, superoxide**Introduction**

The coronary artery plays an important role in supplying fresh nutrients and oxygen to the myocardium and in maintaining the cardiac function. The vascular tone is modulated by several substances released from vascular endothelium and autonomic nerve endings, which act, in

part, on smooth muscle (1, 2). Nitric oxide (NO), synthesized in and released from endothelial and neuronal cells, activates soluble guanylate cyclase (sGC) in smooth muscle cells to generate cGMP, which in turn promotes vasorelaxation (3). On the other hand, activation of G-protein–coupled receptors (GPCRs) such as β -adrenoceptor or prostacyclin (PGI₂) receptor also induces vasorelaxation by stimulating adenylyl cyclase (AC), which catalyzes the increased production of cAMP (3). These two pathways can act synergistically and also serve as backup systems for each other in the case of in-

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Published online in J-STAGE on May 19, 2011 (in advance)
doi: 10.1254/jphs.11031FP

jury to one side.

The performance of coronary vessels during hypoxia or hypoxia/reoxygenation is of great importance in clinical conditions like angina pectoris and atherosclerosis. It is well known that prolonged hypoxia impairs vascular function due to several factors (4). Although the best way to minimize the risk of injury is to supply oxygen, some investigators report reoxygenation leads to further injury (5). In this process of hypoxia/reoxygenation injury, both vascular endothelium and smooth muscle may be affected differently in degree. In fact, previous studies have shown that endothelium-dependent relaxation is attenuated in coronary arteries by hypoxia (6, 7) or hypoxia/reoxygenation (8, 9). On the other hand, the influence of these conditions on coronary smooth muscle function is controversial. Some investigators have demonstrated that NO donor-induced relaxation is unimpaired under the hypoxic (7) or hypoxic/reoxygenated condition (8). In the report by Mingone et al., however, relaxation caused by an NO donor was augmented by hypoxia (9). In addition, findings from the β -receptor-mediated vasorelaxation under hypoxia are also somewhat contradictory: one study showed that hypoxia impairs the relaxation induced by β -receptor agonists (10), while others have implicated that the hypoxic condition enhances the relaxation (11). Therefore, these discrepancies still remain to be clarified.

A crucial feature of the response to hypoxia or hypoxia/reoxygenation is to produce reactive oxygen species (ROS) in the vascular wall. Numerous studies suggest that ROS are central mediators of the adverse events that occur during hypoxia (12) or after hypoxia/reoxygenation (13). Superoxide, the most reactive ROS, is generated in several types of cells including vascular cells. Not only inflammatory cells but also smooth muscle cells in directional coronary atherectomy specimens have been reported to generate superoxide (14). In addition, the research group of Wolin et al. has demonstrated that superoxide production is increased by hypoxia (15) or by hypoxia/reoxygenation (16) in isolated endothelium-denuded bovine coronary arteries.

This study was undertaken to investigate whether hypoxia or hypoxia/reoxygenation affects smooth muscle function in primates by analyzing the drug-induced relaxation in isolated endothelium-denuded monkey coronary arteries and whether superoxide may contribute to any observed response.

Materials and Methods

Animals

Sixteen Japanese monkeys (*Macaca fuscata*) of either sex, weighing 6–12 kg, were used for the present study.

The Animal Care and Use Committee at Shiga University of Medical Science approved the use of monkey materials along with the experimental protocols in this study.

Preparation

Under deep general anesthesia with ketamine (15 mg/kg, i.m.) and sodium pentobarbital (30 mg/kg, i.v.), each monkey was sacrificed by bleeding from the common carotid arteries. The heart was rapidly removed, and coronary arteries (1.0–2.5-mm outside diameter) were isolated, the surrounding tissues cleaned off of them, and each cut helically into strips with special care being taken to preserve the endothelium. Endothelial denudation of the strips was accomplished 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 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 temperature was maintained at $37 \pm 0.3^\circ\text{C}$. 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 a pen recorder. The contractile response to 30 mM K^+ was first obtained, and the arterial strips were washed 3 times with control media and equilibrated. The strips were partially contracted with prostaglandin F_{2a} in a concentration range of 0.1–10 μM . Endothelial integrity was determined by relaxations induced by 10^{-6} M acetylcholine (ACh). Although it is known that relaxations caused by NO donors are potentiated by endothelium denudation in rodent arteries, the phenomenon has not been observed in monkey arteries (17). In fact, the potentiation of vasorelaxing action of nitroglycerin (NTG) did not occur in monkey coronary artery (Supplementary Fig. 1: available in the online version only). Consequently, the present study was performed with endothelium-denuded arteries to focus attention on the smooth muscle function. Concentration–response curves for NTG, isosorbide dinitrate (ISDN), isoproterenol (ISO), and 8-bromoguanosine-3',5'-cyclic monophosphate (8-Br-cGMP) were obtained by adding the drug directly to the bathing media in cumulative concentrations. At the

end of each experiment, 100 μ M papaverine was added to induce the maximal relaxation, which was taken as 100% for relaxations induced by the agonists. After the response to the agonist under the normal condition (normoxia) was obtained, preparations had been exposed for about 60 min to the bathing media with 95% N₂ and 5% CO₂ (hypoxia) or then returned to 95% O₂ and 5% CO₂ for about 30 min (reoxygenation), before the response to the vasodilator agent was obtained at each condition. The partial O₂ pressure in the solution exposed to normoxia, hypoxia, and reoxygenation were in a range of 431–524, 58–71, and 476–532 mmHg, respectively. The effects of superoxide dismutase (SOD) (200 U/mL), tempol (3 mM, membrane-permeable SOD mimetic), apocynin (100 μ M, NADPH oxidase inhibitor), oxypurinol (100 μ M, xanthine oxidase inhibitor), rotenone (10 μ M, mitochondrial electron transport inhibitor), and each combination on the response of the strips treated for 10–30 min was also evaluated. The concentration of each inhibitor was determined based on the previous works (18–20).

Assessment of ex vivo tissue superoxide production and NADPH oxidase activity

Superoxide production in coronary artery segments was measured using the lucigenin-enhanced chemiluminescence method as described previously (18). Segments of the endothelium-denuded coronary artery were placed in the modified Krebs/Hepes buffer (pH 7.4) and the chemiluminescence value was measured. The vessel was dried at 90°C for 24 h and then the dry weight was measured.

To measure the vascular NADPH oxidase activity, the endothelium-denuded coronary vessel was homogenized in a glass homogenizer after the addition of 500 μ L homogenizing buffer (pH 7.8), and the homogenate was subjected to centrifugation (3000 $\times$ g) for 10 min. A 20- μ L aliquot of the supernatant was then added to glass scintillation vials containing lucigenin (50 μ M) and NADPH (100 μ M) in 2 mL homogenizing buffer and the chemiluminescence value was obtained.

Drugs

The following drugs were used: ACh (Daiichi Co., Tokyo); NTG (Nihonkayaku Co., Tokyo); 8-Br-cGMP, ISDN, ISO, SOD, oxypurinol, and rotenone (Sigma Chemical Co., St. Louis, MO, USA); tempol (MP Biomedicals, Aurora, OH, USA); apocynin (Calbiochem, San Diego, CA, USA); PGF_{2 α} (Pharmacia-Upjohn, Tokyo); ketamine (Sankyo, Tokyo); sodium pentobarbital and papaverine hydrochloride (Dainippon-Sumitomo Pharma Co., Osaka). Sodium hydroxide (1 N) was used as a solvent for oxypurinol. Ethanol was used as the

solvent for rotenone and apocynin. PGF_{2 α} was dissolved in sodium bicarbonate buffer (pH 9.2). These solvents at the concentrations used in the present study had no significant effect 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 $\pm$ S.E.M. Statistical analyses were made by Student's unpaired *t*-test for two groups and by Tukey-Kramer multiple comparisons test after one-way analysis of variance for three or more groups. Differences were considered significant at *P* < 0.05.

Results

Influence of hypoxia or hypoxia/reoxygenation on the agonist-induced vasorelaxation

The addition of NTG at concentrations of 10⁻¹⁰–10⁻⁶ M produced a dose-dependent relaxation in coronary arteries without endothelium (Fig. 1A) in which ACh did not produce any relaxation (data not shown). The maximal response was significantly reduced under hypoxia (77.0 $\pm$ 2.3%) or under hypoxia/reoxygenation (79.2 $\pm$ 2.4%) as compared with that under normoxia (88.5 $\pm$ 1.8%). The dose-response curve was also shifted to the right under hypoxia or under hypoxia/reoxygenation (Fig. 1A). Similar results were also obtained for the relaxation induced by ISDN among the three conditions (Fig. 1B). The potencies of impairment for NTG and ISDN were not obviously different between under hypoxia and under hypoxia/reoxygenation. On the other hand, hypoxia or hypoxia/reoxygenation did not affect the relaxation induced by 8-Br-cGMP (Fig. 1C) or ISO (Fig. 1D).

Influence of hypoxia or hypoxia/reoxygenation on the NO donor-induced vasorelaxation in the presence of SOD or tempol

In coronary arteries without endothelium that were preincubated with SOD, the relaxant response to NTG was less potent under hypoxia or under hypoxia/reoxygenation compared with that under normoxia (Fig. 2A), as seen in the absence of SOD. On the other hand, in the presence of tempol, the magnitudes of relaxation by NTG did not significantly differ among these three conditions (Fig. 2B). Similar results were also obtained in the case of ISDN (Fig. 2: C and D).

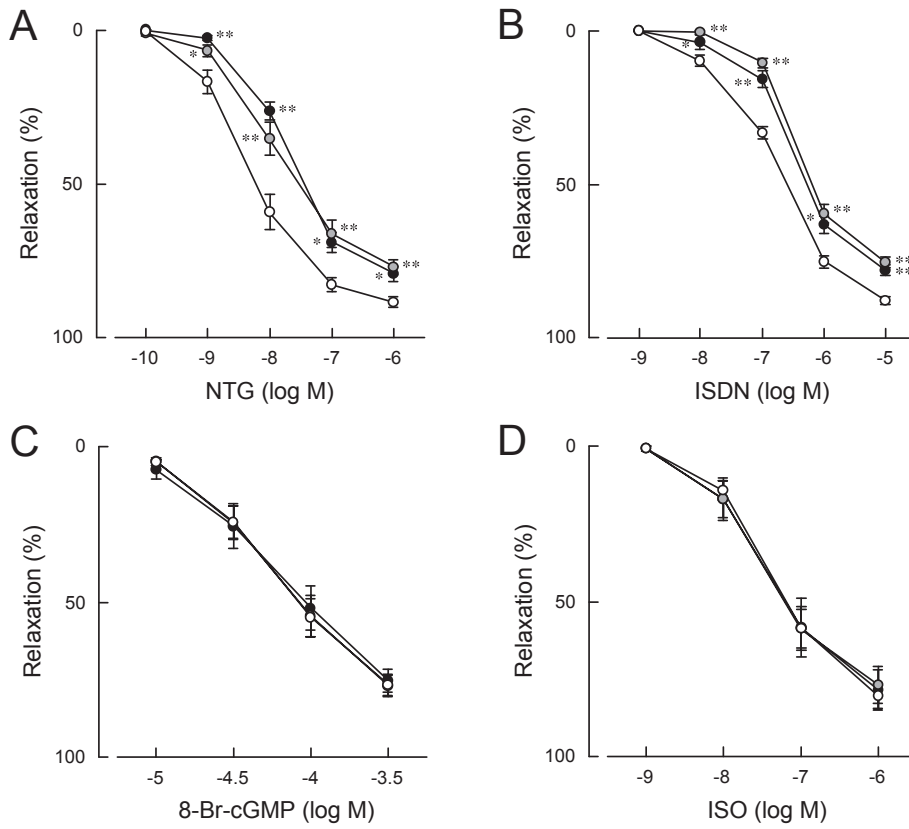


Fig. 1. NTG (A)-, ISDN (B)-, 8-Br-cGMP (C)-, and ISO (D)-induced relaxation of endothelium-denuded monkey coronary arteries under normoxia (white circle), hypoxia (gray circle), and hypoxia/reoxygenation (black circle). Each point and bar represents the mean $\pm$ S.E.M. of 5–7 experiments with arteries from different monkeys. * $P < 0.05$ and ** $P < 0.01$, compared with normoxia.

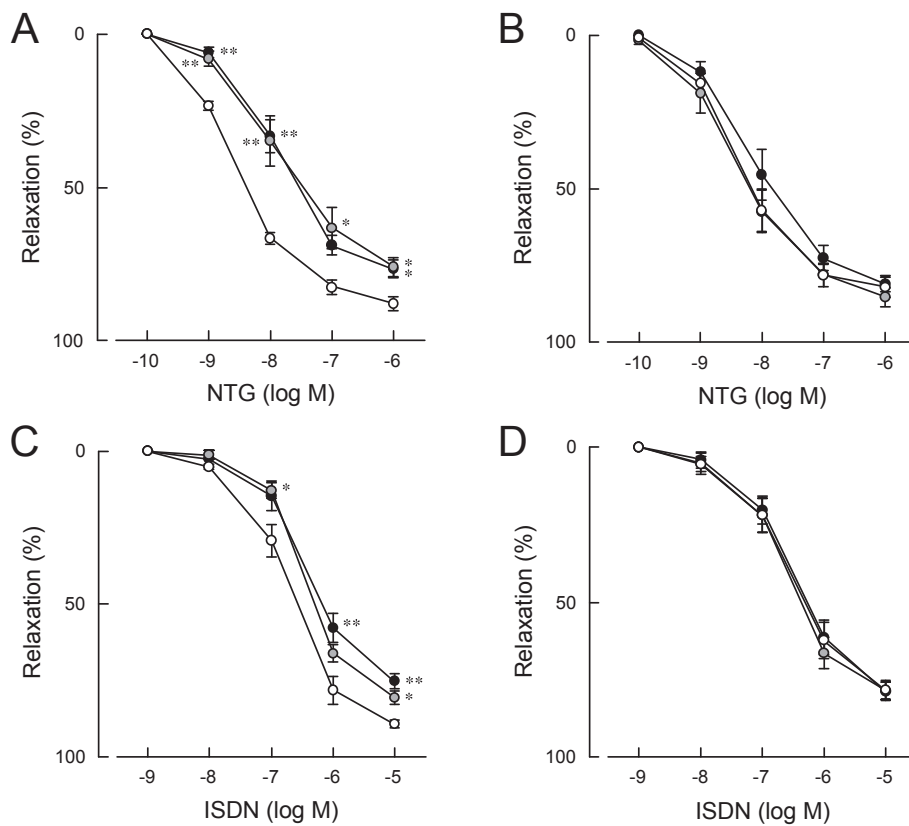


Fig. 2. NTG- and ISDN-induced relaxation of endothelium-denuded monkey coronary arteries in the presence of SOD (A and C) or tempol (B and D) under normoxia (white circle), hypoxia (gray circle), and hypoxia/reoxygenation (black circle). Each point and bar represents the mean $\pm$ S.E.M. of 5 experiments with arteries from different monkeys. * $P < 0.05$ and ** $P < 0.01$, compared with normoxia.

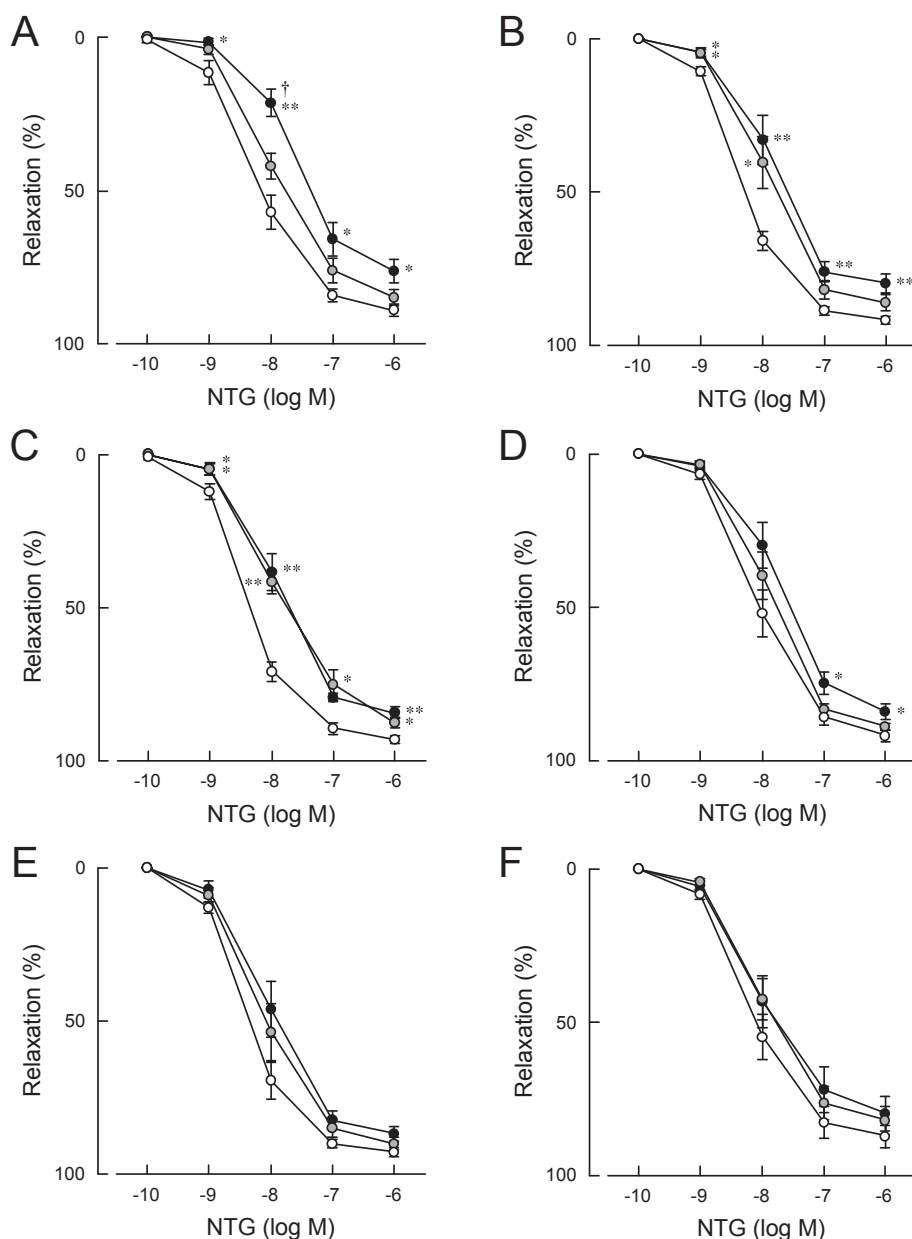


Fig. 3. NTG-induced relaxation of endothelium-denuded monkey coronary arteries in the presence of apocynin (A), rotenone (B), oxypurinol (C), apocynin plus rotenone (D), apocynin plus oxypurinol (E), or apocynin plus rotenone plus oxypurinol (F) under normoxia (white circle), hypoxia (gray circle), and hypoxia/reoxygenation (black circle). Each point and bar represents the mean $\pm$ S.E.M. of 6–7 experiments with arteries from different monkeys. * $P < 0.05$ and ** $P < 0.01$, compared with normoxia; † $P < 0.05$, compared with hypoxia.

Influence of hypoxia or hypoxia/reoxygenation on the NTG-induced vasorelaxation in the presence of various inhibitors

As shown in Fig. 3A, apocynin, an NADPH oxidase inhibitor, improved the impaired relaxation by NTG under hypoxia but not that under reoxygenation. On the other hand, the single-agent treatment of rotenone, a mitochondrial electron transport inhibitor, or oxypurinol, a xanthine oxidase inhibitor, had no obvious influence on it (Fig. 3: B and C). As compared with apocynin alone, more effective restorations against the impairment both under hypoxia and under hypoxia/reoxygenation were observed in a combined pretreatment of apocynin with rotenone or oxypurinol, respectively (Fig. 3: D and E). In

addition, a combined treatment of these three inhibitors more potently reversed the impairment (Fig. 3F).

Superoxide production in endothelium-denuded coronary arteries

As shown in Fig. 4, superoxide production under hypoxia was clearly but not significantly higher than that under normoxia. This increase in superoxide production under hypoxia was inhibited by apocynin pretreatment. On the other hand, reoxygenation further increased superoxide production as compared with that under hypoxia, and production of superoxide was significantly higher under hypoxia/reoxygenation than that under normoxia. This was completely suppressed by a com-

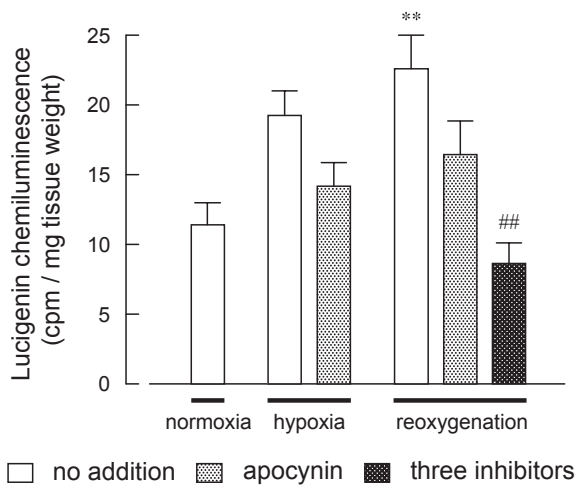


Fig. 4. Superoxide production in endothelium-denuded monkey coronary arteries. Each column and bar represents the mean $\pm$ S.E.M. of 4–5 experiments with arteries from different monkeys. ** $P < 0.01$, compared with normoxia. ## $P < 0.01$, compared with no addition.

bined pretreatment of apocynin with rotenone and oxypurinol, while treatment with apocynin alone produced a partial inhibition.

NADPH oxidase activity in endothelium-denuded coronary arteries

Superoxide production derived from NADPH oxidase ($n = 5$, per group) in coronary arteries was significantly higher under hypoxia (4104 ± 1015 cpm/mg protein) than that under normoxia (1435 ± 362 cpm/mg protein). Apocynin inhibited the NADPH oxidase activity and completely attenuated the superoxide production under hypoxia (1728 ± 395 cpm/mg protein).

Discussion

The major findings in the present study are as follows: 1) hypoxia or hypoxia/reoxygenation affects vasodilation mediated with the NO/sGC signaling pathway but not that with the AC signaling pathway in the isolated endothelium-denuded monkey coronary artery; 2) vascular intracellular superoxide is responsible for this impairment of vasodilation both under hypoxia and under hypoxia/reoxygenation; 3) NADPH oxidase is a key source of its superoxide production under hypoxia, whereas xanthine oxidase or mitochondrial electron transport or both is a key source under reoxygenation.

Several signaling pathways contribute to control of coronary vascular tone. Elevation of intracellular concentrations of second messengers such as cGMP and cAMP is a major target of a variety of vasoactive agonists, such as NO, PGs, and β -adrenergic agents. These

second messengers are produced in the smooth muscle cells by different stimuli through activation of GC or AC and induce relaxation of the smooth muscle cells by activating specific protein kinase PKG or PKA, respectively (3). In the present study using the isolated endothelium-denuded monkey coronary artery, relaxation induced by NO donors was attenuated by hypoxia or hypoxia/reoxygenation, while β -agonist-induced relaxation was not affected. Although many investigators have reported the influence of hypoxia or hypoxia/reoxygenation on the relaxation of coronary artery smooth muscle, the results are not always similar. For example, in contrast with our present data, other researchers have shown that vasorelaxation by NO donors is unimpaired (pig and rat coronary artery) (7, 8) or augmented (bovine coronary artery) (9) by hypoxia or hypoxia/reoxygenation. Previously, we reported that reactivity of coronary artery to vasoactive agents is different among animal species (21, 22). Consequently, we consider that one possible reason for this discrepancy may be due to animal species used in the study (non-primate vs. primate). Anyway, as far as we know, this is the first report that hypoxia or hypoxia/reoxygenation impairs vasorelaxation through the pathway mediated by intracellular cGMP but not by cAMP in monkey coronary arteries. In addition, attenuation of the NTG-induced relaxation by hypoxia or hypoxia/reoxygenation has also been seen in monkey renal artery (M. Tawa et al., unpublished observation), suggesting that the phenomenon may be unique in the primate arteries.

Superoxide is suggested to be involved in the pathogenesis of vascular disorders in several diseases. It is well known that hypoxia or hypoxia/reoxygenation promotes superoxide generation in several arterial smooth muscles. The research group of Wolin et al. has demonstrated that superoxide production is increased by hypoxia (15) or by hypoxia/reoxygenation (16) in isolated endothelium-denuded bovine coronary arteries. These findings are consistent with our current result that the tissue level of superoxide increases in hypoxic and further in reoxygenated endothelium-denuded monkey coronary arteries. In addition, hypoxia- or hypoxia/reoxygenation-induced impairment of vasodilation with NO donors was inhibited by treatment with tempol but not with SOD, suggesting that intracellular, but not extracellular, superoxide increased by hypoxia or hypoxia/reoxygenation plays an important role in the impairment of vasodilation.

Superoxide easily reacts with NO to form peroxynitrite (ONOO^-) and other nitrogen species, thereby causing an effective reduction in NO bioavailability (23). In the present study, vasorelaxation induced by the cGMP analog 8-Br-cGMP was unaffected by hypoxia or hypoxia/reoxygenation, indicating that the sites affected by these

conditions lay upstream of PKG. Thus, we hypothesize that the mechanism of impairment of NO donors–induced vasodilation under hypoxia or hypoxia/reoxygenation is due to excessively generated superoxide which scavenges NO. However, there are increasing evidences indicating that superoxide directly regulates the sGC activity or expression of sGC. The report by Wu and de Champlain has noted an inhibitory effect of superoxide on cGMP formation in vascular smooth muscle cells (24). Other investigators have also reported that exposure of smooth muscle cells to superoxide generating agents causes a decrease in sGC expression and attenuates cGMP formation induced by NO donors (25). In addition, NO-donors used in this study require enzymatic catalysis by mitochondrial aldehyde dehydrogenase 2 (ALDH2) or by P450 in the endoplasmic reticulum to release NO (26). In this regard, recent studies have implicated that ROS like superoxide and peroxynitrite modulate the activity of ALDH2 or P450 (27, 28). Therefore, we cannot rule out the possibility that superoxide has influence on the step of enzymatic catalysis. Further studies are required to demonstrate which steps are critical for the impairment of NO donors–induced vasodilation by superoxide generation under hypoxia or under hypoxia/reoxygenation in endothelium-denuded monkey coronary arteries.

Various potential sources of vascular superoxide generation such as NADPH oxidase, xanthine oxidase, cyclooxygenase, uncoupled NO synthase, and the mitochondrial electron transport chain have been identified (29). Among them, the main source of superoxide production in the vascular smooth muscle is thought to be NADPH oxidase or the mitochondrial electron transport chain (15, 30). However, it has been also shown that xanthine oxidase is localized in vascular smooth muscle cells (31) and plays an important role in hypoxia/reoxygenation injury (32). Although it is controversial whether or not NADPH oxidase and xanthine oxidase generates superoxide intracellularly, some reports have indicated these enzymes as possible sources of intracellular superoxide production (33–35). Therefore, we have taken particular note of these three sources and examined the individual contribution by using inhibitors of various superoxide-generating enzymes. As the result, the impairment of vasorelaxation by NTG was drastically reversed by the NADPH oxidase inhibitor apocynin. Moreover, hypoxia-induced increases in the tissue level of superoxide and NADPH oxidase activity are potently suppressed by apocynin. As the support of our results, Marshall et al. have shown that hypoxia increases superoxide production caused by NADPH oxidase in bovine arterial smooth muscle (36). Rathore et al. have also demonstrated that hypoxia significantly increases NADPH oxidase activity by translocation of the cytosolic

subunit to the plasma membrane in smooth muscle cells (37). Considering these findings, NADPH oxidase mainly contributes to hypoxia-induced superoxide generation and impairment of vasorelaxation with NO donors in the current study. However, apocynin alone did not completely reverse the impairment of NO donor–induced vasorelaxation by hypoxia. There is an investigation demonstrating that another source is also involved in the hypoxia-induced increase in superoxide generation in smooth muscle (15). In fact, more effective inhibition against hypoxic impairment of the vasorelaxation was observed by combining apocynin with the xanthine oxidase inhibitor oxypurinol, the mitochondria electron transport inhibitor rotenone, or both, while neither of these two inhibitors obviously influenced the impaired relaxation when administered alone. However, the possibility that the blockage of one or more sources could result in a compensatory activation of other sources can be easily predicted. Realistically, that is why it is hard to identify the specific source stimulated by hypoxia. Nonetheless, it is clear that NADPH oxidase is a key source but not the only source of hypoxia-induced superoxide generation in endothelium-denuded monkey coronary arteries.

In contrast to the case of hypoxia, apocynin did not recover the impairment of NTG-induced vasorelaxation under reoxygenation. Additionally, the significant increase in the superoxide production by hypoxia/reoxygenation was only partially suppressed by apocynin. These findings indicate that superoxide is also generated from sources other than NADPH oxidase when reoxygenation occurs. Although reoxygenation is widely recognized to increase the superoxide generation in several types of cells, little has been reported about its source in smooth muscle cells under this condition. In the present study, impairment of the NO donor–induced vasorelaxation under reoxygenation was reversed by combination of apocynin with oxypurinol or rotenone. Moreover, the significant increase in superoxide production was also dramatically inhibited by a combined treatment with three inhibitors. Therefore, it is clear that reoxygenation-induced superoxide generation through mitochondria or xanthine oxidase or both also contributes to the impairment of vasorelaxation with NO donors. However, as mentioned above, it is very difficult to specify the source responsible for the superoxide generation under reoxygenation.

Several isoforms of the catalytic subunit of NADPH oxidase have been described. This family of NADPH oxidase consists of seven members termed Nox/Duox that differ in their tissue expression profiles, modes of activation, and physiological functions (38). Among these seven members, four have been identified as central

sources of superoxide generation in the vascular smooth muscle cells: Nox1, Nox2, Nox4, and Nox5 (38). Additionally, activity or expression of these isoforms has been shown to increase within coronary artery smooth muscle cells under several conditions (39–42). On the other hand, recent study has shown that the isoform regulated by hypoxia is Nox4 in pulmonary artery smooth muscle (43). Unfortunately, however, we cannot suggest which isoform is related to the hypoxia-induced superoxide generation in monkey coronary artery smooth muscle at this time. Further study is required to clarify this point.

In conclusion, the present study suggests that increased intracellular superoxide generation evoked by hypoxia or hypoxia/reoxygenation affects vasodilation mediated by the NO/sGC signaling pathway but not by the AC signaling pathway in the primate. The source responsible for the superoxide generation under hypoxia seems to be different from that under reoxygenation: the key source under hypoxia is NADPH oxidase while that under reoxygenation is xanthine oxidase, mitochondria, or both.

Acknowledgment

This work was partly supported by a grant from the Smoking Research Foundation.

References

- Lüscher TF, Richard V, Tschudi M, Yang ZH, Boulanger C. Endothelial control of vascular tone in large and small coronary arteries. *J Am Coll Cardiol*. 1990;15:519–527.
- Feigl EO. Neural control of coronary blood flow. *J Vasc Res*. 1998;35:85–92.
- Lincoln TM, Cornwell TL. Towards an understanding of the mechanism of action of cyclic AMP and cyclic GMP in smooth muscle relaxation. *Blood Vessels*. 1991;28:129–137.
- Saikumar P, Dong Z, Weinberg JM, Venkatachalam MA. Mechanisms of cell death in hypoxia/reoxygenation injury. *Oncogene*. 1998;17:3341–3349.
- Hearse DJ, Bolli R. Reperfusion induced injury: manifestations, mechanisms, and clinical relevance. *Cardiovasc Res*. 1992;26:101–108.
- Vedernikov YP, Gräser T, Leisner H, Tiedt N. Effect of hypoxia on endothelium-dependent relaxation of porcine coronary arteries and veins. *Biomed Biochim Acta*. 1991;50:257–263.
- Hashimoto M, Close LA, Ishida Y, Paul RJ. Dependence of endothelium-mediated relaxation on oxygen and metabolism in porcine coronary arteries. *Am J Physiol*. 1993;265:H299–H306.
- Richard V, Kaeffer N, Tron C, Thuillez C. Ischemic preconditioning protects against coronary endothelial dysfunction induced by ischemia and reperfusion. *Circulation*. 1994;89:1254–1261.
- Mingone CJ, Gupte SA, Iesaki T, Wolin MS. Hypoxia enhances a cGMP-independent nitric oxide relaxing mechanism in pulmonary arteries. *Am J Physiol Lung Cell Mol Physiol*. 2003;285:L296–L304.
- Rubanyi G, Paul RJ. O₂-sensitivity of beta adrenergic responsiveness in isolated bovine and porcine coronary arteries. *J Pharmacol Exp Ther*. 1984;230:692–698.
- Fukuda S, Toriumi T, Xu H, Kinoshita H, Nishimaki H, Kokubun S, et al. Enhanced beta-receptor-mediated vasorelaxation in hypoxic porcine coronary artery. *Am J Physiol*. 1999;277:H1447–H1452.
- Guzy RD, Schumacker PT. Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. *Exp Physiol*. 2006;91:807–819.
- Li C, Jackson RM. Reactive species mechanisms of cellular hypoxia-reoxygenation injury. *Am J Physiol Cell Physiol*. 2002;282:C227–C241.
- Azumi H, Inoue N, Ohashi Y, Terashima M, Mori T, Fujita H, et al. Superoxide generation in directional coronary atherectomy specimens of patients with angina pectoris: important role of NAD(P)H oxidase. *Arterioscler Thromb Vasc Biol*. 2002;22:1838–1844.
- Gao Q, Wolin MS. Effects of hypoxia on relationships between cytosolic and mitochondrial NAD(P)H redox and superoxide generation in coronary arterial smooth muscle. *Am J Physiol Heart Circ Physiol*. 2008;295:H978–H989.
- Mohazzab-H KM, Kaminski PM, Fayngersh RP, Wolin MS. Oxygen-elicited responses in calf coronary arteries: role of H₂O₂ production via NADH-derived superoxide. *Am J Physiol*. 1996;270:H1044–H1053.
- Toda N, Kawakami M, Yamazaki M, Okamura T. Comparison of endothelium-dependent responses of monkey cerebral and temporal arteries. *Br J Pharmacol*. 1991;102:805–810.
- Shinozaki K, Ayajiki K, Nishio Y, Sugaya T, Kashiwagi A, Okamura T. Evidence for a causal role of the rennin-angiotensin system in vascular dysfunction associated with insulin resistance. *Hypertension*. 2004;43:255–262.
- Guzik TJ, Sadowski J, Guzik B, Jopek A, Kapelak B, Przybylowski P, et al. Coronary artery superoxide production and Nox isoform expression in human coronary artery disease. *Arterioscler Thromb Vasc Biol*. 2006;26:333–339.
- Ahmad M, Kelly MR, Zhao X, Kandhi S, Wolin MS. Roles for Nox4 in the contractile response of bovine pulmonary arteries to hypoxia. *Am J Physiol Heart Circ Physiol*. 2010;298:H1879–H1888.
- Toda N. Isolated human coronary arteries in response to vasoconstrictor substances. *Am J Physiol*. 1983;245:H937–H941.
- Fujioka H, Ayajiki K, Shinozaki K, Toda N, Okamura T. Mechanisms underlying endothelium-dependent, nitric oxide- and prostanoind-independent relaxation in monkey and dog coronary arteries. *Naunyn Schmiedeberg's Arch Pharmacol*. 2002;366:488–495.
- Gryglewski RJ, Palmer RM, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature*. 1986;320:454–456.
- Wu L, de Champlain J. Effects of superoxide on signaling pathways in smooth muscle cells from rats. *Hypertension*. 1999;34:1247–1253.
- Gerassimou C, Kotanidou A, Zhou Z, Simoes DC, Roussos C, Papapetropoulos A. Regulation of the expression of soluble guanylyl cyclase by reactive oxygen species. *Br J Pharmacol*. 2007;150:1084–1091.
- Münzel T, Daiber A, Mülsch A. Explaining the phenomenon of nitrate tolerance. *Circ Res*. 2005;97:618–628.
- Wenzel P, Hink U, Oelze M, Schuppan S, Schaeuble K, Schildknecht S, et al. Role of reduced lipoic acid in the redox

- regulation of mitochondrial aldehyde dehydrogenase (ALDH-2) activity. Implications for mitochondrial oxidative stress and nitrate tolerance. *J Biol Chem*. 2007;282:792–799.
- 28 Minamiyama Y, Takemura S, Hai S, Suehiro S, Okada S. Vitamin E deficiency accelerates nitrate tolerance via a decrease in cardiac P450 expression and increased oxidative stress. *Free Radic Biol Med*. 2006;40:808–816.
 - 29 Wolin MS, Gupte SA, Oeckler RA. Superoxide in the vascular system. *J Vasc Res*. 2002;39:191–207.
 - 30 Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, Griendling KK. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2004;24:677–683.
 - 31 Hellsten-Westling Y. Immunohistochemical localization of xanthine oxidase in human cardiac and skeletal muscle. *Histochemistry*. 1993;100:215–222.
 - 32 Saugstad OD. Role of xanthine oxidase and its inhibitor in hypoxia: reoxygenation injury. *Pediatrics*. 1996;98:103–107.
 - 33 Cai H, Griendling KK, Harrison DG. The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. *Trends Pharmacol Sci*. 2003;24:471–478.
 - 34 Paravicini TM, Touyz RM. Redox signaling in hypertension. *Cardiovasc Res*. 2006;71:247–258.
 - 35 Schiffrin EL. Oxidative stress, nitric oxide synthase, and superoxide dismutase. *Hypertension*. 2008;51:31.
 - 36 Marshall C, Marmar AJ, Verhoeven AJ, Marshall BE. Pulmonary artery NADPH-oxidase is activated in hypoxic pulmonary vasoconstriction. *Am J Respir Cell Mol Biol*. 1996;15:633–644.
 - 37 Rathore R, Zheng YM, Niu CF, Liu QH, Korde A, Ho YS, et al. Hypoxia activates NADPH oxidase to increase [ROS]_i and [Ca²⁺]_i through the mitochondrial ROS-PKC ϵ signaling axis in pulmonary artery smooth muscle cells. *Free Radical Biol Med*. 2008;45:1223–1231.
 - 38 Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev*. 2007;87:245–313.
 - 39 Ungvari Z, Csiszar A, Edwards JG, Kaminski PM, Wolin MS, Kaley G, et al. Increased superoxide production in coronary arteries in hyperhomocysteinemia: role of tumor necrosis factor- α , NAD(P)H oxidase, and inducible nitric oxide synthase. *Arterioscler Thromb Vasc Biol*. 2003;23:418–424.
 - 40 Gupte SA, Kaminski PM, George S, Kouznestova L, Olson SC, Mathew R, et al. Peroxide generation by p47phox-Src activation of Nox2 has a key role in protein kinase C-induced arterial smooth muscle contraction. *Am J Physiol Heart Circ Physiol*. 2009;296:H1048–H1058.
 - 41 Ryu J, Lee CW, Shin JA, Park CS, Kim JJ, Park SJ, et al. Fc γ RIIa mediates C-reactive protein-induced inflammatory responses of human vascular smooth muscle cells by activating NADPH oxidase 4. *Cardiovasc Res*. 2007;75:555–565.
 - 42 Guzik TJ, Chen W, Gongora MC, Guzik B, Lob HE, Mangalat D, et al. Calcium-dependent NOX5 nicotinamide adenine dinucleotide phosphate oxidase contributes to vascular oxidative stress in human coronary artery disease. *J Am Coll Cardiol*. 2008;52:1803–1809.
 - 43 Mittal M, Roth M, König P, Hofmann S, Dony E, Goyal P, et al. Hypoxia-dependent regulation of nonphagocytic NADPH oxidase subunit NOX4 in the pulmonary vasculature. *Circ Res*. 2007;101:258–267.