Biphasic modulation of vascular nitric oxide catabolism by oxygen

Xiaoping Liu, Crystal Cheng, Nicholas Zorko, Scott Cronin, Yeong-Renn Chen, and Jay L. Zweier

Davis Heart and Lung Research Institute and Division of Cardiovascular Medicine,
Department of Internal Medicine, The Ohio State University, Columbus, Ohio 43210

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Liu, Xiaoping, Crystal Cheng, Nicholas Zorko, Scott Cronin, Yeong-Renn Chen, and Jay L. Zweier. Biphasic modulation of vascular nitric oxide catabolism by oxygen. Am J Physiol Heart Circ Physiol 287: H2421–H2426, 2004. First published July 22, 2004; doi:10.1152/ajpheart.00487.2004.—Endothelium-derived nitric oxide (NO) plays an important role in the regulation of vascular tone. Lack of NO bioavailability can result in cardiovascular disease. NO bioavailability is determined by its rates of generation and catabolism; however, it is not known how the NO catabolism rate is regulated in the vascular wall under normoxic, hypoxic, and anaerobic conditions. To investigate NO catabolism under different oxygen concentrations, studies of NO and O2 consumption by the isolated rat aorta were performed using electrochemical sensors. Under normoxic conditions, the rate of NO consumption in solution was enhanced in the presence of mitochondrial respiration by NO, the inhibitory effects of NO on aortic O2 consumption increased as O2 concentration decreased. Under anaerobic conditions, however, a paradoxical reacceleration of NO consumption occurred. This increased anaerobic NO consumption was inhibited by the cytchrome c oxidase inhibitor NaNc but not by the free iron chelator deferoxamine, the flavoprotein inhibitor diphenylene iodonium (10 μM), or superoxide dismutase (200 U/ml). The effect of O2 on the NO consumption could be reproduced by purified cytchrome c oxidase (CcO), implying that CcO is involved in aortic NO catabolism. This reduced NO catabolism at low O2 tensions supports the maintenance of effective NO levels in the vascular wall, reducing the resistance of blood vessels. The increased anaerobic NO catabolism may be important for removing excess NO accumulation in ischemic tissues.

ENDOTHELIUM-DERIVED NITRIC OXIDE (NO) is a potent vasodilator (11, 12). To dilate blood vessels, NO needs to diffuse from the endothelial layer of blood vessels into smooth muscle cells to activate soluble guanylate cyclase (sGC). The physiological role of NO is largely dependent on the bioavailability of NO, which is determined by its generation rate and decay rate. To understand the physiological role of NO in blood vessels, it is very important to study the characteristics of NO catabolism in the vascular wall because the rate of NO catabolism can greatly affect NO bioavailability.

It is known that NO can react with O2 in the water, but the reaction rate is relatively slow at physiological concentrations of NO (22). However, the reaction of NO with O2 can be accelerated within biological membranes in the body (14, 17). Furthermore, NO and O2 can interact with each other through heme proteins. This interaction between NO and O2 has important physiological significance, which makes it possible to efficiently regulate the rate of O2 consumption by NO or regulate the rate of NO consumption by O2. For example, O2 can be rapidly reduced by cytochrome c oxidase (CcO) in mitochondria, whereas NO can bind to the binuclear center in CcO to efficiently inhibit the reduction of O2. On the other hand, NO can be consumed by CcO (15, 20), and the consumption can be regulated by O2 (17, 18). This interaction between NO and O2 should happen in the body. In fact, experimental evidences have shown that NO can regulate oxygen metabolism in tissues (23); however, there are still not sufficient direct experimental data to show how oxygen tension affects NO consumption in tissues. In this study, we directly measured the kinetics of NO consumption by rat aortas under different O2 concentrations using a Clark-type NO probe and a Clark-type O2 probe. We addressed the following critical question: How is the rate of NO catabolism regulated by O2 in the aortic wall?

METHODS

Preparation of NO solution. NO solution was prepared as described previously (13, 19). Briefly, NO gas was scrubbed of higher nitrogen oxides by passage first through a U-tube containing NaOH pellets and then through a 1 M deaerated (bubbled with 100% argon) KOH solution in a custom-designed apparatus using only glass or stainless steel (no plastic) tubing and fittings. The purified NO was collected by saturating a deaerated phosphate buffer solution (0.2 M potassium phosphate, pH 7.4) contained in a glass-sampling flask with a septum (Kimble/Kontes; Vineland, NJ).

Preparations of rat aorta. Male Sprague-Dawley rats (3 mo old, 300–350 g) were anesthetized with pentobarbital (100 mg/kg ip). The thoracic aorta (~4 cm in length) was rapidly dissected out and placed into ice-cold phosphate buffer solution (PBS-G, pH 7.4) of the following composition (in mM): 137 NaCl, 2.5 KCl, 0.9 CaCl2, 0.5 MgSO4, 1.5 KH2PO4, 0.8 Na2HPO4, and 5.6 glucose. The blood in the aorta was immediately washed out, and the loosely adhering fat and connective tissue were then removed. After the weight and length of the aorta were measured, the aorta was incubated at 37°C in a beaker containing 10 ml PBS-G for 30 min. This incubation process was repeated one more time with fresh PBS-G. The final aorta was transferred to the electrochemical chamber containing 2 ml PBS-G for measurements of NO and O2 consumption. The use of animals and the animal protocol were approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

Preparations of mitochondrial CcO. Highly purified heart mitochondrial CcO was prepared and assayed according to the methods reported by Yu et al. (25). Submitochondrial particles were prepared (21), used as starting material, and subjected to sequential ammonium sulfate fractionations in the presence of 1.5% sodium cholate. CcO, as prepared, is essentially in the delipidated form and spectrophotometrically free of complex III. It contains 10–12 nmol heme a/mg protein (ratio of 280-nm absorbance to 419-nm absorbance ~2.5–2.7) and

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7–8 µg phospholipid (PL)/mg protein. PL-replenished oxidase activity (0.4 mg asolectin/mg CcO) was confirmed by measuring the oxidation of ferrous cytochrome c and by O2 consumption in the presence of ascorbate and ferric cytochrome c (25). Rates of NO consumption and O2 consumption were measured in the electrochemical chamber containing 2 ml Dulbecco’s PBS (D-PBS; Invitrogen Life Technologies, catalog no. 14190-250).

Electrochemical measurements of NO and O2. The electrochemical system included a Clark NO electrode (ISO-NOP, WPI; Sarasota, FL), a Clark O2 electrode (ISO-OXY-2, WPI), a four-port water-jacketed electrochemical chamber (NOCHM-4, WPI), a Haake DC10-PS/U circulating bath, a magnetic stirrer, and either a CHI electrochemical detector (CH Instruments; Austin, TX) or an Apollo 4000 free radical analyzer (WPI). The NO electrode and O2 electrode were inserted into the water-jacketed chamber containing 2 ml of solution. The solution was constantly stirred with a magnetic bar controlled by the stirrer. The temperature of the chamber was held at 37°C by the circulating bath. To prevent O2 in the atmosphere from dissolving into the solution or prevent NO from volatilizing into the atmosphere, the chamber was sealed with a cap that was pushed down until the solution overflowed out of the holes in the cap. These holes were used for installing an additional electrode or adding samples.

Data analysis. In general, the half-life of NO can be used to determine the rate of NO decay. Half-life is usually represented as \( t_{1/2} \), meaning the time spent as the concentration decreases to half of its initial concentration. If we inject NO to the solution, the initial concentration is the NO concentration at the initial time of the injection. The initial time can be written as \( t_0 \), and the initial concentration can be written as \( c_0 \). If we add 2 µM NO to the solution, \( c_0 \) should be 2 µM. However, when we use a Clark NO electrode to measure NO concentration, the initial signal does not correspond to 2 µM NO, but a number close to 0. This is because the Clark NO electrode needs time to respond to the change in NO concentration. This delay time, which is needed for the electrode to respond to this change in NO concentration, is called the response time. The response time of the commercial Clark NO electrode is about 10 s. Thus the NO concentration curve collected by the Clark NO electrode reaches the peak concentration at around 10 s, not immediately after the authentic NO is added into the solution. This causes a problem in accurately measuring \( t_{1/2} \) in the case that the half-life of NO is close to or <10 s, because \( t_{1/2} \) should be measured from \( t_0 \) but the peak is not at \( t_0 \) for the Clark NO electrode. Because it is not accurate to measure \( t_{1/2} \) from the NO concentration curve obtained from a Clark NO electrode, we will use the half peak width, \( T_{1/2} \), to approximately represent \( t_{1/2} \). Theoretically, \( T_{1/2} \) would be very close to \( t_{1/2} \) if the response time of the electrode is very close to 0 or if the half-life of NO is much greater than the response time of the electrode. To determine whether NO consumption increases between two NO concentration curves, we can simply compare their \( T_{1/2} \). A smaller \( T_{1/2} \) or a greater \( 1/T_{1/2} \) means a faster rate of NO decay; vice versa, a greater \( T_{1/2} \) or a smaller \( 1/T_{1/2} \) means a slower rate of NO consumption. Using \( T_{1/2} \), we can qualitatively or semiquantitatively determine how the rate of NO consumption is affected by different factors. This is usually sufficient for most experimental purposes except for accurate measurements of kinetic constants. In this study, relative decay rates of two NO concentration curves (r) were calculated from the following equation assuming that NO decay follows first-order kinetics:

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r = \frac{1}{T_{1/2}^{i}} - \frac{1}{T_{1/2}^{h}} = T_{1/2}^{i}/T_{1/2}^{h} \tag{1}
\]

where \( T_{1/2}^{i} \) and \( T_{1/2}^{h} \) are the half peak widths of curve 1 (inhibited or enhanced) and curve 2 (control), respectively.

All experimentally measured parameters or values are given as means ± SE. The number of samples in each experimental group is indicated in the related text.
mitochondria play an important role in the aortic O₂ consumption and NO consumption. It was surprising that the rate of NO consumption did not continuously decrease but increased under anaerobic conditions after oxygen tension dropped below 1 Torr (1.03 ± 0.13 Torr, n = 12). On the basis of this result, we further tested whether the increased anaerobic NO consumption was activated by the anaerobic conditions alone or if it still required other stimulation. In the experiments, the cap was lifted, so that a headspace existed between the surface of the solution and the bottom of the cap. O₂ in the solution was removed by pure argon gas, and the aorta was kept under anaerobic conditions for about 20, 70, and 140 min, respectively. The cap was then reinserted into the solution, and NO was injected under anaerobic conditions. It was interesting to note that the first NO injection always produced NO decay at a slower rate (peak a in A–C), but the NO decay rate increased by four to nine times (calculated from $T_{1/2}$) in subsequent injections of NO (peak b in A–C).

The above experimental results imply that CcO may be involved in aortic NO consumption under anaerobic conditions. Therefore, experiments with isolated CcO were performed in a closed chamber to test whether CcO would mimic the behavior of the aorta in metabolizing NO. In these experiments, 2 mM CcO was mixed with 200 mM ascorbate to make effectively reduced CcO in solution. To make sure that NO consumption by the CcO-ascorbate system was mainly caused by the reduced CcO rather than ascorbate, additional experiments for NO consumption by 200 mM ascorbate alone were performed.

Fig. 2. Prechallenge with NO increased anaerobic NO consumption by the aorta. After the aorta was subjected to anaerobic conditions for about 20 min (A), 70 min (B), and 140 min (C), 2 µM NO was injected into the solution. The rate of NO decay with the first NO addition in each experiment was relatively slow (peak a in A–C), but the NO decay rate increased by four to nine times (calculated from $T_{1/2}$) in subsequent injections of NO (peak b in A–C).

Fig. 3. Anaerobic NO consumption of the aorta was not saturated by treatment with a large amount of NO. After the aorta was treated with 2 µM NO under anaerobic conditions (peak a), the aortic NO consumption increased (peak b). With repeated injections of 2 or 10 µM NO into the solution, for a total NO concentration of more than 110 µM, the increased anaerobic NO consumption was not reduced but slightly enhanced.

Experimental results show that they have no inhibition on the anaerobic NO consumption (data not shown). Because there are no O₂ and ROS available to oxidize NO under anaerobic conditions, the rate of NO oxidation should decrease, but we observed that the rate of NO consumption increased. Thus it is likely that the enhanced anaerobic NO consumption is caused by either of NO reduction or NO binding. Therefore, we examined whether the anaerobic NO consumption by the aorta is caused by NO binding or NO reduction. In the experiment, a large amount of NO was added into the solution to test whether the anaerobic NO consumption could be saturated by NO. If the anaerobic NO consumption resulted from the binding of NO to some active sites (metal centers) in vascular proteins, the rate of NO consumption would be very slow after all these sites were saturated with NO. Vice versa, if the anaerobic NO consumption was caused by NO reduction, NO would be continuously consumed. It was observed (n = 5) that the rate of anaerobic NO consumption was not reduced at all, but slightly enhanced (Fig. 3), after more than 110 µM NO was added into the solution. Further experiments show that the anaerobic NO consumption was not inhibited by the ferric iron chelator deferoxamine (DFO; 250 µM, n = 5) but inhibited by the mitochondrial protein CcO inhibitor NaCN (1 mM). In an identical control experiment, it was seen that in the absence of NaCN the consumption rate actually continues to slightly increase (Fig. 4).
performed. It was observed that 200 μM ascorbate slightly increased NO consumption, which was only about 6% (5.7% ± 0.8%, n = 5) of the rate of NO consumption by the 2 μM CeO + 200 μM ascorbate reaction system. Therefore, we concluded that NO consumption by the CeO-ascorbate system was primarily caused by the reduced CeO. As shown in Fig. 5A, the behavior of reduced CeO in consuming NO was very similar to that of the aorta. After CeO-ascorbate was added into the solution (designated by the arrow), O₂ in the solution was consumed by the CeO-ascorbate system, and the rate of NO consumption increased by nearly three times (3.1 ± 0.17, n = 5; Fig. 5B). The rate decreased as O₂ concentration decreased. After NO consumption reached its minimum rate at these low O₂ concentrations, the rate of NO consumption by CeO increased under anaerobic conditions. Like anaerobic NO consumption by aortas, the rate of anaerobic NO consumption increased about 50% in the

Fig. 6A, and the relative rate of NO consumption after each NO injection is shown in Fig. 6B. Thus CeO closely mimicked the characteristic biphasic O₂-dependent process of NO catabolism observed in the aorta as well as its inhibition by NaCN.

**DISCUSSION**

After the aorta was loaded into the chamber, it consumed O₂ at a constant rate (Fig. 1A). By injecting NO into the solution containing the aorta, we could simultaneously observe both the NO inhibition of aortic O₂ consumption and the effect of O₂ concentrations on aortic NO consumption. Our results show that NO can inhibit the aortic O₂ consumption, which is consistent with previous results from mitochondria (5, 9, 16, 17), cells (1, 4, 10), and tissues (3, 24). In the meantime, the rate of aortic NO consumption also depends on O₂ concentration. It has been reported that the rate of NO consumption increased in the presence of mitochondria and the rate of mitochondrial NO consumption decreases as O₂ concentration decreases (17, 18). Aortic NO consumption follows this pattern. The rate of NO consumption increased about 50% in the
presence of the aorta compared with that in the absence of the aorta (Fig. 1, A and B) under normoxic conditions. At low O₂ concentrations (<50 μM), NO markedly inhibited aortic O₂ consumption, and the rate of aortic NO consumption also markedly decreased with O₂ concentration. Thus mitochondria are likely to be involved in the interaction between NO and O₂ in the aorta. Two pathways for NO consumption by mitochondria have been suggested recently: one is to directly react with O₂ within membrane (17), and the other is to directly react with CcO (15, 20). Because removal of endothelium from aortas did not appreciably change NO decay rates, this implies that intact endothelium is not required for the NO consumption by the aorta, although it may contribute to NO consumption.

It is surprising that the rate of aortic NO consumption did not continuously decrease as O₂ concentration dropped to 0; instead, it increased after O₂ tension dropped below 1 Torr. However, low O₂ tension is only a necessary condition for the acceleration of anaerobic NO consumption. To induce the acceleration, a preinjection of NO was required under anaerobic conditions (Fig. 2). Because O₂ does not exist under anaerobic conditions, it is unlikely that the anaerobic NO consumption was caused by the direct reaction between NO and O₂. In fact, even if there was remaining O₂ in the aorta, the NO consumption by the direct reaction with O₂ can be ignored because the reaction rate of NO with O₂ is very low, because O₂ concentration is nearly 0. However, the reactions between NO and O₂⁻ or other ROS are rapid, so a small amount of remaining O₂ may have large effects on NO consumption. To test whether anaerobic consumption was caused by O₂⁻ and other ROS, we used SOD, a SOD mimic, and DPI in experiments. It was observed that SOD, a SOD mimic, and DPI have no inhibition on the anaerobic NO consumption. This result confirmed that O₂ and O₂-derived oxidants are not involved in anaerobic NO consumption.

It has been reported that NO can be reduced into N₂O by reduced CcO under anaerobic conditions (2, 6, 8, 26). To test whether CcO is involved in anaerobic NO consumption, the CcO inhibitor NaCN and the free iron chelator DFO were used in our experiments. The results show that anaerobic NO consumption by the aorta can be inhibited by NaCN but not by DFO, suggesting that CcO is involved in aortic NO consumption under anaerobic conditions. In the saturation experiments for testing whether anaerobic NO consumption is caused by NO binding or NO reduction, we added a large amount of NO (~110 μM) to the solution under anaerobic conditions (Fig. 3). It was observed that the rate of anaerobic NO consumption did not decrease but slightly increased after treatment of a high amount of NO. Considering that the volume of the aorta segment was about 28 times smaller than the volume of the solution in the chamber, the aorta must contain more than 3 mM (28 × 110 μM) metal centers for binding all the added NO if all the added NO are binding on the metal centers of vascular proteins in the aortic wall. This required amount of proteins is much more than the total amount of all proteins in the aortic wall. Therefore, these experimental results suggest that anaerobic NO consumption is not caused by simple binding but rather by continuous reductions.

To further confirm the role of CcO in the aortic NO catabolism, we directly measured the effect of O₂ concentration on the rate of NO consumption by CcO. It was observed that CcO has very similar properties to the aorta in its consumption of NO under different O₂ concentrations (see Figs. 1 and 5) and with regard to its inhibition of NO consumption by NaCN (see Figs. 4 and 6). This result further indicates that CcO is involved in aortic NO consumption. The overall reaction for the reduction of NO by reduced CcO has been suggested as the following equation (7, 26):

$$2\text{NO} + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$$  (2)

Although NaCN has been generally used as an inhibitor of CcO, NaCN can also block other heme proteins. Therefore, a possible involvement of other heme proteins cannot be excluded. Moreover, there may have other mechanisms for explaining anaerobic NO consumption. For example, if we assume that NO dismutase activity can be induced from CcO under anaerobic conditions, the acceleration of anaerobic NO consumption can be also explained. However, this concept needs more work for verification. The detailed pathway for anaerobic NO consumption and the detailed mechanism about
how the mitochondrial respiratory chain affects anaerobic NO consumption remain to be further explored.

In summary, the rate of NO catabolism in the aortic wall markedly decreases as O₂ concentrations drop below 50 µM but increases under anaerobic conditions after treatment with NO. Mitochondria play an important role in the regulation of NO catabolism in the aortic wall not only in the presence of O₂ but also under anaerobic conditions. Reduced NO catabolism at low O₂ tensions supports the maintenance of effective NO levels in the vascular wall reducing the resistance of blood vessels, whereas increased anaerobic NO catabolism is an interesting phenomenon that may play an important role in the removal of accumulated NO in ischemic tissues.

GRANTS

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REFERENCES


