Characterization of the Effects of Oxygen on Xanthine Oxidase-mediated Nitric Oxide Formation*

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Under anaerobic conditions, xanthine oxidase (XO)-catalyzed nitrite reduction can be an important source of nitric oxide (NO). However, questions remain regarding whether significant XO-mediated NO generation also occurs under aerobic conditions. Therefore, electron paramagnetic resonance, chemiluminescence NO-analyzer, and NO-electrode studies were performed to characterize the kinetics and magnitude of XO-mediated nitrite reduction as a function of oxygen tension. With substrates xanthine or 2,3-dihydroxybenz-aldehyde that provide electrons to XO at the molybdenum site, the rate of NO production followed Michaelis-Menten kinetics, and oxygen functioned as a competitive inhibitor of nitrite reduction. However, with flavin-adenine dinucleotide site-binding substrate NADH as electron donor, aerobic NO production was maintained at more than 70% of anaerobic levels, and binding of NADH to the flavin-adenine dinucleotide site seemed to prevent oxygen binding. Therefore, under aerobic conditions, NADH would be the main electron donor for XO-catalyzed NO production in tissues. Studies of the pH dependence of NO formation indicated that lower pH values decrease oxygen reduction but greatly increase nitrite reduction, facilitating NO generation. Isotope tracer studies demonstrated that XO-mediated NO formation occurs in normoxic and hypoxic heart tissue. Thus, XO-mediated NO generation occurs under aerobic conditions and is regulated by oxygen tension, pH, nitrite, and reducing substrate concentrations.

Nitric oxide (NO) is a free radical endogenously produced in biological tissues and is an important regulator of numerous biological functions (1–4). NO can also cause cellular injury by means of reaction with superoxide to form the potent oxidant peroxynitrite (5–7). Specific nitric oxide synthase (NOS) enzymes have been generally considered to be the primary source of NO in biological systems. These enzymes metabolize arginine to citrulline with the formation of NO, and oxygen is required for this process (8, 9). Under severe hypoxic conditions, such as those that occur in ischemic tissues, the NO production from NOS is impaired because of the lack of oxygen. In ischemic tissues, other factors including marked acidosis also impair the function of NOS. More recently, it has been demonstrated that there is another enzymatic pathway of NO generation that does not require oxygen. It has been shown that under anaerobic conditions, XO-mediated nitrite or nitrate reduction can be a prominent source of NO (10–14). This pathway is also enhanced under the acidic conditions that occur during ischemia. Such XO-mediated NO generation can serve as an alternative source of NO under the near anoxic conditions that occur with no-flow ischemia (13, 14).

Xanthine oxidase (XO) is a flavoprotein enzyme that is widely distributed in various mammalian tissues. In addition to its FAD binding site, it also has molybdenum and iron sulfur centers. XO plays important roles in both physiological and pathological conditions. It is well known as an important source of superoxide and reactive oxygen species generation (15). More recently, XO-mediated nitrite reduction to NO has been reported under anaerobic conditions. The magnitude and kinetics of this process have been characterized, and it has been shown that under conditions occurring during no-flow ischemia, myocardial XO and nitrite levels are sufficient to generate NO at levels comparable with or exceeding those from maximally activated nitric oxide synthases (13, 14).

XO has different site-specific electron donors. Xanthine and 2,3-dihydroxybenz-aldehyde (DBA) serve as electron donors to XO by binding to the molybdenum site, the site at which XO-mediated nitrite reduction occurs (13). However, NADH reduces XO at the FAD site of the enzyme, which is the site that also reduces oxygen. Previous studies on XO-mediated nitrite reduction under anaerobic conditions showed that xanthine and DBA are more effective electron donors than NADH (13). Under aerobic conditions, it is unclear whether significant XO-mediated NO generation would occur because oxygen would be expected to effectively compete with nitrite for reduction by the enzyme. In addition, under aerobic conditions, substrate preference and control could be very different from that in the absence of oxygen.

Thus, a number of questions remain regarding the magnitude and mechanism of XO-mediated NO generation in the presence of oxygen and the significance of this process in aerobic biological systems. To investigate the mechanism and effect of oxygen on XO-mediated nitrite reduction, electron paramagnetic resonance (EPR) spectroscopy, chemiluminescence NO analyzer, and NO electrode studies were performed to characterize the kinetics and magnitude of XO-mediated nitrite reduction as a function of oxygen tension. We observe that XO-mediated NO production does occur under aerobic condi-
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MATERIALS AND METHODS

Materials—Xanthine oxidase from buttermilk (xanthine, oxygen oxidoreductase; EC1.1.3.22), superoxide dismutase (superoxide, superoxide oxidoreductase; EC1.15.1.1), xantherine, oxyxpurinol, diphenyleneiodonium chloride (DPI), sodium nitrite, β-nicotinamide adenine dinucleotide (β-NADH), and 2,3-dihydroxybenz-aldehyde (DHA) were obtained from Sigma. N-methyl-n-glucamine diethiocarbamate (MGD) was synthesized using carbon disulfide and N-methyl-N-glucamine. Ferrous ammonium sulfate was purchased from Aldrich Chemical Co (99.997%). Dulbecco's phosphate buffered saline (PBS) was obtained from Invitrogen.

EPR Spectroscopy—EPR measurements were performed using a Bruker-IBM ER 300 spectrometer operating at X-band. Measurements were performed using a TM110 microwave cavity at ambient temperature with a modulation frequency of 100 kHz, modulation amplitude of 2.5 G, and microwave power of 20 mW. Samples for EPR spin-trapping measurements of NO were prepared as follows. The reaction solution for nitrite reduction was placed in a purging vessel from the reaction mixture in the purging vessel from the spin trap and thus NO generation was determined from the integration of solutions containing known concentrations of NO into vessel I with purging to vessel II, which contained the same amount and concentration of Fe-MGD spin trap (using the same gas mixture as the experimental sample).

Electrochemical Measurements—Aerobic Nitric Oxide Formation from Xanthine Oxidase

RESULTS

NO-mediated NO Generation from Nitrite under Aerobic Conditions—NO is paramagnetic and binds with high affinity to the water-soluble spin-trap Fe²⁺-MGD, forming a mononitrosyl iron complex with characteristic triplet spectrum at g = 2.04, with hyperfine splitting A₉ = 12.8 gauss. From the intensity of the observed spectrum, measurement of NO generation can be performed (19-21). Under aerobic conditions, XO can catalyze the reduction of oxygen, leading to the formation of superoxide anion, O₂⁻, which can react with nitric oxide forming peroxynitrite. To prevent the interference of NO formation and trapping was performed by double integration of the observed EPR signal with comparison to that from a similar aqueous NO-Fe-MGD standard. Quantitation of NO formation and trapping was performed by double integration of the observed EPR signal with comparison to that from a similar aqueous NO-Fe-MGD standard. 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superoxide with the measurement of NO production, SOD was added to quench the \( \cdot O_2^- \) in the solution. NO generated by XO-catalyzed nitrite reduction was purged out using compressed air from purging vessel I to purging vessel II (which contained the spin-trap \( \cdot Fe^{2+}-(MGD) \)). Mixtures of SOD (400 units/ml), XO (0.02 mg/ml), and its reducing substrate xanthine (0.1 mM) also gave rise to no signal (Fig. 2A). In the absence of SOD, preparations of XO (0.02 mg/ml) with nitrite (1 mM) and xanthine (0.1 mM), or NADH (1 mM), or DBA (0.5 mM), a large NO signal was seen (Fig. 2, C, D, and E). Thus, typical XO-reducing substrates including NADH, 2,3-dihydroxybenzaldehyde, and xanthine acted as electron donors for XO-catalyzed nitrite reduction and triggered NO generation under aerobic conditions. As reported previously, under anaerobic conditions xanthine is the most effective reducing substrate, whereas NADH is a less effective electron donor for XO-mediated nitrite reduction to NO (13). However, under aerobic conditions, NADH stimulated XO-mediated nitrite reduction occurs with a higher magnitude of NO generation than that stimulated by xanthine or DBA (Fig. 2).

Electrochemical NO measurements were performed to further confirm the occurrence of NO generation from XO-mediated nitrite reduction under aerobic conditions. Prior to the addition of XO, no detectable NO generation was seen from nitrite (1 mM) in the presence of SOD (400 units/ml) with xanthine (0.02 mM) or NADH (1 mM) as reducing substrates. However, after addition of XO (0.02 mg/ml), NO generation was triggered from xanthine or NADH (Fig. 3, A and B) in the presence of SOD. Under aerobic conditions, the magnitude and rate of NO generation in the presence of NADH (Fig. 3A) was considerably higher than that with xanthine (Fig. 3B). In contrast, under anaerobic conditions, the magnitude of NO generation in the presence of similar concentrations of xanthine was much higher than that with NADH (13). Thus, both EPR spin-trapping and NO electrode studies demonstrated that NADH is the most effective electron donor for XO-mediated NO generation under aerobic conditions.

To quantitate the rate of XO-mediated NO generation, further studies were performed with a chemiluminescence NO
analyzer. NO was purged out from the solution by compressed air. It was observed that the NADH-stimulated rate of NO generation is higher than the rate of NO generation stimulated by xanthine or DBA under aerobic conditions (Fig. 4).

Kinetics of XO-catalyzed Nitric Oxide Generation in the Presence of Site-specific Reducing Substrates—All of the typical reducing substrates of XO can stimulate NO formation from nitrite under aerobic conditions as well as under anaerobic conditions; however, NADH, which is the least effective reducing substrate under anaerobic conditions, was the most potent reducing substrate in the presence of oxygen. To quantitatively describe the effect and further investigate the mechanism of nitrite reduction, chemiluminescence measurements of the rate of NO production were performed using different site-specific reducing substrates under different oxygen tensions. NO formation from XO-catalyzed nitrite reduction was measured with continuous purging with air, 10, 5, or 2% oxygen, corresponding to oxygen concentrations in solution at 37 °C of 214, 102, 51, and 20 μM, respectively. Both xanthine and DBA provide electrons to XO at the molybdenum site of the enzyme. For xanthine or DBA as reducing substrates, the rate of XO-mediated NO formation was determined as a function of nitrite concentration, and Michaelis-Menten kinetics were observed. Lineweaver-Burk plots characterized the shift of $K_m$ value by oxygen and demonstrated that oxygen is a competitive inhibitor to nitrite in XO-mediated nitrite reduction (Fig. 5, A and B). With xanthine as reducing substrate, the $K_m$ values of nitrite, obtained from fitting to the Michaelis-Menten equation, increased from 11.9, 23.4, and 39.2 to 58.6 mM with the increase of $pO_2$ upon purging with 2, 5, 10% oxygen or air (21% oxygen), whereas $V_{max}$ values remained at about 9.0 nmol s$^{-1}$ mg$^{-1}$ (Fig. 5A). With DBA as reducing substrate, the $K_m$ values for nitrite increased from 10.0, 17.3, and 30.2 to 48.5 mM with the increase of $pO_2$, whereas $V_{max}$ values remained at about 4.2 nmol s$^{-1}$ mg$^{-1}$ (Fig. 5B).

NADH reacts with XO at the FAD site of the enzyme. With NADH as electron donor and purging with air, NO generation rates from nitrite do not follow Michaelis-Menten kinetics (Fig. 6B). Aerobic NO generation rates from nitrite were maintained at more than 70% of anaerobic levels (Fig. 6A). For 1 mM nitrite with XO (0.02 mg/ml), NADH (1 mM), and SOD (400 units/ml) for reactions in 21, 10, 5, or 2% oxygen or in argon, the rates of NO generation were 0.22 ± 0.01, 0.25 ± 0.01, 0.27 ± 0.01, and 0.29 ± 0.02 or 0.30 ± 0.03 nmol s$^{-1}$ mg$^{-1}$, respectively. The rate of NO generation decreased with the increase of $pO_2$ when NADH was the reducing substrate; however, even in air, prominent NO production was still present.

Effects of Site-specific Inhibitors on XO-mediated NO Generation under Aerobic Conditions—The effects of site-specific inhibition of XO were studied to investigate the reaction mechanisms in the process of the XO-catalyzed nitrite reduc-
tion under aerobic conditions. Oxypurinol binds to the molybdenum site of XO. Over the range of oxygen tensions studied, it was observed that oxypurinol inhibited the NO generation triggered by xanthine, DBA, or NADH. Addition of the FAD site-specific inhibitor, DPI, strongly inhibited XO-mediated NO generation, with NADH serving as reducing substrate; with xanthine or DBA as reducing substrates, NO generation was increased more than six times (Fig. 7). This result suggests that disruption of the flavin by DPI inhibited the binding and transfer of electrons to oxygen by XO, thus greatly inhibiting NO-mediated oxygen reduction. Thus, DPI blocked oxygen reduction by the enzyme and this, in turn, greatly increased its competitive reaction of nitrite reduction.

Effects of pH on XO-catalyzed NO Generation—To assess the NO formation under different physiological or pathological conditions and to further characterize the mechanism of XO-catalyzed nitrite reduction, experiments were performed to measure the effect of different pH values on the magnitude of XO-mediated NO generation under aerobic conditions. As shown in Table I, it was observed for each of the three reducing substrates, xanthine, NADH, and 2,3-dihydroxybenz-aldehyde, that pH 5 is the condition of maximal NO generation. Under aerobic conditions, NO generation increased more than three times as the pH value decreased from 7.4 to 6.0, and another three times as pH decreased from 6 to 5.0 (Table I).

XO-mediated NO Generation from Nitrite in Heart Tissue—In mammalian organs under normoxic conditions, O₂ concentration ranges from 10 to <0.5%, with values of ~14% in arterial blood and ~5% in the myocardium. During mild hypoxia, myocardial O₂ levels drop to ~1–3% or lower (22, 23). To determine whether nitrite can be reduced to form NO in tissues and to ascertain the role of XO in this process under aerobic conditions, studies were performed in heart tissue in the presence of [15N]nitrite, as described under.

![Fig. 6](image)

**Fig. 6.** XO-mediated NO generation from nitrite with NADH as reducing substrate under anaerobic or aerobic conditions. Initial rates of NO generation were measured by chemiluminescence NO analyzer, as described in Figs. 1 and 4. A, the effects of nitrite concentration on the rate of NO generation from 0.02 mg/ml XO and 1 mM NADH in the presence of 0.02–4 mM nitrite and 400 units/ml SOD purged with argon. B, as in A, but purged with air.

![Fig. 7](image)

**Fig. 7.** Effect of site-specific inhibitors on XO-mediated nitrite formation under aerobic conditions. Rates of NO generation were measured by chemiluminescence NO analyzer from samples purged with compressed air from preparations of 0.02 mg/ml XO in the presence of 1.0 mM nitrite and 400 units/ml SOD with either 0.02 mM xanthine, 0.5 mM DBA, or 1.0 mM NADH. Control, without inhibitor; Control + DPI (0.05 mM); Control + oxypurinol (0.05 mM).

![Fig. 8](image)

**Fig. 8.** EPR measurement of NO generation from [15N]nitrite in heart tissue. The NO formed in the reaction solutions was continuously purged to a trap vessel containing 1.0 ml of 2 mM (MGD)₂-Fe²⁺ using 5% (A, B, C) or 2% (D) oxygen/nitrogen mixture gas. Samples were taken after 60 min, and the spectra of the (MGD)₂-Fe²⁺-NO adducts formed are shown. A, spectra of NO trapped from 20 μM [15N]nitrite, 5 mM N⁵-nitro-L-arginine, and 400 units of SOD/ml in HBSS buffer, pH 7.0, at 37 °C. B, as in A with 1 g of heart tissue added. C, as in B, but heart tissue was pretreated with 100 μM oxypurinol. D, as in B, but the reaction was performed in 2% instead of 5% oxygen.

**Table I.**

| NO generation rate | pH 5.0 | pH 6.0 | pH 7.4 | pH 8.0 |
|--------------------|-------|-------|-------|-------|
| NADH (1 mM)        | 2.44 ± 0.22 | 0.68 ± 0.04 | 0.22 ± 0.01 | 0.04 ± 0.01 |
| Xanthine (20 μM)   | 1.89 ± 0.23 | 0.56 ± 0.07 | 0.15 ± 0.02 | 0.05 ± 0.01 |
| DBA (0.5 mM)       | 1.16 ± 0.16 | 0.36 ± 0.05 | 0.08 ± 0.01 | 0.03 ± 0.01 |
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“Materials and Methods.” Rat heart tissue was isolated, cut into pieces (−50 mg), suspended in HBSS (pH 7.0), and purged with 5% oxygen/95% nitrogen. The tissue was rinsed to remove blood, thus preventing foam formation during purging. This also served to minimize the intracellular nitrite/nitrate concentrations. NOS-inhibitor N”-nitro-l-arginine (5 mM) was added to prevent possible NO generation from l-arginine. NO production from [15N]nitrite in the heart tissue was measured by EPR spectroscopy. 15NO gives a characteristic doublet 15NO-Fe2+–MGD spectrum, rather than the triplet observed with naturally abundant 14NO, thus enabling direct and selective detection of nitrite-derived NO formation. In the absence of tissue, no signal was seen from 20 µM nitrite with 400 units of SOD and 5 mM NOS-inhibitor oxypurinol, the 15NO-Fe-MGD signal was quenched more than 60% (Fig. 8A). However, upon addition of 1 g of heart tissue, a 15NO signal was seen (Fig. 8B). With the addition of the XO inhibitor oxyxynurino1, the 15NO-Fe-MGD signal was quenched more than 60% (Fig. 8C). In the presence of 2% oxygen, the level of NO generation was more than 50% higher than that at 5% (Fig. 8D). Thus, XO-mediated nitrite reduction can be a source of NO in heart tissue under conditions of tissue normoxia, and it is further increased with mild hypoxia.

**DISCUSSION**

It has been reported recently that XO catalyzes reduction of nitrite to NO under hypoxic conditions (10–14), and the kinetics of this process have been characterized. It is clear that XO-mediated nitrite and nitrate reduction occurs and can be an important source of NO, particularly under conditions of limited tissue perfusion and resulting acidosis (13, 14). However, questions remained regarding whether XO-mediated NO generation also occurs in the presence of oxygen, and the regulation, mechanism, and quantitative importance of this process in biological systems had not been characterized. Therefore, we performed a series of studies using EPR, chemiluminescence NO analyzer, and NO electrode techniques to measure the magnitude and kinetics of XO-mediated NO formation under different oxygen tensions.

Experimental data obtained using each of these methods confirmed that XO does reduce nitrite to NO under aerobic conditions. Three typical reducing substrates of XO triggered NO generation from XO-mediated nitrite reduction (Figs. 2–5); however, the kinetics of XO-mediated NO formation are quite different in the presence of molybdenum-site binding substrates xanthine or DBA, compared with that in the presence of the FAD-site binding substrate NADH (Figs. 5 and 6). With xanthine or DBA as reducing substrates that donate electrons to XO at the molybdenum site of enzyme, the rate of NO production followed typical Michaelis-Menten kinetics, and this can be expressed in the form of the Michaelis-Menten equation,

\[
V_{\text{NO}} = \frac{V_{\text{max}} \cdot [\text{nitrite}]}{K_m + [\text{nitrite}]}
\]

where terms are defined as follows

\[
V_{\text{max}} = \frac{2E_0[S]}{k_{1} + k_{2} + \frac{1}{k_{3} + k_{4} + k_{5} + k_{6}}[S]}
\]

It was observed that over a broad range of nitrite concentrations, Equation 7 provided a good fit to the experimental data measuring the rate of NO generation from XO in the presence of molybdenum-site binding, reducing substrates xanthine or DBA under different oxygen tensions (Fig. 5). Although the apparent \(K_m\) value of nitrite for XO increased with higher concentrations of oxygen, the apparent \(V_{\text{max}}\) remained almost constant. These results clearly show the competitive inhibitory effect of oxygen on XO-mediated nitrite reduction when molybdenum-site binding reducing substrates, xanthine or DBA, are the electron donors.

Under aerobic conditions, with xanthine or DBA as reducing substrates, XO-mediated NO production is less than the 10% of NO production under anaerobic conditions (13). With the FAD-site binding reducing substrate, NADH, as electron donor, XO-mediated NO production is maintained at more than 70% of the anaerobic levels, and the XO-catalyzed NO generation rate only changes from −0.30 nmol mg⁻¹ s⁻¹ under anaerobic conditions to −0.22 nmol mg⁻¹ s⁻¹ under aerobic conditions in the presence of the same enzyme and substrate concentrations (Fig. 6). From analysis of the data with NADH under aerobic conditions, it was seen that this XO-mediated nitrite reduction did not follow Michaelis-Menten kinetics. NADH serves as electron donor to XO at the FAD site, the same site as that for oxygen binding, whereas nitrite reduction takes place at the molybdenum site of the enzyme (13). With NADH as reducing substrate, the possible XO-mediated NO generation may occur through two possible processes as shown below.

In Process I, XO is in the reduced state. With FAD site free, both oxygen and nitrite can accept electrons from reduced XO. Thus, under aerobic conditions, oxygen is a strong competitive inhibitor to XO-mediated nitrite reduction in Process I. NO

\[
E_{\text{red}} + O_2 \overset{k_{5}}{\underset{k_{7}}{\rightleftharpoons}} E_{\text{red}}O_2 \overset{k_{9}}{\rightarrow} E_{\text{red}} + O_2^- \quad \text{(Eq. 4)}
\]

\[
E_{\text{red}} + O_2 \overset{k_{5}}{\underset{k_{7}}{\rightleftharpoons}} E_{\text{red}}O_2 \overset{k_{9}}{\rightarrow} E_{\text{red}} + O_2^- \quad \text{(Eq. 5)}
\]

Where \(E_{\text{red}}\) is the fully oxidized enzyme, \(E_{\text{red}}\) is the two-electron reduced enzyme, and \(E_{\text{red}}\) is the one-electron reduced enzyme. \(S\) refers to the molybdenum reducing substrates of XO (such as xanthine and DBA), and \(P\) is the corresponding product. It should be noted that, for each xanthine oxidized, two molecules of nitrite can be reduced to NO. The total enzyme concentration, \([E]\), can be defined as follows:

\[
E'_{\text{red}} = [E_{\text{red}}] + [E_{\text{red}}S] + [E_{\text{red}}\text{nitrile}] + [E_{\text{red}}\text{nitrile}] + [E_{\text{red}}]\]

\[
+ [E_{\text{red}}O_2] + [E_{\text{red}}O_2] \quad \text{(Eq. 6)}
\]

From Equations 1–6, the rate of NO generation can be derived, and this can be expressed in the form of the Michaelis-Menten equation,

\[
V_{\text{NO}} = \frac{V_{\text{max}}[\text{nitrite}]}{K_m + [\text{nitrite}]}
\]

where terms are defined as follows

\[
V_{\text{max}} = \frac{2E_0[S]}{k_1 + k_2 + \frac{1}{k_3 + k_4 + k_5 + k_6}[S]}
\]
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**Scheme I**

| Process I | Process II |
|-----------|-----------|
| Nitrile → NO | Nitrile → NO |
| Mo e | Mo e |
| 4FeS e | 4FeS e |
| FA DH(H) | NADH-FAD(H) |
| O₂ | O₂ → O₂⁻ | O₂⁻ + H₂O₂ |

Generation through Process I would decrease greatly in the presence of oxygen, and it would follow the same kinetics as shown in Eq. 7. However, in Process II, the FAD site is occupied by the binding of NADH, thus oxygen reduction is totally blocked; meanwhile, at the molybdenum site, XO-mediated nitrite reduction is unaffected. Thus, the rate of XO-mediated nitrite reduction would be similar in the presence or absence of oxygen as in Process II. The kinetics of this process of NADH-driven XO-mediated nitrite reduction, in the absence of oxygen, have been detailed previously (13). Under aerobic conditions, less than 30% of the nitrite reductase activity of XO is inhibited, which suggests that most nitrite reduction happens while the FAD site is occupied with NADH.

NADH is necessary for many biochemical reactions within the body and is found in every living cell. Brain cells contain about 50 μg of NADH per gram of tissue, and heart cells contain 90 μg of NADH per gram of tissue (24). With molybdenum-site binding electron donors xanthine or DBA, nitrite reduction is greatly inhibited by the presence of oxygen, whereas with NADH, XO-mediated NO generation remains at more than 70% of anaerobic levels. The relatively high concentration of NADH in biological systems and its inhibitive effects on the binding of oxygen strongly suggest that NADH would be the major electron donor for XO-catalyzed NO production under aerobic conditions.

One can estimate the rate of XO-mediated NO production that would occur from NADH-mediated nitrite reduction in the heart under normal physiological conditions. In myocardial tissue, NADH concentration has been reported to be around 90 μg/g tissue, corresponding to an intracellular concentration of ~300 μM; with low flow ischemia, levels rise to above 1 mM (24). Tissue oxygen levels are normally <5% in the heart (22). Xanthine oxidoreductase (XOR) is present in the heart at levels of ~30 μg/g tissue (19). Tissue nitrite levels of ~10 μM have been measured in the heart (20). The process of XO-mediated nitrite reduction with NADH as substrate has a $K_m$ for nitrite of 2.25 mM (13). Well below this nitrite $K_m$ value, the NO formation rate would vary linearly with nitrite concentration. Therefore, under normal aerobic conditions in the heart, the rate of NADH-driven XOR-mediated NO generation would be predicted to be ~60 pmol/s, assuming ~70% of the rate of NO generation from XOR under anaerobic conditions.

The effects of site-specific inhibitors were studied to further investigate the reaction site involved in the process of XO-mediated nitrite reduction under aerobic conditions. Interestingly, DPI, the inhibitor of FAD site-related function, greatly increased NO generation under aerobic conditions with xanthine or DBA used as reducing substrate (Fig. 7). It is known that oxyurinol blocks the binding of xanthine, DBA, and nitrite, whereas DPI inhibits the reduction of XO by NADH. With xanthine or DBA as reducing substrates, the presence of DPI inhibits XO-mediated oxygen reduction at the FAD side and thus increases the capability of the enzyme for nitrite reduction at the molybdenum site (Fig. 7). Both the reduction of nitrite and the oxidation of xanthine and DBA take place on the molybdenum site of XO. Thus, the damage or mutation of the FAD site of XO prevents the binding of oxygen and converts this enzyme from primarily oxygen radical generation to nitric oxide generation in the presence of nitrite and the substrates xanthine or DBA. The potential effects of DPI in stimulating NO generation from XO should be taken into account when DPI is used in biological systems, especially when high concentrations of nitrite are present.

Previous studies demonstrated that XO-mediated nitrite reduction takes place by means of an acid-catalyzed mechanism. When pH decreased from 8.0 to 6.0, NO generation rate increased about six times under anaerobic conditions (13). In contrast, the maximum XO-catalyzed oxygen-free radical generation occurs at pH 8–9 (25–27). The present study shows that, under aerobic conditions, XO-mediated NO generation rates increase more than 10 times when pH values fall from 8.0 to 6.0, and further increase about 3-fold as pH values decrease from 6.0 to 5.0 (Table I). When the pH was lowered, a more rapid increase of XO-mediated NO generation rate was observed under aerobic conditions than under anaerobic conditions. This would be expected, because under aerobic conditions, the acidosis would significantly increase XO-mediated nitrite reduction and simultaneously inhibit the competitive reaction of oxygen reduction, thus facilitating NO generation under aerobic conditions.

Under aerobic conditions, XO-mediated reduction of oxygen leads to the production of superoxide anion or hydrogen peroxide, but XO can also catalyze nitrite reduction to NO. The simultaneous production of NO and superoxide can form the potent oxidant peroxynitrite. In the setting of inflammatory disease or pharmacological treatment with organic nitrates or NO-donating compounds, nitrite concentrations can rise by more than an order of magnitude (28–32). Without the protection of antioxidants or antioxidant enzymes, accumulated nitrite can become an important source of peroxynitrite production that can damage cells or tissues. Superoxide dismutase in biological systems is an extremely potent antioxidant enzyme that is responsible for the elimination of cytotoxic active oxygen by catalyzing the dismutation of the superoxide radical to oxygen and hydrogen peroxide (33–36). Because NO is readily inactivated by superoxide, the bioactivity of NO is dependent upon the local activity of SOD (37). Also, there are numerous peroxynitrite scavengers, such as uric acid and NADH, in biological systems (38, 39). Reducing substrate and nitrite concentrations as well as pH regulate XO-mediated NO production under aerobic conditions, and the biological effects of this production are determined by local SOD and antioxidant levels.

These results suggest that under aerobic conditions, NADH would be the main electron donor for XO-catalyzed NO production in mammalian cells and tissues. During ischemia, the myocardial NADH/NAD⁺ concentration ratio can increase more than 10-fold (40), xanthine levels can rise to the level of 10–100 μM, with nitrite levels of about 10 μM (19, 20); the low oxygen pressure and acidosis greatly facilitate XO-mediated NO generation and limit superoxide production. The magnitude of XO-mediated NO generation can approach that of the maximal NO production from NOS (13). Even with mild to moderate levels of hypoxia, as can occur with subtotal coronary lesions or regional ischemia in the presence of collateral flow, this process would be stimulated. This could allow NO to accumulate and exert a vasodilator role during ischemia. Upon reperfusion, the accumulated NO would react with XO-derived...
superoxide, giving a burst of peroxynitrite production that can mediate protein nitration and cellular injury (7).

Thus, XO-mediated NO generation occurs under aerobic conditions as well as under anaerobic conditions. With substrates such as xanthine or DBA that bind to the molybdenum site of the enzyme, oxygen serves as a competitive inhibitor of nitrite reduction, whereas with NADH, which binds at the FAD site, oxygen exerts only a modest inhibition of nitrite reduction. This process of aerobic XO-mediated NO generation is modulated by oxygen tension, pH, nitrite levels, and reducing substrate concentrations. It would be expected to be particularly important under disease conditions, where high levels of nitrite accumulate in tissues, and under conditions such as partial or regional ischemia, where cellular acidosis and hypoxia occur along with elevated tissue levels of NADH and other XO substrates.

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Characterization of the Effects of Oxygen on Xanthine Oxidase-mediated Nitric Oxide Formation
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