Xanthine Oxidase Catalyzes Anaerobic Transformation of Organic Nitrates to Nitric Oxide and Nitrosothiols

CHARACTERIZATION OF THIS MECHANISM AND THE LINK BETWEEN ORGANIC NITRATE AND GUANYLYL CYCLASE ACTIVATION*

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Organic nitrates have been used clinically in the treatment of ischemic heart disease for more than a century. Recently, xanthine oxidase (XO) has been reported to catalyze organic nitrate reduction under anaerobic conditions, but questions remain regarding the initial precursor of nitric oxide (NO) and the link of organic nitrate to the activation of soluble guanylyl cyclase (sGC). To characterize the mechanism of XO-mediated biotransformation of organic nitrate, studies using electron paramagnetic resonance spectroscopy, chemiluminescence NO analyzer, NO electrode, and immunohistochemical were performed. The XO reducing substrates xanthine, NADH, and 2,3-dihydroxybenzaldehyde triggered the reduction of organic nitrate to nitrite anion (NO$_2^-$). Studies of the pH dependence of nitrite formation indicated that XO-mediated organic nitrate reduction occurred via an acid-catalyzed mechanism. In the absence of thiols or ascorbate, no NO generation was detected from XO-mediated organic nitrate reduction; however, addition of l-cysteine or ascorbate triggered prominent NO generation. Studies suggested that organic nitrite (R-O-N$^-$) is produced from XO-mediated organic nitrate reduction. Further reaction of organic nitrite with thiols or ascorbate leads to the generation of NO or nitrosothiols and thus stimulates the activation of sGC. Only flavin site XO inhibitors such as diphenyleneiodonium chloride (DPI) have shown strong inhibition of the bioactivation of organic nitrate (6, 10). Xanthine oxidase (XO) is a ubiquitous flavoenzyme in mammalian cells. XO plays a variety of important roles in normal physiology and disease, and it is the only identified enzyme that can mediate organic nitrate and inorganic nitrate/nitrite reduction (11–14). It has been reported that XO can catalyze organic nitrate reduction to nitrite anion (NO$_2^-$) under anaerobic conditions (13), but questions remain concerning the initial product, the precursor of NO, and the link of organic nitrate to the activation of soluble guanylyl cyclase (sGC). Thus, there is a need to characterize the process of XO-mediated organic nitrate bioactivation, and this should provide broader insights regarding bioactivation of organic nitrates by other enzymes as well.

Normally, NO$_2^-$ has been assumed to be the initial product of organic nitrate metabolism and the precursor of NO that accounts for organic nitrate bioactivity. However, controversies remain whether inorganic nitrite is an active intermediate in the vascular metabolism of organic nitrate to form NO (15–20). It has been questioned how the low micromolar to nanomolar levels of GTN in tissues can produce sufficient inorganic nitrite to serve as an intermediate to generate the levels of NO required to induce significant vasodilatory effects (21). Additional studies have shown that the magnitude and time course of GTN-mediated NO$_2^-$ formation in tissues make it impossible for GTN to act via liberation of nitrite (15, 19, 22–24). Thus, the link between organic nitrate and sGC activation remains elusive and is the most puzzling issue regarding the mechanism of organic nitrate biotransformation.

The literature is replete with conflicting observations concerning the role of thiols in the biotransformation of organic nitrate. Previous studies show that the bioactivation of organic nitrate involves thiols or sulfhydryl-containing compounds, and the re-

DBA, 2,3-dihydroxybenzaldehyde; NO, nitric oxide; BSA, bovine serum albumin; GSH, glutathione; GST, glutathione S-transferase; MGD, N-methyl-D-glucamine dithiocarbamate; EPR, electron paramagnetic resonance; DPI, diphenyleneiodonium chloride; PBS, phosphate-buffered saline; GSNO, S-nitrosothioglutathione.
repeated administration of organic nitrate causes sulfydryl depletion and consequent tolerance to further vasodilatation (25–28). However, other investigators have shown that there is no correlation between the concentration of endogenous thiols and the state of tolerance (29, 30). Therefore, the molecular mechanism of the interaction between organic nitrate and thiols to generate NO and S-nitrosothiol remains unclear.

To characterize the mechanism of XO-catalyzed organic nitrate reduction and elucidate the precise molecular mechanism of organic nitrate bioactivation in biological systems, electron paramagnetic resonance (EPR) spectroscopy, chemiluminescence NO analyzer, NO electrode, and immunoassay studies were performed under anaerobic conditions to characterize the initial product, the precursor of NO and nitrosothiol, and to determine the link between organic nitrate and sGC activation. These results demonstrate that XO catalyzes the bioactivation of organic nitrate to form NO and nitrosothiols in a thioldependent reaction.

EXPERIMENTAL PROCEDURES

Materials—Xanthine oxidase from buttermilk (xanthine: oxygen oxidoreductase; EC 1.1.3.22), xanthine, oxypurinol, DPL, sodium nitrite, Griess Reagent, β-NADH, bovine serum albumin (BSA), and 2,3-dihydroxybenzaldehyde (DBA) were obtained from Sigma. Soluble guanylyl cyclase was obtained from Alexis Biochemical Corp. (San Diego, CA). Direct cGMP assay kit was obtained from Assay Designs, Inc. (Ann Arbor, MI), and cGMP production was quantified by immunoassay according to the protocol provided by the company. GTN was synthesized as described previously (9). N-Methyl-D-glucamine dithiocarbamate (MGD) was synthesized using carbon disulfide and N-methyl-D-glucamine (31, 32). Ferric ammonium sulfate was purchased from Aldrich Chemical Co. (99.997%). Dulbecco’s phosphate-buffered saline (PBS) was obtained from Invitrogen. Millipore ultra free centrifugal filter (nominal molecular weight limit 10,000) was obtained from Fisher.

EPR Spectroscopy—EPR measurements were performed using a Bruker EMX spectrometer with HS resonator operating at X-band. Measurements were performed at ambient temperature with a modulation frequency of 100 kHz, modulation amplitude of 2.5 G, and a microwave power of 20 milliwatts. NO generated by XO-catalyzed reaction frequency of 100 kHz, modulation amplitude of 2.5 G, and a weight limit 10,000 was obtained from Fisher.

Electrochemical Measurements—Electrochemical measurements of NO generation by XO under anaerobic conditions were performed by using NO analyzer as described under “Experimental Procedures.”

RESULTS

Inorganic Nitrite Generation from XO-mediated Organic Nitrate Reduction—It has been reported that XO can catalyze organic nitrate reduction to NO2− (13). To investigate the mechanism of NO2− generation, as well as its possible role as an intermediate of NO in the process of XO-mediated organic nitrate reduction, the rate of nitrite formation from XO-catalyzed GTN and ISDN reduction was measured under anaerobic conditions (Fig. 1, A and B, respectively). In the presence of GTN (10 μM) or ISDN (100 μM) and reducing substrates DBA (0.5 mM), xanthine (20 μM), or NADH (1.0 mM),
after addition of XO, the reaction mixture was sampled every 2 min, and its nitrite concentration was determined by NO analyzer with reduction of nitrite to NO using 1% KI under acidic conditions. With FAD site-binding substrate NADH as an electron donor, nitrite concentrations in the reaction mixture linearly increase for the first 10 min and then gradually plateau by 30 min (Fig. 1, c). With xanthine or DBA that provides electrons to XO at the molybdenum site, the increase in nitrite concentrations plateau within 20 min, and a quicker decrease in nitrite generation rates is seen (Fig. 1, a and b).

For xanthine as reducing substrate, the initial rate of XO-mediated nitration formation was determined as a function of GTN or ISDN concentration, and Michaelis-Menten kinetics were observed with correlation coefficient $\chi^2 > 99\%$ (Fig. 2, A and B). The apparent $K_m$ and $V_{max}$ values are shown inside each curve. The apparent $K_m$ values of GTN (0.49 mM) and ISDN (1.64 mM) are far higher than the clinical levels of organic nitrates in tissues or blood. Thus, at pharmacological levels in the range of 10–100 $\mu$m, the rate of XO-mediated organic nitrate reduction would be expected to increase linearly with the given organic nitrate dose. From this kinetic data, it is possible to predict the magnitude of XO-mediated nitrite formation as a function of organic nitrate and to determine the quantitative importance of this mechanism of nitrite generation and its subsequent NO generation in a given biological system where these substrate levels are known.

Under ischemic conditions, in addition to marked hypoxia, marked intracellular acidosis also occurs, and pH values in tissues, such as the heart, can fall to levels of 6.0 or below. In order to assess the nitrite formation under different physiological or pathological conditions and to further characterize the mechanism of XO-catalyzed organic nitrate reduction, experiments were performed to measure the effects of different pH values on the magnitude of nitrite generation. Because the apparent $K_m$ values of organic nitrates is far higher than the pharmacological levels of organic nitrates in tissues or blood, we evaluated the effect of pH on XO-mediated organic nitrate reduction with a typical pharmacological level of 10 $\mu$m GTN or 100 $\mu$m ISDN. As shown in Table I, it was observed for each of the three reducing substrates (xanthine, NADH, and DBA) that under acidic conditions, increased XO-mediated nitrite generation occurs. In contrast, under alkaline conditions, decreased nitrite generation was seen.

**Nitric Oxide Generation from XO-mediated GTN or ISDN Reduction**—It is well known that organic nitrates such as GTN and ISDN are prodrugs requiring metabolism to generate bioactive NO. In order to investigate whether XO can directly catalyze the reduction of GTN and ISDN to NO and quantitate the rates of NO generation, EPR spectroscopy was applied to measure GTN- and ISDN-mediated NO generation under anaerobic conditions. NO generated by organic nitrate was purged out using argon to a purging vessel that contained the spin-trap Fe$^{2+}$-MGD. NO is paramagnetic and binds with high affinity to the water-soluble spin trap Fe$^{2+}$-MGD forming the mononitrosyl iron complex NO-Fe$^{2+}$-MGD with characteristic triplet spectrum at $g = 2.04$ and hyperfine splitting $a_N = 12.8$. From the intensity of the observed spectrum, quantitative measurement of NO generation can be performed (36–38). With ISDN (100 $\mu$m) or GTN (10 $\mu$m), in the presence of XO (0.04 mg/ml) and NADH (1 mM), no signal was seen (Fig. 3, spectra A and B). This demonstrates that no significant NO formation occurs.

It has been reported that sulphhydryl (–SH) compounds are needed for GTN activation and that repeated administration of GTN causes sulphhydryl depletion and consequent tolerance to further vasodilatation (25–28). To test whether sulphhydryl compounds play a role in XO-mediated transformation of organic nitrates, L-cysteine (5 mM) was added, and this triggered marked NO generation from 100 $\mu$m ISDN with 0.28 $\mu$m NO trapped over 30 min (Fig. 3, spectrum C) and from 10 $\mu$m GTN with 0.22 $\mu$m NO trapped over 30 min (Fig. 3, spectrum D). The XO molybdenum site inhibitor oxypurinol (100 $\mu$m) could not inhibit this NO generation from GTN or ISDN (data not shown); however, the FAD site inhibitor DPI (100 $\mu$m) effectively blocked XO-dependent NO formation (Fig. 3, spectrum E). This suggests that organic nitrate reduction takes place at the FAD site of XO.

In order to further investigate XO-mediated GTN and ISDN reduction and quantitate the rates of NO generation, studies were performed using a chemiluminescence NO analyzer. NO was purged from the solution by argon and then reacted with ozone in the analyzer to form an excited-state NO$_2$, which emits light. This method provides direct measurement of the rate of NO generation as a function of time. With GTN (10 $\mu$m), in the presence of xanthine (20 $\mu$m) or NADH (1 $\mu$m) with XO (0.04 mg/ml), no measurable rate of NO formation was observed (Fig. 4, trace D). To test the effect of sulphhydryl compounds on GTN reduction, L-cysteine (5 mM) was added, and prominent NO generation was triggered (Fig. 4, traces A and B). In the absence of XO, cysteine alone reacted with GTN to produce NO, but only trace production was seen (Fig. 4, trace C). XO-mediated ISDN reduction was similar to that of GTN reduction. In the presence of XO (0.04 mg/ml), ISDN (0.1 mM), and xanthine or NADH as reducing substrate, no detectable NO was generated (Fig. 5, trace C). However, after addition of L-cysteine, prominent NO generation was seen (Fig. 5, traces A and B). With ISDN and cysteine alone, no detectable NO production was seen (data not shown).

To further confirm these observations and quantitate the NO concentration that accumulates, NO generation was measured by electrochemical detection. Prior to the addition of XO, no detectable NO was seen. After the addition of XO (0.04 mg/ml), NO was triggered from L-cysteine (5 mM) with ISDN (1 mM or 0.1 mM) and NADH (1 mM) in the presence of L-cysteine (5 mM).
**Xanthine Oxidase-mediated Organic Nitrate Biotransformation**

The initial nitrite generation rates were calculated as described in Fig. 1. Measurements were performed with XO (0.04 mg/ml) in the presence of GTN (10 μM) or ISDN (100 μM) with reducing substrate.

|                  | pH 6.0     | pH 7.4     | pH 8.0     |
|------------------|------------|------------|------------|
| NADH (1 mM) + GTN (10 μM) | 0.11 ± 0.02 | 0.06 ± 0.01 | 0.04 ± 0.01 |
| NADH (1 mM) + ISDN (100 μM) | 0.21 ± 0.03 | 0.10 ± 0.01 | 0.06 ± 0.01 |
| Xanthine (20 μM) + GTN (10 μM) | 0.56 ± 0.07 | 0.31 ± 0.03 | 0.17 ± 0.02 |
| Xanthine (20 μM) + ISDN (100 μM) | 0.77 ± 0.07 | 0.46 ± 0.06 | 0.22 ± 0.03 |
| DBA (0.5 mM) + GTN (10 μM) | 0.33 ± 0.05 | 0.17 ± 0.03 | 0.10 ± 0.02 |
| DBA (0.5 mM) + ISDN (100 μM) | 0.56 ± 0.07 | 0.32 ± 0.04 | 0.16 ± 0.02 |

(Fig. 6, traces A and B), whereas no NO generation was observed without L-cysteine (Fig. 6, trace C).

It is commonly known that the combination of ascorbate with GTN or ISDN reduces the occurrence of nitrate tolerance. To investigate the role of ascorbate in the activation of organic nitrate, NO generation from XO-mediated ISDN reduction was detected with or without ascorbate. Without XO, no detectable NO was generated with ascorbate (1 mM), ISDN (0.1 mM), and xanthine (20 μM) or NADH (1 mM) (Fig. 7, trace C). However, upon addition of XO (0.04 mg/ml), prominent NO generation was triggered from ISDN (100 μM) using xanthine or NADH as reducing substrates (Fig. 7, traces A and B).

**Assay of Nitrosothiol Formation from XO-mediated GTN or ISDN Reduction**—It has been reported that thiol such as glutathione (GSH) react with labile organic nitrite esters, as could be formed from reduction of organic nitrates, to give rise to corresponding S-nitrosothioles either chemically or enzymatically, and these S-nitrosothioles, in turn, can serve as a precursor for NO formation (39–43). To investigate whether the initial product of XO-mediated reduction of organic nitrate is organic nitrite (R-O-NO) and whether this organic nitrite can be the nitrosothiol precursor, studies were performed to measure the S-nitrosothiol (GSNO) production from XO-catalyzed organic nitrate reduction in the presence of GSH (Fig. 8).

Following incubation at 37 °C under anaerobic conditions (argon), the reaction mixture was sampled at the time indicated, and the detection and quantification of nitrosothioles in the solution were performed using the Saville Assay, which is based on mercury ion-mediated heterolysis cleavage of the...
Fig. 6. Electrochemical measurement of NO generation from XO-mediated ISDN reduction. The time course of NO generation was measured using an electrochemical NO sensor under anaerobic conditions at 37 °C in PBS (10% BSA), pH 7.4. The arrow shows the time at which XO (0.04 mg/ml) was added. Trace A shows the data from ISDN (1 mM) in the presence of NADH (1 mM) and L-cysteine (5 mM); trace B shows the data from ISDN (100 μM) in the presence of NADH (1 mM) and L-cysteine (5 mM); trace C shows the data from ISDN (1 mM) in the presence of NADH (1 mM).

Fig. 7. Measurement of the rate of NO generation from Xo-catalyzed ISDN reduction in the presence of ascorbate. Measurements were performed using a chemiluminescence NO analyzer under anaerobic conditions at 37 °C in PBS (10% BSA), pH 7.4. Trace A shows the rate of NO generation by XO (0.04 mg/ml), xanthine (20 μM), ISDN (100 μM), and ascorbate (1 mM). Trace B shows the rate of NO generation by XO (0.04 mg/ml), NADH (1 mM), ISDN (100 μM), and ascorbate (1 mM). Trace C shows the rate of NO generation by ascorbate (1 mM), xanthine (20 μM), and ISDN (100 μM).

Fig. 8. Nitrosothiol production from XO-mediated GTN or ISDN reduction. GTN (A, 100 μM) or ISDN (B; 1 mM) was incubated in PBS (10% BSA), pH 7.4, under anaerobic conditions at 37 °C with the following: a, NADH (1 mM), GSH (5 mM), and XO (0.04 mg/ml); b, NADH (1 mM), GSH (5 mM), and oxypurinol; c, NADH (1 mM), GSH (5 mM), XO (0.04 mg/ml), and DPI (100 μM); or d, NADH (1 mM), GSH (5 mM). The reaction mixture was sampled at 10, 15, 20, 25, and 30 min. Detection and quantification of nitrosothiols were performed as described under “Experimental Procedures.”

S-NO bond. Nitrosium ion is released, which is in turn measured by the Griess Reaction. The concentrations of GSNO were determined from the increase in the nitrite concentration induced by treatment of samples with HgCl₂. Without XO, GTN (100 μM) or ISDN (1 mM) chemically react with GSH (5 mM) with formation of low levels of GSNO of 0.21 μM (Fig. 8A, d) or 0.11 μM (Fig. 8B, d), respectively, after a 30-min incubation. With the addition of XO (0.04 mg/ml), GSNO production from GTN quickly increased to 1.38 μM within 20 min and then gradually plateaued (Fig. 8A, a). GSNO production from ISDN and XO showed similar kinetics with 2.05 μM GSNO production after 20 min. Using the molybdenum site inhibitor oxyxypurinol, XO-dependent nitrosothiol production was not inhibited (Fig. 8, b). When the FAD site inhibitor DPI (100 μM) was used, it completely inhibited XO-dependent nitrosothiol production (Fig. 8, c). This suggests that organic nitrate reduction takes place at the FAD site of XO.

Effect of XO-mediated GTN or ISDN Reduction on sGC Activity—In order to determine whether XO-mediated organic nitrate biotransformation can induce sGC activation, enzyme-linked immunoassays were performed to determine the cGMP concentrations in the reaction mixture. After incubation of XO (0.04 mg/ml) with GTN (10 μM) or ISDN (100 μM) in reaction buffer (10 ng of sGC, 10% BSA, 5 mM EDTA, 2 mM MgCl₂, 1 mM GTP, and 1 mM NADH or 20 μM xanthine in 1 ml of PBS) under anaerobic conditions for 10 min, measurements of the sGC product cGMP were performed. Without L-cysteine, with NADH or xanthine as XO-reducing substrates, no cGMP could be detected, suggesting that sGC was not activated (Fig. 9, A). However, for similar conditions with the addition of L-cysteine (5 mM), a significant amount of cGMP production was generated from XO-mediated reduction of GTN (Fig. 9, B) or ISDN (Fig. 9, C). Using the molybdenum site-specific inhibitor oxyxypurinol, XO-mediated sGC activation by organic nitrate was largely inhibited (>95%) when xanthine was used as the reducing substrate, but not when NADH was used (Fig. 9, D). When the FAD site inhibitor DPI was used, it inhibited XO-mediated sGC activation by GTN regardless of the type of reducing substrate present (Fig. 9, E), further confirming that organic nitrates are reduced at the FAD site.

DISCUSSION

Organic nitrates (R-ONO₂) such as GTN and ISDN have been widely used clinically in the treatment of myocardial ischemia. Over the last decade, their efficacy has been attributed to their metabolic conversion to NO. However, the molec-
ular mechanism of this organic nitrate biotransformation has not been fully defined. Questions remain concerning the initial product, the precursor of NO, and the link of organic nitrate to the activation of sGC. There is further uncertainty regarding how this process occurs under the markedly hypoxic conditions in ischemic tissues. Recently, XO has been reported to be able to mediate organic nitrate and inorganic nitrate/nitrite reduction under anaerobic conditions (11–13). To investigate the mechanism of XO-mediated organic nitrate reduction and thus further elucidate the precise molecular mechanism of bioactivation, we performed a series of studies using EPR spectroscopy, chemiluminescence NO analyzer, immunobassay, and NO electrode techniques to measure the magnitude of various products (NO₂⁻, NO, and R-S-NO) derived from XO and their effects on activation of sGC.

It was observed that each of the typical XO reducing substrates xanthine, DBA, and NADH could act as electron donors to support this XO-mediated GTN/ISDN reduction (Figs. 1 and 2). However, the time-dependent nitrite concentrations in the reaction mixture showed progressively decreased nitrite generation rates (Figs. 1 and 2). This is consistent with the previous report of suicide inhibition in the process of XO-mediated GTN/ISDN reduction (13).

For xanthine as a reducing substrate, the rate of XO-mediated nitrite formation was determined as a function of GTN or ISDN concentrations, and Michaelis-Menten kinetics were observed (Fig. 2, A and B). From these kinetic data, comparing the kinetics of XO-mediated inorganic nitrate (NO₃⁻) reduction (12), with the same substrate levels, nitrite formation rates are about 5 times higher for ISDN reduction and about 30 times higher for GTN reduction than from nitrate anion (NO₃⁻) reduction. Similar to NO₂⁻ reduction (12), XO-mediated reduction of organic nitrates also takes place via an acid-catalyzed mechanism (Table 1).

Traditionally, it has been assumed that NO₂⁻ production is the first step in the process of organic nitrate biotransformation and that it is the precursor of NO or nitrosohiol. It has been assumed that organic nitrates are converted to NO₂⁻ by reaction with sulfhydryls (–SH); nitrite then liberates NO via nitrous acid, and NO combines with thiol to generate a nitrosothiol that activates sGC (3).

\[
\begin{align*}
R-SH + R-O-NO₂ & \rightarrow R-S-NO₂ + ROH \\
R-S-NO₂ + R-SH & \rightarrow R-S-S-R + H^+ + NO₂ \\
NO₂ + H^+ & \rightarrow HONO \\
HONO & \rightarrow NO \\
R-SH + NO & \rightarrow R-S-NO \\
\end{align*}
\]

RECTIONS 1–5

However, controversy remains regarding whether this nitrite can be an active intermediate in the vascular metabolism of organic nitrates to form NO (15–20). Normally, the concentrations of NO₂⁻ in the serum or tissues range from 1 to 10 μM, and it has been questioned how micromolar to nanomolar levels of GTN, the clinically relevant concentrations, can produce sufficient NO₂⁻ to serve as an intermediate for the generation of sufficient NO to exert vasodilatory effects (21). Studies in whole tissue or in isolated mitochondria have shown that clinically used GTN levels cannot produce sufficient NO₂⁻ as intermediate of NO to exhibit vasodilatory effects (15, 19, 22–24). However, long term high dose administration of organic nitrates can highly elevate nitrate anion (NO₃⁻) or NO₂⁻ concentrations in tissues (44), and these can be important sources of NO during ischemia (11, 12, 14, 45–47).

It is well known that sulfhydryl (–SH) compounds are needed in GTN activation and that the repeated administration of GTN causes sulfhydryl depletion and consequent tolerance to further vasodilatation. In this study, it was shown that the presence of l-cysteine or ascorbate triggered significant NO generation from XO-mediated reduction of GTN/ISDN, whereas no detectable NO was generated without the addition of thiols or ascorbate (Figs. 3–7). Of note, at pH 7.4, the presence of sulfhydryls or ascorbate does not increase NO generation from XO-mediated NO₂⁻ reduction. All these experimental results suggest that NO₂⁻ is not the critical intermediate of NO in the process of XO-mediated reduction of GTN or ISDN.

It has been reported that thiols such as GSH can react with organic nitrates to form the corresponding S-nitrosothiols either chemically or enzymatically, and this S-nitrosothiol, in turn, can serve as a precursor to NO formation (39–43). In this study, besides observation of NO generation in the process of XO-mediated organic nitrate reduction, GSNO production was also observed in the presence of GSH. This XO-dependent GSNO production was inhibited in the presence of the FAD site inhibitor DPI (Fig. 8). All these results suggest that organic nitrite (R-O-NO) is the initial product in the process of XO-mediated organic nitrate reduction and the precursor of NO and nitrosohiol. The presence of l-cysteine or ascorbate can reduce organic nitrite further, triggering significant NO generation. Of note, even with this competitive reduction, the maximum rate of NO production is an order of magnitude lower than the rate of nitrite production from organic nitrite hydrolysis (Figs. 3–7). This suggests that under hydrophilic conditions, most of the organic nitrite (R-O-NO) will quickly hydrolyze to produce NO₂⁻ (Figs. 1 and 2). However, under hydrophobic conditions, NO and nitrosohiols could be major products of the reaction of organic nitrite (R-O-NO) with sulfhydryl compounds either chemically (39) or enzymatically as catalyzed by glutathione S-transferase (GST) (41, 42, 48).

Activation of sGC by XO-mediated organic nitrate reduction was investigated by employing enzyme-linked immunobassay. Our results showed that sulfhydryl compounds or ascorbate is needed for the activation of sGC. Without sulfhydryl compounds or ascorbate, sGC is not activated by GTN or ISDN (Fig. 9). The flavin-binding XO inhibitor DPI inhibited XO-mediated organic nitrate reduction and sGC activation, indicating that organic nitrate reduction occurs at the flavin site.

The following reactions define the steps in the reaction mechanism of XO-mediated biotransformation of organic nitrate:

\[
\begin{align*}
E_{\text{ox}} + \text{RONO}_2 + 2\text{H}^+ & \rightarrow \text{R-O-NO} + \text{H}_2\text{O} + E_{\text{red}} \\
\text{R-O-NO} + \text{H}_2\text{O} & \rightarrow \text{R-OH} + \text{HNO}_2 \\
\text{R-O-NO} + \text{R-SH or ascorbate...} & \rightarrow \text{NO} + \text{ROH} \\
\text{R-O-NO} + \text{R-SH} & \rightarrow \text{R-OH} + \text{R-S-NO} \\
\end{align*}
\]

REATIONS 6–9

\[
E_{\text{ox}} \text{ is the oxidized enzyme, and } E_{\text{red}} \text{ is the reduced enzyme.}
\]

Organic nitrite (R-O-NO) is the initial product in the process of organic nitrate biotransformation and the precursor of NO and nitrosohiol. Thus, organic nitrite (R-O-NO) is the link between organic nitrate and activation of sGC.

Several enzyme systems are involved in the biotransformation of organic nitrate, including GST (49, 50), cytochrome P450 systems (6, 51, 52), xanthine oxidoreductase (13), and mitochondrial aldehyde dehydrogenase (53). These enzymes can be classified into two groups: (a) flavoenzymes, including xanthine oxidoreductase and cytochrome P450/P450 reductase systems; and (b) enzymes that have sulfhydryl (–SH) groups in the active site, such as GST and mitochondrial aldehyde dehydrogenase.
It has been demonstrated previously that the activity of XO in the postischemic rat heart is 16.8 milliunits/g protein (36), which corresponds to 0.013 mg XO/g protein or ~3.4 μg/g cell water. The total XO and xanthine dehydrogenase activity, however, is 10-fold above this value. During ischemia, xanthine levels rise from near zero to values on the order of 10–100 μM. Tissue or blood sulfhydryl compound concentrations are typically in the range of 0.5–2 mM under normal physiological conditions (54, 55). Thus, pharmacological levels of GTN (10–25 μM) or ISDN (100–250 μM) in the heart could generate about 1–2 m/s NO from xanthine oxidoreductase-mediated reduction under anaerobic conditions, which is comparable to maximal NO production from tissue NO synthases. In the rat liver, XO activity is about 10-fold above the levels in heart (56), and thus NO generation from organic nitrite in liver could be 10-fold higher.

Previous studies have shown that free flavin can mediate organic nitrate reduction. This study further demonstrated that the flavin site of XO catalyzes reduction of organic nitrate to organic nitrite. Our results suggest that multiple flavoenzymes may be able to similarly catalyze this reaction and may also be involved in the biotransformation of organic nitrates to form NO.

Besides flavoenzymes, GST and mitochondrial aldehyde dehydrogenase also have been reported to play important roles in organic nitrate bioactivation. Both GST and mitochondrial aldehyde dehydrogenase have sulfhydryl (~SH) groups in the active site. Previous studies have shown that nucleophile attack by the active site sulfhydryl groups of GST or mitochondrial aldehyde dehydrogenase (mtALDH) on organic nitrate (R-O-NO₂) can result in the formation of E-S-NO₂ (57–59), whereas compared with organic nitrate (R-O-NO₂), organic nitrite (R-O-NO) would be predicted to much more readily react with sulfhydryl compounds to form nitrosothiols or NO.

R-O-NO + R-SH (GST, mtALDH . . .) → R-OH + R-S-NO

These two enzymatic systems may synergistically act in the process of organic nitrate biotransformation. First, flavoenzymes would be required to catalyze the reduction of organic nitrate to produce organic nitrite; then enzymes such as GST and mitochondrial aldehyde dehydrogenase could further catalyze reaction of organic nitrite and thiols to produce nitrosothiols.

Overall, this study demonstrates that XO can reduce GTN and ISDN to their respective organic nitrates (R-O-NO). These organic nitrates (R-O-NO) then react chemically or enzymatically with thiols or other reducing substrates to form nitrosothiols or NO. Furthermore, the depletion of thiols or inhibition of crucial enzyme(s) may cause nitrate tolerance. Nitrite anion (NO₂⁻) is the hydroslysis product of organic nitrite, which is a byproduct rather than an important intermediate in the process of organic nitrate bioactivation. Thus, organic nitrite (R-O-NO) is the link between organic nitrate and activation of sGC.

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