Characterization of the Magnitude and Kinetics of Xanthine Oxidase-catalyzed Nitrite Reduction

EVALUATION OF ITS ROLE IN NITRIC OXIDE GENERATION IN ANOXIC TISSUES*

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Xanthine oxidase (XO)-catalyzed nitrite reduction with nitric oxide (NO) production has been reported to occur under anaerobic conditions, but questions remain regarding the magnitude, kinetics, and biological importance of this process. To characterize this mechanism and its quantitative importance in biological systems, electron paramagnetic resonance spectroscopy, chemiluminescence NO analyzer, and NO electrode studies were performed. The XO reducing substrates xanthine, NADH, and 2,3-dihydroxybenz-aldehyde triggered nitrite reduction to NO, and the molybdenum-binding XO inhibitor oxy-purinol inhibited this NO formation, indicating that nitrite reduction occurs at the molybdenum site. However, at higher xanthine concentrations, partial inhibition was seen, suggesting the formation of a substrate-bound reduced enzyme complex with xanthine blocking the molybdenum site. Studies of the pH dependence of NO formation indicated that XO-mediated nitrite reduction occurred via an acid-catalyzed mechanism. Nitrite and reducing substrate concentrations were important regulators of XO-catalyzed NO generation. The substrate dependence of anaerobic XO-catalyzed nitrite reduction followed Michaelis-Menten kinetics, enabling prediction of the magnitude of NO formation and delineation of the quantitative importance of this process in biological systems. It was determined that under conditions occurring during no-flow ischemia, myocardial XO and nitrite levels are sufficient to generate NO levels comparable to those produced from nitric oxide synthase. Thus, XO-catalyzed nitrite reduction can be an important source of NO generation under ischemic conditions.

Nitric oxide is an important regulator of a variety of biological functions, and it also plays important roles in the pathogenesis of cellular injury. It has been generally accepted that NO is solely generated in biological tissues by specific nitric oxide synthases (NOSs) that metabolize arginine to citrulline with the formation of NO. However, previous studies have also demonstrated that NOS-independent generation of NO from nitrite occurs in ischemic tissues, such as the heart, demonstrating that nitrite can be a source rather than a product of NO, particularly under acidic conditions (1–3). Although it is clear that NO formation occurs secondary to nitrite reduction (3), questions remain regarding the source of the reducing equivalents required and the role of non-NOS enzymes in this process (1).

Xanthine oxidase (XO) is a ubiquitous enzyme in mammalian cells that plays important roles in both physiological and pathological conditions. It is involved in the catabolism of purine and pyrimidines, oxidizing hypoxanthine to xanthine and xanthine to uric acid. XO also reduces oxygen to superoxide and hydrogen peroxide and is one of the key enzymes responsible for superoxide-mediated cellular injury (4). It has a central role in the process of injury that occurs upon reoxygenation of hypoxic cells and tissues (5–7). XO contains critical flavin, iron, sulfur, and molybdenum sites and has some structural similarity with microbial nitrite reductase (8). Recently, it has been reported that XO catalyzes reduction of nitrite to NO under hypoxic conditions (8–10), but a number of important questions have remained regarding the mechanism, substrate specificity, and magnitude of this process.

XO exhibits broad specificity, accepting a variety of reducing substrates (11). It was first reported that NADH, but not xanthine, can act as an electron donor to XO and catalyze nitrite reduction (8, 9). Xanthine or hypoxanthine was found to inhibit this NO formation from XO (9). However, more recently, it was reported that xanthine can serve as a reducing substrate to stimulate nitrite reduction (10). In contrast to this, other investigators reported that XO in the presence of xanthine does not reduce nitrite to NO (12). In addition, in these studies there are major differences regarding the rates of NO formation and Km values of XO for nitrite or the requisite reducing substrate. Thus, questions remain regarding the substrate source required as well as the rate of this XO-catalyzed process of NO formation as a function of a given substrate concentration.

Considering the important physiological roles of NO in blood pressure regulation, vascular tone, neural signaling, and immune function (13–15) and the functions of the derivatives of NO and superoxide in biological systems, it is important to determine the mechanism, magnitude, and role of XO-catalyzed NO generation.

To characterize this XO-catalyzed pathway of NO production and its quantitative importance in biological systems, electron paramagnetic resonance (EPR) spectroscopy, chemiluminescence NO analyzer, and NO electrode studies were performed.

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The abbreviations used are: NOS, nitric-oxide synthase; XO, xanthine oxidase; XDH, xanthine dehydrogenase; NADH, nicotinamide adenine dinucleotide; DBA, 2,3-dihydroxybenz-aldehyde; NO, nitric oxide; MGD, N-methyl-D-glucamine dithiocarbamate; EPR, electron paramagnetic resonance; DPI, diphenyleneiodonium chloride; FAD, flavin-adenine dinucleotide; PBS, phosphate-buffered saline.

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The mechanism of NO formation was shown to occur due to nitrite reduction at the molybdenum site, with either NADH or xanthine serving as reducing substrates. The kinetic parameters for nitrite, NADH, and xanthine were determined, enabling prediction of the magnitude of NO formation and delineation of the quantitative importance of this process in biological systems.

**EXPERIMENTAL PROCEDURES**

**Materials**—Xanthine oxidase from buttermilk (xanthine: oxygen oxidoreductase) (EC 1.1.3.22), xanthine, oxyxpurinol, diphenyleneiodonium chloride (DPI), sodium nitrite, beta-NADH, and 2,3-dihydroxybenzaldehyde were obtained from Sigma. N-Methyl-γ-gluamine dithiocarbamate (MGD) was synthesized using carbon disulfide and N-methyl-γ-gluamine, as described (16). Ferrous ammonium sulfate was purchased from Aldrich (99.997%). Dulbecco’s phosphate-buffered saline (PBS) was obtained from Life Technologies Inc.

**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. NO formation was measured by spin trapping using the ferrous iron complex of MGD, Fe-MGD. Solid ferrous ammonium sulfate and MGD (molar ratio, 1:5) were added to the deoxygenated (argon-purged) PBS buffer with a final concentration of 2 mM iron. Experiments were performed under anaerobic conditions achieved by argon purging in a glass-purging vessel followed by transfer under argon to a quartz flat cell that was then sealed. Quantitation of NO trapping was determined from the intensity of NO adduct signal recorded after mixing of Fe-MGD with aqueous solutions equilibrated with NO gas of known concentration (3).

**Chemiluminescence Measurements**—The rate of the NO production was measured using a Sievers 270B nitric oxide analyzer interfaced through a DT2821 A to D board to PC. In the analyzer, NO is reacted with ozone forming excited-state NO$_2^-$, which emits light. Mixing of reagents and separation of NO from the reaction mixture were done at a controlled temperature of 37 °C in a glass-purging vessel equipped with a heating jacket. An ice-water cooling condenser was attached to the top of the vessel to reduce the outflow of vapors during purging. Additionally, an ice-cooled chemical trap filled with 1.0 M NaOH was placed between the purging vessel and NO analyzer. The release of NO was quantified by analysis of the digitally recorded signal from the photomultiplier tube using specially designed data acquisition and analysis software developed in our laboratory. Calibration of the magnitude of NO production was determined from the integral of the signal over time compared with that from nitrite concentration standards added to acetate acid containing 1% potassium iodide (3). To enhance sensitivity for measurements of low levels of NO formation, a high quality, low noise photomultiplier tube was installed in the analyzer.

**Electrochemical Measurements**—Electrochemical measurements of NO generation by XO were carried out at 37 °C using a CHI 832 electrochemical detector with a Faraday cage (CH Instruments, Inc., Cordova, IN) and WPI NO electrode (Word Precision Instruments, Sarasota, FL). The electrochemical detector continuously recorded the current through the working electrode, which is proportional to the NO concentration in the solution. The sensor was calibrated before and after experiments with known concentrations of NO, using NO gas-equilibrated solutions. The electrode was placed in a closed, water-jacketed glass electrochemical cell with ports for gas purging. All measurements were performed at 37 °C with prepurging of argon gas into the solution for 20 min before measurements, and then continuous flow of argon above the solution was maintained to assure anaerobic conditions throughout the measurements.

**Statistical Analysis and Kinetic Fitting**—Values are expressed as mean ± S.D. of at least three repeated measurements, and the statistical significance of difference was evaluated by Student’s t test. A p value of 0.05 or less was considered to indicate statistical significance. Kinetic fits to the experimental data were performed on a PC using Table Curve 2D v4 (Jandel Scientific).

**RESULTS**

**Role of Reducing Substrates in XO-mediated NO Generation**—NO is paramagnetic and binds with high affinity to the water-soluble spin trap Fe$^{3+}$-MGD, forming a mononitrosyl iron complex with characteristic triplet spectrum at $g = 2.04$ with hyperfine splitting $a_N$ = 12.8. From the intensity of the observed spectrum, quantitative measurement of NO generation can be performed (17–19). This technique was applied to measure nitrite-mediated NO generation under anaerobic conditions. In the absence of nitrite, mixtures of XO (0.1 mg/ml) and its reducing substrate xanthine (20 μM) gave rise to no signal (Fig. 1A). In the absence of XO but the presence of nitrite (1 mM), only a trace signal was seen (Fig. 1B). Whereas xanthine (20 μM) and nitrite only gave rise to a trace signal (Fig. 1C), upon addition of the XO reducing substrates NADH (1 mM), 2,3-dihydroxybenzaldehyde (DBA) (200 μM), or xanthine (20 μM), a marked increase in the NO signal was seen (Fig. 1, D–F). Thus, all of the typical types of XO reducing substrates, including NADH, 2,3-dihydroxybenzaldehyde, and xanthine, acted as electron donors for XO-catalyzed nitrate reduction and...
triggered large amounts of NO generation under anaerobic conditions.

To further confirm the existence of the XO-catalyzed mechanism of NO generation in the absence of any possible effects or perturbation by the spin trap, studies were performed measuring NO generation using a specific electrochemical NO sensor. Prior to the addition of XO, no detectable NO generation was seen from nitrite (1 mM) in the presence of xanthine (10 μM) or NADH (1 mM). However, after addition of XO (0.015 mg/ml), prominent NO generation was triggered from xanthine or NADH (Fig. 2, traces A and B). The magnitude and rate of NO generation in the presence of xanthine (Fig. 2, trace A) was considerably higher than that with NADH (Fig. 2, trace B), whereas with xanthine and nitrite in the absence of XO, no NO generation was observed (Fig. 2, trace C). Of note, the substrate DBA also stimulated NO generation from nitrite in the presence of XO, with magnitude and rate approaching that seen from xanthine (data not shown).

Thus, both EPR spin trapping and NO electrode studies demonstrate that XO can generate NO production in the presence of substrates that reduce the enzyme. To confirm that XO-mediated NO generation occurs and to directly measure the rate of this NO generation, further studies were performed using a chemiluminescence NO analyzer. Mixing of reagents was performed at 37 °C in the glass-purging vessel. NO was purged from the solution with argon gas and then reacted with ozone in the analyzer to form excited-state NO2, which emits light. This method provides direct measurement of the rate of NO generation as a function of time (3). With nitrite (1 mM) alone or nitrite in the presence of xanthine (5 μM) or NADH (1 mM), no measurable rate of NO formation was observed (Fig. 3, trace C). However, with the addition of XO (0.02 mg/ml) in the presence of xanthine or NADH, prominent NO generation was triggered (Fig. 3, traces A and B). Additional control experiments performed with XO in the presence of xanthine or NADH but in the absence of nitrite confirmed that nitrite was required for NO generation. Again, a higher rate of XO-mediated NO generation was seen with xanthine than with NADH. As was seen with the electrode measurements, the substrate DBA (40 μM) also stimulated NO generation from nitrite in the presence of XO with a rate approaching that seen with xanthine (data not shown).

Kinetics of XO-catalyzed Nitrite Reduction—The rate of NO formation derived from XO-catalyzed nitrite reduction was measured under anaerobic conditions using the NO analyzer. The concentration dependence of each reducing substrate was first determined in the presence of a fixed nitrite concentration of 1 mM. Each of the typical reducing substrates, xanthine, DBA, and NADH, acted as electron donors to support XO-catalyzed nitrite reduction, and each of these reactions followed Michaelis-Menten kinetics (Fig. 4, A–C). For each reducing substrate, the apparent values of $K_m$, $K_{cat}$, and $V_{max}$ were determined by fitting the data to the Michaelis-Menten equation, and the values for each reducing substrate are shown inside each curve. For each of these reducing substrates, the rate of XO-mediated NO formation was also determined as a function of nitrite concentration (Fig. 4, D–F). Again, typical Michaelis-Menten kinetics were observed as a function of nitrite concentration, and the apparent $K_m$, $K_{cat}$, and $V_{max}$ values are shown inside each curve. From these kinetic data, it is possible to predict the magnitude of XO-catalyzed NO formation as a function of nitrite and reducing substrate concentration and to determine the quantitative importance of this mechanism of NO generation in a given biological system where these substrate levels are known.

Determination of the Mechanism and Reaction Site of Nitrite Reduction—The effects of site-specific inhibitors of XO were studied to investigate the reaction sites involved in the process of the XO-catalyzed nitrite reaction observed with different reducing substrates. Oxpurinol binds to the molybdenum site of XO. It was observed that oxypurinol inhibited XO-catalyzed nitrite reduction regardless of the type of reducing substrate present. Near total inhibition of NO generation was seen in the presence of either xanthine or NADH (Fig. 5). Because oxypurinol inhibits substrates binding at the molybdenum site of the enzyme, this suggests that nitrite binds to the reduced molybdenum site. DPI, which acts at the FAD site, inhibited XO-dependent nitrite reduction only when NADH was used as the reducing substrate, and it did not inhibit NO generation when xanthine was used (Fig. 5). This suggests that NADH donates electrons to FAD, and then electrons are transported back to
FIG. 4. Kinetics of NO generation from XO as a function of reducing substrate or nitrite concentration. Initial rates of NO generation were measured by chemiluminescence NO analyzer as described in Fig. 3. A shows the effect of [NADH] on the rate of NO generation from 0.04 mg/ml XO and 1.0 mM nitrite. B shows the effect of [xanthine] on the rate of NO generation from 0.02 mg/ml XO and 1.0 mM nitrite. C shows the effect of [2,3-dihydroxybenz-aldehyde] on the rate of NO generation from 0.02 mg/ml XO and 1.0 mM nitrite. D shows the rate of NO generation by 0.04 mg/ml XO and 1.0 mM NADH in the presence of 0.2–4 mM nitrite. E shows the rate of NO generation by 0.02 mg/ml XO, 5 mM xanthine in the presence of 5 μM–2.5 mM nitrite. F shows the rate of NO generation by 0.02 mg/ml XO, 40 μM 2,3-dihydroxybenz-aldehyde in the presence of 0.5 mM–5 mM nitrite. For each of these graphs, the corresponding fits (solid lines) $K_m$, $V_{max}$, and $K_{cat}$ data were obtained using the Michaelis-Menten equation.
reduce the molybdenum that in turn reduces nitrite to NO. When xanthine or aldehydes are the electron donors, both XO reduction (by xanthine or aldehydes) and oxidation (by nitrite) takes place at the molybdenum site, so that only oxypurinol could inhibit XO-dependent NO formation.

**Inhibitive Effect of High Xanthine Levels on NO Generation**—It has been reported that excess xanthine (>100 μM) can exert inhibition of XO catalytic function due to binding of xanthine to reduced forms of the enzyme generated in the process of XO-catalyzed oxygen reduction. This kind of substrate-bound reduced XO complex inhibits intramolecular electron transport from the molybdenum center to FAD (20, 21). In view of the questions regarding the role and potency of xanthine as a reducing substrate for the process of XO-mediated NO formation, NO analyzer and EPR spin trapping studies were performed to detect XO-catalyzed NO generation under anaerobic conditions in the presence of high concentrations of xanthine (10–100 μM). Although XO-catalyzed NO generation followed classic Michaelis-Menten kinetics with low concentrations of xanthine (<20 μM, Fig. 4B), higher concentrations of xanthine showed significant concentration-dependent inhibition of NO formation (Fig. 6). From fitting of the data in Fig. 6A, Kᵢ was calculated to be 55 μM. These results suggest that a xanthine-reduced XO complex is formed and inhibits the maximum rate of NO generation from XO-catalyzed nitrite reduction. It is likely that excessive xanthine inhibits XO-catalyzed nitrite reduction by binding to reduced forms of the enzyme, in turn blocking the binding of nitrite to the molybdenum site.

**NO Generation under Conditions Occurring in Ischemic Tissues**—To determine the magnitude of NO generation that can occur in biological tissues that typically contain low nitrite concentrations, measurements of XO-derived NO generation were performed in the presence of 5–40 μM nitrite, the range of concentrations observed in myocardial tissue (1–3). A process of xanthine-stimulated NO generation was seen that was similar to that observed with higher nitrite levels (Fig. 7). Maximum rates of NO generation of 18, 31, 56, and 135 pmol/s/mg were observed for 5, 10, 20, and 40 μM nitrite, respectively, at xanthine concentrations ranging from 1 to 10 μM. With further increase in xanthine levels, progressive inhibition was seen, as was observed with 1 mM nitrite (Fig. 6).

**Effects of pH on XO-catalyzed NO Generation**—Under ischemic conditions, marked intracellular acidosis occurs, and pH values in tissues, such as the heart, can fall to levels of 6.0 or below (2). In order 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 NO generation. Measurements were performed with 0.02 mg/ml of XO in the presence of 1 mM nitrite. As shown in Table I, it was observed for each of the three substrates xanthine, NADH, and DBA that under acidic conditions increased XO-catalyzed NO generation occurs. In contrast, under alkaline conditions, prominent inhibition was seen.

**DISCUSSION**

In view of the critical role of NO in normal physiology and disease, it is of crucial importance to understand the biochemical mechanisms of NO formation that occur in biological cells and tissues. In addition to the formation of NO from specific NOS enzymes, it is clear that nitrite derived from either NO metabolism or dietary sources can be an important source of NO formation, particularly under conditions of limited tissue perfusion and resulting acidosis. Although NO can be formed by the simple process of nitrite disproportionation that is accelerated under acidic conditions, it has been previously ob-

![Fig. 5](image-url)  
**Fig. 5.** Effect of site-specific inhibitors on XO-mediated NO formation. Rates of NO generation were measured by chemiluminescence NO analyzer as described in Fig. 3. The inhibitive effect of oxypurinol, which binds to the molybdenum site, and DPI, which modifies the flavin, was determined for xanthine (X)- or NADH-mediated NO generation. For the left set of bars, experiments were performed with 0.5 mM nitrite, 5 μM xanthine, and 0.02 mM NADH, and for the right set of bars, experiments were performed with 1.0 mM nitrite, 1.0 mM NADH, and 0.04 mg/ml XO. Control, without inhibitor; DPI, 20 μM; oxypurinol, 20 μM.

![Fig. 6](image-url)  
**Fig. 6.** Inhibition of XO-mediated NO generation by high xanthine concentrations. A, rates of NO generation were measured by chemiluminescence NO analyzer as described in Fig. 3 from 1.0 mM nitrite in the presence of different xanthine concentrations (1–100 μM), with XO (0.02 mg/ml) under anaerobic conditions, pH 7.4, 37 °C. The points show the measured experimental values ± S.D., and the line shows a least squares fit of the data to the equation with competitive inhibition (Equation 9). A good fit was obtained with a correlation coefficient r² > 0.98. B, NO generation measured by EPR spin trapping with (MGD)$_2$-Fe$^{2+}$ after 30 min in anaerobic conditions, pH 7.4, 37 °C. Production of NO by XO (0.02 mg/ml)-catalyzed nitrite (1.0 mM) reduction was measured as a function of xanthine concentration (10–100 μM). With both techniques, significant inhibition was seen with high xanthine concentrations.
were measured by chemiluminescence NO analyzer with 5, 10, 20, or 40 µM nitrite and 0.02 mg/ml of XO, with measurements performed as described in Fig. 3. With low nitrite concentrations, typical of cellular levels, the process of NO generation was generally similar to that observed with higher, mM levels. Maximum rate of NO generation was seen with low xanthine concentrations followed by progressive inhibition at high substrate levels. The points show the measured experimental values with ± S.D., and the line shows a least squares fit of the data to the rate equation with competitive inhibition (Equation 9). A good fit was obtained for each curve with correlation coefficients \( r^2 > 0.97 \). From these fits of the data to Equation 9, the \( V_{max} \) values were determined to be: 18.9, 33.6, 61.6, and 148.9 pmol/mg, and the \( K_m \) values were determined to be 0.012, 0.026, 0.053, and 1.0 µM for 5, 10, 20, and 40 µM nitrite, respectively, whereas the \( K_i \) value remained almost constant at 53 µM.

**Table 1**

| pH | NO generation rate (nmol · mg⁻¹ · s⁻¹) |
|----|--------------------------------------|
| 6.0 | Xanthine (10 µM) 2.15 ± 0.10 | 1.87 ± 0.09 | 0.34 ± 0.03 |
| 7.4 | NADH (1 mM) 0.7 ± 0.05 | 0.3 ± 0.03 | 0.11 ± 0.01 |
| 8.0 | DBA (0.1 mM) 1.96 ± 0.10 | 0.76 ± 0.05 | 0.45 ± 0.04 |

**Fig. 7. XO-mediated NO formation with cellular nitrite levels.** Initial rates of NO generation as a function of xanthine concentration were measured by chemiluminescence NO analyzer with 5, 10, 20, or 40 µM nitrite and 0.02 mg/ml of XO, with measurements performed as described in Fig. 3. With low nitrite concentrations, typical of cellular levels, the process of NO generation was generally similar to that observed with higher, mM levels. Maximum rate of NO generation was seen with low xanthine concentrations followed by progressive inhibition at high substrate levels. The points show the measured experimental values with ± S.D., and the line shows a least squares fit of the data to the rate equation with competitive inhibition (Equation 9). A good fit was obtained for each curve with correlation coefficients \( r^2 > 0.97 \). From these fits of the data to Equation 9, the \( V_{max} \) values were determined to be: 18.9, 33.6, 61.6, and 148.9 pmol/mg, and the \( K_m \) values were determined to be 0.012, 0.026, 0.053, and 1.0 µM for 5, 10, 20, and 40 µM nitrite, respectively, whereas the \( K_i \) value remained almost constant at 53 µM.

**Observed** that in ischemic tissues, nitrite is also reduced to form NO. Questions have remained regarding the role of non-NOS enzymes in this process of nitrite reduction (2).

Although it has recently been reported that XO can reduce nitrite to NO, questions remain regarding the role of non-NOS enzymes in this process of nitrite reduction (2).

Although there has been a recent report that XO can reduce nitrite to NO, questions remain regarding the biological importance of this pathway of NO production, as well as the mechanism, magnitude, and substrate specificity of this process. It was first reported that NADH, but not xanthine, can act as an electron donor to XO and catalyze nitrite reduction (8, 9). Xanthine or hypoxanthine was found to inhibit this NO formation from XO (9). However, more recently, it was reported that xanthine can serve as a reducing substrate to stimulate nitrite reduction (10). In contrast to this, other investigators reported that XO in the presence of xanthine does not reduce nitrite to NO (12). In addition, in these studies, there were large differences regarding the rates of NO formation and \( K_m \) values of XO for nitrite or the required reducing substrate. In view of these uncertainties, it has not been possible to ascertain the biological relevance and importance of this pathway of NO generation. Therefore, we performed a series of studies using EPR, chemiluminescence NO analyzer, and NO electrode techniques to measure the magnitude and kinetics of NO formation that arises due to XO-mediated nitrite reduction.

Data obtained using each of these three methods confirmed that XO does reduce nitrite to NO under anaerobic conditions. It was observed that each of the typical reducing substrates xanthine, DBA, and NADH could act as electron donors to support this XO-mediated nitrite reduction (Figs. 1–3). The results of these studies, along with the inhibition seen with oxypurinol, suggested that reduced XO was the direct electron donor to nitrite, with nitrite binding and reduction occurring at the molybdenum site. Whereas NADH-stimulated NO generation was inhibited by the flavin modifier DPI, NO generation stimulated by xanthine or DBA was unaffected. Thus, whereas xanthine or DBA directly reduces the molybdenum center, NADH initially reduces the flavin, which subsequently transfers electrons to the molybdenum.

From past studies, questions remain regarding the \( K_m \) and \( V_{max} \) values observed for XO-mediated NO generation under anaerobic conditions as a function of nitrite and reducing substrate concentration. Initial studies reported a \( K_m \) of 22.9 mM for nitrite reduction in the presence of NADH (9). However, a subsequent study reported a \( K_m \) value of 2.4 mM (8). A more recent study measuring NADH depletion rather than NO generation reported a \( K_m \) of 16 mM (10). In our studies, we observed that the \( K_m \) for nitrite was consistently about 2.3 mM in the presence of 1.0 mM NADH. This observation agrees closely with the report of Zhang et al. (8). In contrast with the first two studies that report only enzyme reduction by NADH and the recent study reporting a \( K_m \) of 36 mM for nitrite in the presence of xanthine, we observed that the \( K_m \) for nitrite is 2.4 ± 0.2 mM for each of the three types of substrates studied, NADH (1 mM), xanthine (5 µM), and DBA (40 µM). Possible reasons for variable results of the prior studies could relate to the conditions used for NO purging from the solutions, leak of oxygen into the measurement system, or partial enzyme inactivation or denaturation. Indeed, in the most recent report, Godber et al. (10) acknowledge that phase equilibration and gas flow rates delayed the delay in measurements for 2 min after initiation of the reaction. In their system, they measure the spontaneous liberation of NO into the gas phase and this enabled NO detection in the NO generation performed by NO electrode yielded similar values.

Although xanthine was the highest efficiency reducing substrate of XO-catalyzed nitrite reduction, excessive xanthine exhibited inhibition of NO production. Previous studies reported that enzyme inactivation resulted from NO-induced conversion of XO to its relatively inactive desulfo-form (10). However, this could not explain why these investigators observed that XO kept its activity (95%) when NADH acted as reducing substrate (10). Furthermore, we also observed that the presence of excess NADH (>10 mM) or DBA (>2 mM) had no inhibitory effect on XO-catalyzed NO generation. In a previous study, we showed that ONOO⁻ markedly inhibits XO activity in a dose-dependent manner, whereas NO from NO gas in concentrations up to 200 µM had no effect (22). So inactivation of XO (10) could be caused by ONOO⁻ formation triggered exposure to oxygen at the time of spectrophotometric activity assay. Our results suggest that excessive xanthine acts to...
inhibit XO by binding to the molybdenum site of the reduced enzyme (20, 21), thus blocking the binding of nitrite at this enzyme site. This xanthine-mediated inhibition, which has also been demonstrated by Godber et al. (10), may explain the prior failure to detect XO-mediated NO generation from nitrite in studies in which 150 μM xanthine were used (12).

It has been reported that purine and aldehyde substrate hydroxylation takes place via a base-catalyzed mechanism and that substrate must be protonated for hydroxylation (10). The rate of NO reduction by purine and aldehydes greatly increases when the pH value is increased from 6.0 to 8.0, and this increased rate of XO reduction will lead to an increased rate of nitrite reduction. However, our experiments showed that acidic conditions promote XO-catalyzed nitrite reduction. NO generation increased as the pH was decreased from 8.0 down to 7.4 or from 7.4 down to 6.0, suggesting that nitrite reduction takes place via an acid-catalyzed mechanism, presumably due to nitrite protonation. HNO_2 concentration increases when the pH decreases, and it could be the direct binding substrate of XO. Although the decrease of pH would decrease the rate of XO reduction by reducing substrates, it would greatly increase the speed of XO oxidation by nitrite/HNO_2.

From the studies performed, it can be clear that XO can catalyze the process of NO generation from nitrite under anaerobic or markedly hypoxic conditions similar to those occurring in ischemic tissues. The key questions are, what is the magnitude of this process, and whether the levels of NO produced are likely to have functional significance. To address these critical questions, a kinetic model can be constructed that enables prediction of the magnitude of XO-catalyzed NO formation and understanding the quantitative importance of this mechanism of NO generation in biological systems. The following equations define the steps in the reaction mechanism.

\[
E_{OX} + S \xrightarrow{k_1} E_{OX} \cdot S \xrightarrow{k_2} E_{red} + P \quad \text{(Eq. 1)}
\]

\[
E_{red} + \text{nitrite} \xrightarrow{k_3} E_{red} \cdot \text{nitrite} \xrightarrow{k_4} E'_{red} + NO \quad \text{(Eq. 2)}
\]

\[
E'_{red} + \text{nitrite} \xrightarrow{k_5} E'_{red} \cdot \text{nitrite} \xrightarrow{k_6} E_{OX} + NO \quad \text{(Eq. 3)}
\]

where \(E_{OX}\) is the fully oxidized enzyme, \(E_{red}\) is the 2-electron reduced enzyme, and \(E'_{red}\) is the 1-electron reduced enzyme. \(S\) refers to the reducing substrate of XO, such as xanthine, and \(P\) is the corresponding product. It should be noted that for each xanthine oxidized, 2 molecules of nitrite could be reduced to NO. The total enzyme concentration, \([E]\), can thus be defined as follows.

\[
[E] = [E_{OX}] + [E_{OX}S] + [E_{red}] + [E_{red} \text{ nitrite}] + [E'_{red} + [E'_{red} \text{ nitrite}] \quad \text{(Eq. 4)}
\]

From Equations 1–4, the rate of NO generation can be derived, and this can be expressed in the form of the Michaelis-Menten equation.

\[
V_{[NO]} = \frac{k_{cat}[E][S]}{K_m + [S]} \quad \text{(Eq. 5)}
\]

where terms are defined as follows.

\[
k_{cat} = \frac{2[NO_2]}{(k_2 + k_4 + k_{k3} + k_{k6}) + (\frac{1}{k_2} + \frac{1}{k_4} + \frac{1}{k_5})[NO_2]}\]

To consider the inhibitive effect of xanthine, two more equations must be considered.

\[
E_{red} + X \xrightarrow{k_7} E_{red}X \quad \text{(Eq. 6)}
\]

\[
E'_red + X \xrightarrow{k_8} E'_redX \quad \text{(Eq. 7)}
\]

The total enzyme concentration \([E]\) is defined as follows.

\[
[E] = [E_{OX}] + [E_{OX}X] + [E_{red} + [E_{red} \text{ nitrite}] + [E_{red} + [E'_{red} \text{ nitrite}] + [E_{red}X] \quad \text{(Eq. 8)}
\]

The rate of NO generation can be expressed as follows,

\[
V_{[NO]} = \frac{2[E][NO_2]}{(k_2 + k_4 + k_{k3} + k_{k6}) + (\frac{1}{k_2} + \frac{1}{k_4} + \frac{1}{k_5})[NO_2]} \quad \text{(Eq. 9)}
\]

where terms are defined as follows.

\[
K_m = \frac{k_2 + k_4}{k_3 + k_5 + k_{k3} + k_{k6}} \quad \text{and} \quad \frac{1}{k_2} + \frac{1}{k_4} + \frac{1}{k_5}\]

It was observed that over a broad range of physiological nitrite concentrations from 5 to 40 μM nitrite that Equation 9 provided a good fit to the experimental data measuring the rate of NO generation from XO in the presence of xanthine (Fig. 7). Also, at higher nitrite levels of 1 mM, a good fit of the experimental data was obtained (Fig. 6). From Equation 9, it is predicted that \(K_m\), \(V_{max}\), and \(K_i\) can vary as a function of nitrite concentration. The equation for \(K_i\) expression can be simplified as follows:

\[
K_i = a + b[NO_2], \quad \text{(Eq. 10)}
\]

where \(a\) and \(b\) are constants determined from fitting of the experimental data. From the experimental data, \(a = 53 \mu M\) and \(b = 2 \times 10^{-3}\). Thus, the \(K_i\) expression predicts that \(K_i\) will remain almost constant, with a value of 53 μM at low nitrite concentrations (<1 mM), but would greatly increase at high nitrite levels of 5 mM and above, consistent with the results of Godber et al. (10).

Our experiments also showed that nitrite reduction took place via an acid-catalyzed mechanism; thus, we suggest that protonated nitrite (HNO_2) may directly bind to reduced XO. Decrease of pH increases the HNO_2 concentration but will decrease \([E_{red}]\) because xanthine and aldehydes donate electrons to XO via a base-catalyzed mechanism (10). Overall NO generation depends on the combination of these opposite effects of NO on XO reduction and nitrite donation.

It has been previously demonstrated that the activity of XO in the postsischemic rat heart is 16.8 milliunits/g of protein (23), which corresponds to 0.013 mg of XO/g of protein or ~3.4 μg/g
of cell water. The total XO and xanthine dehydrogenase (XDH) activity, however, is 10-fold above this value. In the ischemic heart, xanthine levels rise from near zero to values on the order of 10–100 μM, and nitrite levels are ~10 μM (1, 2, 23). At normal pH values of 7.4, the rate of nitrite degradation due to simple chemical disproportionation is ~0.05 ps/M, as previously reported (3), whereas the rate of XO-catalyzed nitrite reduction would be ~100 ps/M. When pH decreases to 6.0, the rate of nitrite disproportionation will increase to 4 ps/M (3), whereas the rate of XO-catalyzed nitrite reduction would be estimated to increase to ~115 ps/M. If XO catalyzes this process or is converted to XO, the magnitude of this NO generation rate of nitrite disproportionation will increase to 4 ps/M (3), whereas typical tissue levels of nitrite are at least 2 orders of magnitude higher than that estimated for XO alone.

Thus, XO can be an important source of NOS-independent NO generation. Under anaerobic conditions, XO reduces nitrite to NO at the molybdenum site of the enzyme with xanthine, thereby generating NO from xanthine oxidoreductase would be substantially higher than that estimated for XO alone.

Overall, it is clear that XO-mediated NO generation can potentially be an important source of NO under ischemic conditions in biological tissues that contain substantial levels of the enzyme along with nitrite and reducing substrates. In tissues such as the liver and gastrointestinal tract, which contain high levels of the enzyme, this could be even more pronounced than for the example of the heart considered above (32). Beyond the obligatory need for the enzyme, the levels of tissue nitrite and enzyme reducing substrates have a critical role in controlling this process. Nitrite is required, and overall it is the most limiting substrate, because its $K_m$ is ~2.5 mM, whereas typical tissue levels of nitrite are at least 2 orders of magnitude below this value. A number of factors that increase tissue nitrite levels, such as prior activation of constitutive or inducible NOS in inflammatory conditions, dietary sources, pharmacological sources, or bacterial sources, could all modulate this pathway of NO generation. This pathway also requires a reducing substrate, such as NADH or xanthine. Xanthine was the most effective substrate, triggering NO generation under anaerobic conditions with a $V_{max}$ 4-fold higher than that of NADH (Fig. 4). Although only low xanthine concentrations are required, because its $K_m$ value is about 1.5 μM, high levels of xanthine, above 20 μM, resulted in prominent substrate-mediated inhibition (Fig. 6). If particularly high levels of xanthine accumulate, this pathway would be inhibited, and perhaps this may serve a regulatory role to prevent overproduction of NO.

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