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Oxygen Concentration Regulates NO-dependent Relaxation of Aortic Smooth Muscles

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Nitric oxide (NO) functions as an endothelium-derived relaxation factor and regulates vascular resistance. Recent studies in this laboratory (*Arch. Biochem. Biophys.* **323**, 27–32, 1995) revealed that the lifetime of NO significantly increased at physiologically low levels of oxygen concentrations and, hence, this gaseous radical strongly inhibited mitochondrial electron transport for a fairly long duration at low oxygen concentrations. The present work describes the effect of oxygen concentration on NO-induced relaxation and guanylate cyclase (GC) activity of endothelium-denuded aorta of the rat. Both NO and 2,2′-(hydroxynitrosohydrazono)bis-ethanamine (NOC18), an NO donor, induced the relaxa-tion of endothelium-denuded helical segments of rat aorta which were contracted by norepinephrine. NO-dependent relaxation of arterial specimens was enhanced by lowering oxygen concentration in the medium with concomitant increase in their cGMP levels. Anoxia induced the relaxation of the aorta by some NO-enhanceable and methylene blue-insensitive mechanism. These results suggested that local concentrations of oxygen might play important roles in the regulation of NO-dependent GC activity and vascular tonus of resistance arteries.

Keywords: cGMP, guanylate cyclase, nitric oxide, hypoxia, aorta, smooth muscle

Abbreviations: Ach, acetylcholine; EDRF, endothelium-derived relaxing factor; GC, guanylate cyclase; MB, methylene blue; NE, norepinephrine; NO, nitric oxide; NOC18, 2,2′-(hydroxynitrosohydrazono)bis-ethanamine; NOS, nitric oxide synthase; $O_2^{\bullet-}$, superoxide; SOD, superoxide dismutase; TCA, trichloroacetic acid

INTRODUCTION

NO synthesized by NO synthase (NOS) functions as an endothelium-derived relaxing factor (EDRF), inhibits platelet aggregation,[1–3] protein kinase C and calpain,[4,5] and regulates intracellular calcium status.[6] NO generated in vascular endothelial cells readily reaches to smooth muscle cells and affects their functions including the activity of guanylate cyclase (GC) and potassium channels.[1,7–9]

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Hypoxia stimulates the release of EDRF from rabbit aorta and resistance arteries[10] and induces their relaxation.[11] NO synthesized by endothelial NOS in the perfused heart increased cGMP levels in coronary effluent during the early phase of hypoxia.[12] Furthermore, anoxia induced relaxation of arteries by some mechanism which was mediated by either prostaglandin, adenosine, EDRF or by opening ATP-sensitive potassium channels.[13,14] Exposure of aortic specimens to anoxia for 5 min decreased their relaxation response to the second exposure to anoxia.[15] These observations suggest that the extent of release of EDRF is affected by local oxygen concentrations and that endothelial cells function as a sensor for oxygen. Although NO activates calcium-dependent[16] and cGMP-dependent potassium channels,[8,9] the effect of oxygen concentration on NO-dependent vascular events remains to be elucidated.

Because NO rapidly reacts with molecular oxygen ($k = 6.6 \times 10^6\,M^{-2}\,s^{-1}$), the lifetime of NO might be longer at low oxygen than at its high concentration.[17,18] Therefore, changes in local oxygen concentration might affect biological functions of NO in and around resistance arteries. In fact, recent studies revealed that the lifetime of NO greatly depended on the concentration of environmental oxygen and that NO reversibly inhibited mitochondrial electron transport more strongly and for a longer duration at low oxygen than at its high concentration.[19] Biological functions of NO, such as modulation of mitochondrial functions,[19–21] inhibition of tumor cell respiration[22,23] and superoxide ($O_2^{\bullet-}$) generation by neutrophils,[24] and induction of apoptosis of HL-60 cells[25] were also enhanced by decreasing oxygen concentration.

The present work shows that NO-dependent activation of GC and relaxation of the aorta are also enhanced by lowering oxygen concentration in the medium. The results show for the first time that low oxygen concentration enhances NO-dependent generation of cGMP and vasodilation of the artery.

MATERIALS AND METHODS

Chemicals

NO gas (> 99.0%) was obtained from Sumitomo Seika Co. Ltd. (Osaka). NOC18 was obtained from Dojindo Co. Ltd. (Kumamoto). Norepinephrine (NE) and bovine hemoglobin were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and obtained from Nacalai Tesque Co. (Kyoto).

Preparation of NO Solution

NO solution was prepared at room temperature by bubbling the medium used for experiments with argon gas and then with NO gas for 10 and 30 min, respectively. NO concentrations were determined as described previously.[19] NO-containing medium was added to the reaction mixture using a gas-tight syringe.[19,21]

Preparation and Analysis of Endothelium-denuded Aortic Strips

Under ether anesthesia, the thoracic aorta was excised from a male Wistar rat (300 g). The aorta was cut helically in 2 mm wide and 10 mm long strips by the method of Muramatsu *et al.*[26] Endothelial cells were denuded by rubbing with a filter paper. The specimens were mounted by an isotonic transducer (NIHON KOHDEN JD-112S, Tokyo) under 0.5 g of resting tension in a chamber equipped with an oxygen electrode. Experiments were performed in HEPES-buffered solution containing 127 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 11.1 mM glucose and 20 mM HEPES (pH 7.4). Oxygen concentration in the medium was controlled by infusing argon or oxygen. Aortic strips in the buffer solution responded to acetylcholine (Ach) and NO similarly as in bicarbonate-buffered solution.[27] Contraction and relaxation of the specimens and oxygen concentration in the medium were continuously

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monitored during the experiments. Prior to each experiment, the specimens were preincubated in oxygenated medium at 37°C for 2 h. At different oxygen concentrations, the specimens were contracted by 10^{-7} M NE and relaxed by varying concentrations of either NO or NOC18 in the presence or absence of methylene blue (MB).

Assay for ATP Contents of Aortic Specimens

Endothelium-denuded aortic specimens were preincubated in oxygenated medium for 2 h at 37°C. After changing the medium, the specimens were incubated with 10^{-7} M NE for 15 min. When infusing gas was replaced by either oxygen or argon gas, oxygen concentration reached a plateau within 5 min. After incubation under varying concentrations of oxygen for 30 min, the specimens were frozen in liquid nitrogen and stored at −80°C. The frozen specimens were homogenized in 1 ml of ice-cold 0.9 M perchloric acid and centrifuged at 3000× *g* for 10 min at 2°C. The supernatant fractions were neutralized with 5 M K_2CO_3 and centrifuged at 3000× *g* for 10 min.[28] The supernatant fractions were assayed for ATP using ATP Bioluminescence Assay Kit CLS II (Boehringer Mannheim). After solubilization in 0.1 N NaOH, proteins in the precipitated fractions were assayed by the method of Lowry.[29]

Assay for Guanylate Cyclase Activity

Endothelium-denuded aortic specimens were incubated for 5 min under varying oxygen concentrations. After incubation with varying concentrations of NOC18 for 1 min, the specimens were quickly frozen in liquid nitrogen, homogenized in 1 ml of ice-cold 6% trichloroacetic acid (TCA). The homogenates were centrifuged at 2000× *g* for 20 min at 4°C. The supernatant fractions were washed 4 times with 5 vol of water-saturated diethyl ether to remove TCA. The aqueous fractions were dried under nitrogen stream at 60°C.[30] The amounts of cGMP in the dried extracts were determined using an enzyme immunoassay system BIOTRAK (Amersham LIFE SCIENCE).

RESULTS

Effect of Oxygen Concentration on NE-induced Contraction of Aortic Specimens

Unless otherwise stated, vascular resistance was measured in an oxygen-saturated mediums (600 μM) in the presence of 10^{-7} M NE. Although the resistance of the specimen remained unchanged by lowering oxygen concentration in the medium up to 50 μM, it decreased significantly at oxygen concentration lower than 40 μM (Figure 1). Although hypoxia may affect ATP-dependent cellular events, no appreciable decrease in cellular ATP level was found even after 30 min exposure to 10 μM (Figure 2). Thus, the following experiments were performed at oxygen concentrations higher than 40 μM.

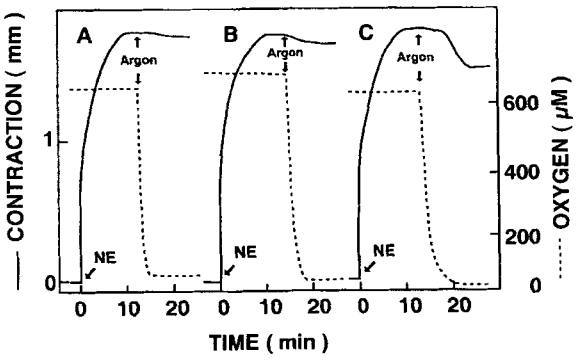

FIGURE 1 Effect of oxygen concentration on the resistance of endothelium-denuded aorta. Aortic specimens were incubated in HEPES-buffer at 37°C and their contraction and relaxation were monitored by an isotonic transducer. The specimens were contracted by adding 10^{-7} M NE. Vascular contraction (solid line) and oxygen concentration (broken line) were monitored continuously as described in the text. Arrows indicate the time for infusing argon gas. Oxygen concentration was decreased to 50 (A), 40 (B) and 20 μM (C), respectively.

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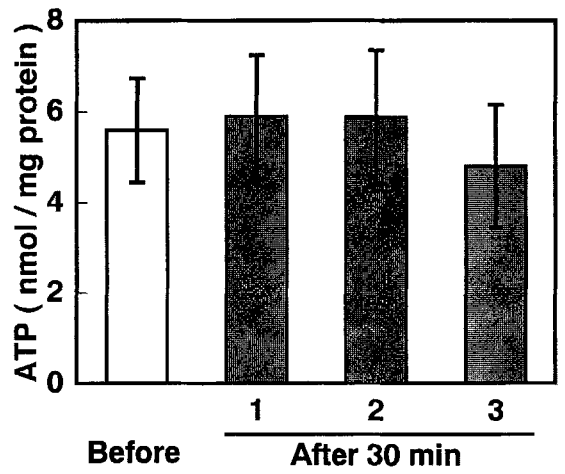

FIGURE 2 Effect of oxygen concentration on ATP contents in endothelium-denuded aorta. The specimens were incubated in an oxygenated medium containing 10^{-7} M NE for 15 min. They were incubated further at 500 (1), 40 (2) or 10 μM (3) oxygen for 30 min. Then, ATP content in the specimens was assayed as described in the Methods. Data are mean ± SD derived from 4–6 separate experiments.

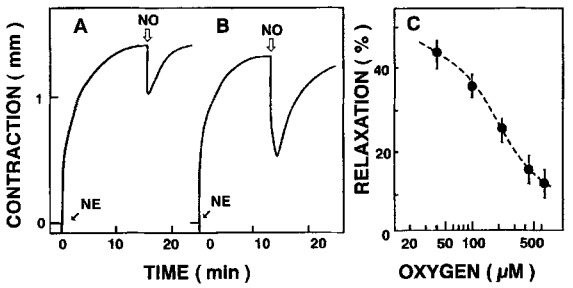

FIGURE 3 Effect of oxygen concentration on NO-induced relaxation. Experimental conditions were the same as described in Figure 1 except for oxygen concentration. NO concentration used was 1.3 nM. (A) 690 μM oxygen; (B) 44 μM oxygen; (C) NO-dependent relaxation under different oxygen concentrations. Data are mean ± SD derived from 5–6 separate experiments.

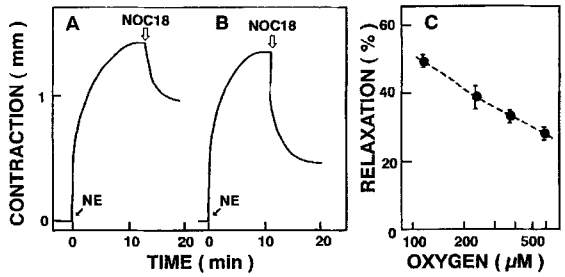

FIGURE 4 Effect of oxygen concentration on NOC18-induced relaxation. Experimental conditions were the same as described in Figure 1. NOC18 concentration used was 1 μM. (A) 600 μM oxygen; (B) 100 μM oxygen. (C) NOC18-dependent relaxation under different oxygen concentrations. Data are mean ± SD derived from 3–5 separate experiments.

Effect of NO and Oxygen Concentration on Aortic Relaxation

In order to study the effect of oxygen tension on NO-dependent relaxation, aortic specimens were contracted by 10^{-7} M NE. When exposed to NO, the specimens rapidly relaxed reversibly. The extent of relaxation depended on NO concentration. At oxygen concentration of 500 μM, only a weak relaxation was induced by a low dose of NO (Figure 3). The degree of NO-dependent relaxation increased by decreasing oxygen concentration in the medium. At 100 μM oxygen, the NO concentration required for the half-maximum relaxation was 10 nM.

Relaxation of the specimen was also observed with NOC18, an NO releasing reagent (Figure 4). Because NOC18 constantly releases NO (1.10 ± 0.04 nM/min/μM NOC18 at 37°C), relaxation continued for a fairly long time during the experiments. NO-dependent relaxation also depended on oxygen concentration. At oxygen concentration of 100 μM, NOC18 concentration required for the half-maximum relaxation was 1.4 μM.

Effect of Oxygen Concentration on NO-dependent cGMP Formation

After incubation of the specimens with NOC18 under 25–500 μM oxygen, arterial levels of cGMP were determined. The amount of cGMP in the specimen was increased by NOC18 in a concentration-dependent manner (Figure 5). The amount of *de novo* synthesized cGMP was also increased by lowering oxygen concentration.

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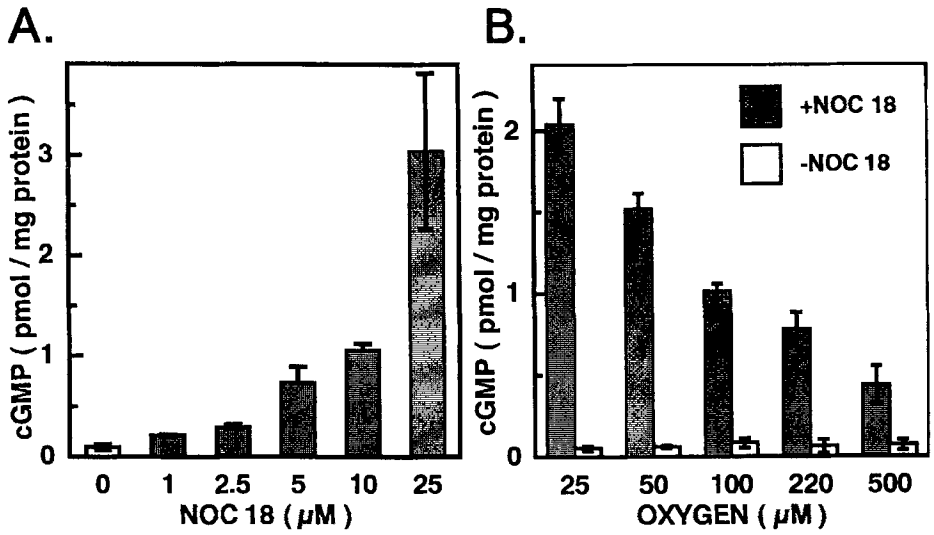

FIGURE 5 Effect of NOC18 on cGMP formation in endothelium-denuded aorta. (A) After incubation for 5 min at 37°C and 100 μM oxygen, varying concentration of NOC18 was added in the medium. After 1 min, arterial levels of cGMP were determined as described in the Methods. (B) Oxygen concentration-dependent generation of cGMP at 10 μM NOC18. Data are the mean ± SD derived from 3–5 separate experiments.

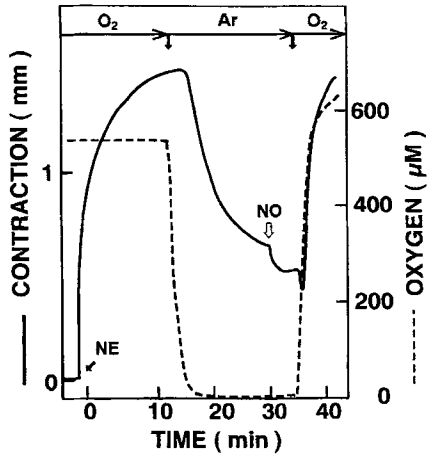

FIGURE 6 Effect of oxygen concentration on NO-induced relaxation in the presence of MB. Experimental conditions were the same as described in Figure 3 except that 20 μM MB was present in the mixture. At the arrows, oxygen concentration in the medium (dotted line) was decreased to 5 μM by infusing argon. The NO concentration used was 25 nM.

cGMP-independent Relaxation Induced by Anoxia

NO induced relaxation of the aorta by some MB-insensitive mechanism. This relaxation has been postulated to occur by a mechanism involving calcium-dependent potassium channels regulated by cGMP-dependent protein kinase.[16] To confirm this possibility, effect of anoxia on the relaxation of the aorta was examined. Anoxia strongly induced relaxation of the specimens (Figure 6). Under anoxic conditions, NO further induced the relaxation even in the presence of MB.

DISCUSSION

The present work shows that NO enhanced the activity of GC and induced relaxation of endothelium-denuded aortic samples by a mechanism which was enhanced by low oxygen concentration. This observation suggests that changes in local concentration of oxygen play important roles in NO-dependent regulation of arterial tonus.

Because NO reacts with molecular oxygen at a rate constant of $6.6 \times 10^6 M^{-2} s^{-1}$, the lifetime and biological actions of NO increased with concomitant decrease in oxygen concentration.[19–25] Thus, it is not surprising that NO-dependent

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relaxation of endothelium-denuded aorta was also augmented by lowering oxygen concentration in the medium. If the lifetime of NO was determined by oxygen concentrations, the following equation would be possible at the steady-state of relaxation by NOC18:

$$k_g[\mathrm{NOC18}] = k_1[\mathrm{NO}]^2[\mathrm{O_2}], \quad (1)$$

where k_g and k_1 are the rate constants for NO generation by NOC18 ($1.38 \times 10^{-5}\,s^{-1}$) and the reaction between NO and oxygen ($6.6 \times 10^6\,M^{-2}\,s^{-1}$), respectively. From Equation (1), NO concentration required for the half-maximum relaxation was calculated to be 21 μM at 100 and 1.4 μM of oxygen and NOC18, respectively. However, when added as NO solution, its concentration required for the induction of half-maximum relaxation was only 10 nM. The reason why NO and NOC18 showed such difference in the activity to induce vasodilatation is not clear at present. Because NOC18 is an organic cation in nature, it may behave differently in the presence and absence of biological specimens. This possibility should be studied further. In this context, Rubanyi and Vanhoutte[31] reported that superoxide dismutase (SOD) but not catalase augmented Ach-dependent relaxation of canine coronary artery by some hyperoxia inhibitable mechanism. Thus, generation of $O_2^{\bullet-}$ in the artery might also be one of the reason for the short lifetime of NO. In fact, $O_2^{\bullet-}$ production in the coronary artery was decreased by hypoxia as assayed by lucigenin chemiluminescence.[32]

If NO reacted predominantly with $O_2^{\bullet-}$, an equation at the steady-state of relaxation by NOC18 under 100 μM oxygen is expressed as follows:

$$k_g[\mathrm{NOC18}] = k_2[\mathrm{NO}][\mathrm{O_2^{\bullet-}}], \quad (2)$$

where k_2 is the rate constant for the reaction of NO and $O_2^{\bullet-}$ ($6.7 \times 10^9\,M^{-1}\,s^{-1}$).[33] If steady-state concentration of NO was 10 nM, the steady-state concentration of $O_2^{\bullet-}$ required for maintaining the half-maximal relaxation by NOC18 would be 0.38 pM. Thus, fairly small concentrations of $O_2^{\bullet-}$ would be sufficient for the inactivation of NO. Recent studies revealed that $O_2^{\bullet-}$ is generated in the homogenates of calf pulmonary arterial smooth muscles[34] and that regulation of $O_2^{\bullet-}$ production by local oxygen concentrations might be an important factor for determining vascular resistance.[35] These observations are consistent with our hypothesis that changes in local oxygen concentrations play important roles in NO-dependent regulation of arterial tonus.

The present work also shows that NO induced relaxation of arterial specimens even in anoxic and MB-containing medium. Under anoxic conditions, the duration of NO-dependent relaxation was prolonged in the presence of MB, suggesting the involvement of cGMP-independent process. It has been reported that MB inhibits NO-dependent activation of GC by generating $O_2^{\bullet-}$.[36] However, cGMP-dependent relaxation might be a major mechanism for NO-induced relaxation particularly under low oxygen concentrations. The mechanism for cGMP-independent relaxation of resistance arteries should be studied further.

Relaxation of the arterial specimens depended on oxygen concentration. Although cellular ATP levels generally decrease under hypoxic and anoxic conditions,[37] they remained unchanged under physiologically low oxygen concentrations (> 10 μM) in the glucose containing medium. Under the present experimental conditions (> 40 μM oxygen), tissue ATP levels remained unchanged during the experiments. Thus, the enhanced relaxation of arterial specimens by physiologically low oxygen concentration might not be due to decrease in ATP levels. At present, the anoxia-induced vasorelaxation is most likely due to the perturbation of cellular energy metabolism.

In conclusion, NO-induced relaxation of the endothelium-denuded aorta is regulated by oxygen tension. The extent of the relaxation and cGMP synthesis was greater at physiologically

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low than high oxygen tensions. The work suggests that local oxygen tension is a critical factor that reversibly regulates the NO-dependent relaxation of resistance arteries.

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