THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 26, pp. 17855–17863, June 27, 2008

Nitric Oxide Production from Nitrite Occurs Primarily in Tissues Not in the Blood

*CRITICAL ROLE OF XANTHINE OXIDASE AND ALDEHYDE OXIDASE**

Received for publication, March 5, 2008, and in revised form, April 17, 2008 Published, JBC Papers in Press, April 18, 2008, DOI 10.1074/jbc.M801785200

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Recent studies have shown that nitrite is an important storage form and source of NO in biological systems. Controversy remains, however, regarding whether NO formation from nitrite occurs primarily in tissues or in blood. Questions also remain regarding the mechanism, magnitude, and contributions of several alternative pathways of nitrite-dependent NO generation in biological systems. To characterize the mechanism and magnitude of NO generation from nitrite, electron paramagnetic resonance spectroscopy, chemiluminescence NO analyzer, and immunoassays of cGMP formation were performed. The addition of nitrite triggered a large amount of NO generation in tissues such as heart and liver, but only trace NO production in blood. Carbon monoxide increased NO release from blood, suggesting that hemoglobin acts to scavenge NO not to generate it. Administration of the xanthine oxidase (XO) inhibitor oxypurinol or aldehyde oxidase (AO) inhibitor raloxifene significantly decreased NO generation from nitrite in heart or liver. NO formation rates increased dramatically with decreasing pH or with decreased oxygen tension. Isolated enzyme studies further confirm that XO and AO, but not hemoglobin, are critical nitrite reductases. Overall, NO generation from nitrite mainly occurs in tissues not in the blood, with XO and AO playing critical roles in nitrite reduction, and this process is regulated by pH, oxygen tension, nitrite, and reducing substrate concentrations.

Nitric oxide is a free radical produced in biological tissues that exerts a large number of critical regulatory functions and also plays an important role in the pathogenesis of cellular injury (1). In addition to NO generation from specific NO synthases (NOSs),[3] it is now clear that nitrite can also be an important source of NO, particularly under acidic conditions (2). However, the mechanism of nitrite reduction in biological systems remains highly controversial. Furthermore, it is unknown whether NO formation from nitrite occurs primarily in tissues or in blood. Questions remain regarding the mechanism, magnitude, and contributions of the several proposed pathways of nitrite-dependent NO generation in biological systems.

Recent studies reported a novel role for hemoglobin as a nitrite reductase reducing nitrite to NO and eliciting vasodilation. Based on these studies, erythrocytes were proposed to be the major intravascular site of nitrite reduction and storage (3–8). However, it is well known that hemoglobin is an extremely effective NO scavenger (9, 10). The mechanism by which NO escapes trapping by the high concentrations of hemoglobin in the blood remains a mystery with resultant controversy regarding the relative importance of nitrite reduction in blood and tissues. Therefore there is a great need to elucidate the relative importance and molecular mechanisms of NO formation from nitrite in blood and tissues.

Initially nitrite was considered as a product of NO metabolism, not a source of NO, in tissues. In 1995, we observed that nitrite can be a prominent source of NO in biological tissues under conditions of intracellular acidosis, as occur following the onset of ischemia with a lack of tissue perfusion (2). Several alternative pathways of nitrite-dependent NO generation have been observed occurring in biological systems. NO formation can occur by the simple process of nitrite disproportionation (2, 11–14). Several enzymatic systems have been reported to be involved in nitrite reduction with NO generation in biological systems. Xanthine oxidase (XO) has a similar structure to bacterial nitrite reductase (15). XO reduces nitrite to NO at the molybdenum site of the enzyme with xanthine, NADH, or aldehyde substrates serving to provide the requisite reducing equivalents (16–20). This NOS-independent NO production could serve as a source of NO under ischemic conditions where NO production from NOS is significantly impaired (17–19). Aldehyde oxidase (AO) is a cytosolic enzyme that plays an important role in the biotransformation of drugs and xenobiotics (21). The amino acid sequences of AO and XO are remarkably similar, with ~86% homology, and they belong to the same family of molybdenum-containing proteins with two iron-sulfur clusters, a flavin cofactor, and a molybdopterin cofactor (22, 23).

* This work was supported, in whole or in part, by National Institutes of Health Grants HL63744, HL65608, and HL38324 (to J. L. Z.). This work was also supported by Grant BGIA-0565249B from the Ohio Valley Affiliate of the American Heart Association (to H. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[3] The abbreviations used are: NOS, nitric-oxide synthase; Hb, hemoglobin; AO, aldehyde oxidase; XO, xanthine oxidase; XOR, xanthine oxidoreductase; sGC, soluble guanylyl cyclase; CP, cytochrome P-450; MGD, *N*-methyl-D-glucamine dithiocarbamate; EPR, electron paramagnetic resonance; PBS, phosphate-buffered saline.

However, no prior research has been done to investigate whether AO has the similar ability to catalyze nitrite reduction to NO. Furthermore, the mechanism, magnitude, and quantitative importance of AO-mediated nitrite reduction in biological systems has not been studied. In addition to the molybdenum enzymes XO and AO, ubiquinone/cytochrome bc1 complex of the mitochondrial electron chain also has been identified as a site where nitrite is reduced (24, 25). Nitrite can also accept electrons from cytochrome P-450 (CP) of liver microsomes (26, 27). In the presence of excessive nitrite, significant NO generation from CP was detected, suggesting that CP-mediated nitrite reduction can be a source of NO (28). It is now certain that there are multiple pathways of NO generation from nitrite in biological systems, but questions remain concerning the localization, magnitude, mechanism, and biological significance of these NOS-independent sources of NO under physiological or pathological conditions.

To characterize the mechanism and magnitude of NO generation from nitrite in tissues and blood, electron paramagnetic resonance (EPR) spectroscopy, chemiluminescence NO analyzer, NO electrode, and immunoassays of cGMP formation were performed. These studies demonstrate that NO is generated largely from nitrite reduction in tissues not in the blood. Hemoglobin in red cells is observed to function as a trap rather than a functional source of NO. The molybdenum enzymes XO and AO are shown to be important sources of nitrite-derived NO in tissues with this nitrite-dependent NO generation regulated by pH, oxygen tension, nitrite, and reducing substrate concentrations.

EXPERIMENTAL PROCEDURES

Materials—XO, NADH, sodium nitrite, bovine hemoglobin, oxypurinol, dithiothreitol, raloxifene chloride, clotrimazole, rotenone, antifoam, and N^G-methyl-L-arginine were obtained from Sigma-Aldrich. Soluble guanylyl cyclase (sGC) was obtained from Alexis Biochemical Corporation (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. *N*-Methyl-D-glucamine dithiocarbamate (MGD) was synthesized using carbon disulfide and *N*-methyl-D-glucamine (29, 30). Ferrous ammonium sulfate was purchased from Aldrich (99.997%). [^{15}N]Nitrite was obtained from Cambridge Isotope Laboratories, Inc. 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.

Rat Tissues and Blood Preparation—Male Sprague-Dawley rats (250–300 g) were heparinized with 500 units of heparin and anesthetized with intraperitoneal pentobarbital at a dose of 30–35 mg/kg. Rat blood was obtained from the abdominal aorta using a 10-ml syringe containing 0.1 ml of heparin. The livers, hearts, and aorta rings were excised and washed with PBS to remove any residual blood and then minced into small pieces (~0.01 g/piece). This minced tissue was then rinsed again with PBS to assure wash out of any remaining RBCs. The reaction mixture was placed in a purging vessel with 50 μl of antifoam to prevent foaming during the gentle purging and maintained at 37 °C.

Hemoglobin Preparation—Oxyhemoglobin was obtained from hemoglobin (2 mM) with the addition of excess sodium dithionate, followed by separation on a Sephadex G-25 column (31). Deoxyhemoglobin was obtained from oxyhemoglobin with the purging of argon.

EPR Spectroscopy—EPR measurements were performed using a Bruker EMX spectrometer with an HS resonator operating at X-band. The measurements were performed at ambient temperature with a modulation frequency of 100 kHz, modulation amplitude of 2.5 G, and microwave power of 20 milliwatt. ^{15}NO generated from the reaction solution was purged out using argon to a vessel that contained 5 ml of the Fe^{2+}-MGD spin trap solution, as previously described (19). This set-up was designed to isolate the reaction solution from the spin trap and thus avoid any possible perturbation caused by Fe^{2+}-MGD complexes on the reaction system. Fe^{2+}-MGD complexes were prepared by adding solid ferrous ammonium sulfate and MGD (molar ratio, 1:5) to the deoxygenated (argon-purged) solution with a final concentration of 2 mM in iron. Quantitation of NO formation and trapping were performed by double integration of observed EPR signal in comparison with that from a similar aqueous NO-iron-MGD standard (19).

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 a 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 controlled temperature in a glass purging vessel equipped with heating jacket. 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. After an initial 30 s of equilibration of the flow from the purging vessel to the detector, the signal provides the rate of NO formation over time (32). Calibration of the magnitude of NO production was determined from the integral of the signal over time compared with that from nitrite concentration standards (14, 18).

Compared with measurements performed in argon, the efficiency of NO measurement in air or 10, 5, or 2% oxygen/nitrogen, was 91.9, 92.5, 96.3, or 98.6%, respectively, The results showed that the chemiluminescence linearly increased with NO generation when purging with any of these gas mixtures ($\gamma^2 > 99\%$) (19).

Purification of AO from Rat Liver—Liver AO was purified as we recently reported (33). AO activity was determined by the oxidation of AO substrate; one unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of substrate 4-(dimethylamino) cinnamaldehyde/min at 30 °C (33).

Immunoassay of Guanylyl Cyclase Activity—Activation of soluble sGC was investigated by employing enzyme-linked immunoassay. After incubation of tissues pieces (0.2 g/ml) with 10 μM nitrite and with 1 ml of reaction buffer (10 ng of sGC, 5 mM EDTA, 2 mM $MgCl_2$, and 1 mM GTP in 1 ml of PBS) at 37 °C under anaerobic conditions, the tissues in the samples were

removed using Millipore filter (centrifuged 2000 × g, 10 min, at 4 °C). Comparative study in blood instead of tissues was performed by incubation of nitrite (10 μM) with 10 ng of sGC, 2 mM $MgCl_2$, and 1 mM GTP in 1 ml of blood. The measurements of cGMP in the solution were performed using direct cGMP assay kit by immunoassay according to the manufacturer's product protocol. The standard curve was obtained with known amounts of cGMP.

Statistical Analysis—The values are expressed as the means of at least three repeated measurements and reported ± S.D., unless noted otherwise. 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.

RESULTS

NO Generation from Tissues, Blood, and Hemoglobin—To investigate the mechanism and magnitude of NO generation from nitrite in tissues and blood, EPR spectroscopy was applied to directly measure NO generation under anaerobic conditions. Rat tissue or blood was subjected to hypoxia in the presence of isotopically labeled nitrite. Rat tissue (liver, heart, or vessels) was isolated and cut into pieces (~0.01 g) suspended in PBS and purged with argon. NO production from [^{15}N]nitrite in anoxic tissue or blood was measured by EPR spectroscopy. ^{15}NO generated was purged from the reaction vessel using argon to a secondary vessel containing the spin trap Fe^{2+}-MGD. ^{15}NO is paramagnetic and binds with high affinity to the water-soluble spin trap Fe^{2+}-MGD forming the mononitrosyl iron complex that exhibits a characteristic doublet ^{15}NO-Fe^{2+}-MGD spectrum, rather than the triplet observed with natural abundance ^{14}NO, enabling direct and selective detection of nitrite-derived NO formation. From the intensity of the observed spectrum, quantitative measurement of NO generation can be performed (2, 34, 35).

With 5 ml of rat blood in the reaction vessel, without the addition of nitrite, no NO generation was detected (Fig. 1*A*). Following the addition of 100 μM nitrite, still no signal was seen. Even with addition of 500 μM nitrite, only a trace NO signal appeared after 30 min (Fig. 1*B*). After gently purging the blood with CO gas for 2 min, followed by the addition of nitrite (500 μM), the trapped NO signal was more than 2-fold higher than that without prepurging of CO (Fig. 1*C*). This experiment indicated that with binding of CO to hemoglobin, which blocks its heme group, significantly increased NO release was detected from blood. In contrast to the very low level of NO production in blood, high level production was seen from the tissues studied. When 1 g of minced aortic ring, heart, or liver was suspended in 5 ml of PBS (pH 7.0), the addition of nitrite (100 μM) triggered marked NO generation over 30 min (Fig. 1, *D–F*). Over 30 min, the NO generation was greater in liver (211 ± 34 nM), heart (132 ± 21 nM), or aorta (82 ± 11 nM) than in blood (5 ± 1 nM without CO, or 15 ± 2 nM in the presence of CO, both with 5-fold higher nitrite levels than in the tissue studies).

To further quantitate the rates of NO generation from nitrite, 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 excited state NO_2, which emits light. For the purging vessel and flows used, it takes about 30 s for NO to reach the detector. Only after this 30-s delay is the signal recorded by the detector proportional to the rate of NO generation in the reaction chamber. This method provides direct measurement of the rate of NO generation as a function of time. With the addition of nitrite (100 μM), prominent NO formation was seen from 1 g of minced liver, heart, or aortic ring in 5 ml of PBS (pH 7.0) (Fig. 2*A*, *traces a–c*). However, in the presence of 5 ml of blood, with the addition of 100 μM nitrite, no significant NO generation was seen. With a 5-fold higher nitrite concentration (500 μM) added to 5 ml of blood under anaerobic conditions, still only trace amount of NO generation was seen (Fig. 2*B*, *trace b*). Further studies were performed using blood (5 ml) pretreated with carbon monoxide. After gentle purging with CO gas for 2 min, the addition of nitrite (500 μM) triggered greatly increased NO release from the blood (Fig. 2*B*, *trace a*).

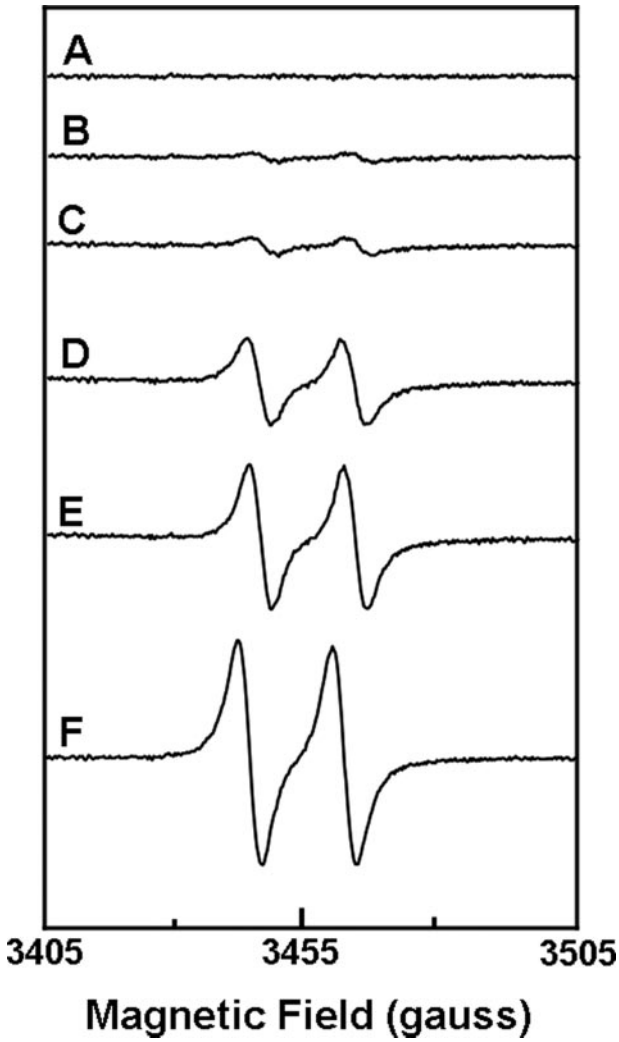

FIGURE 1. **EPR measurement of NO generated from nitrite under anaerobic conditions.** *A*, [^{15}N]nitrite (500 μM). *B*, blood (5 ml) and [^{15}N]nitrite (500 μM). *C*, blood (5 ml) pretreated with CO and [^{15}N]nitrite (500 μM). *D*, aorta ring (1 g of tissue), and [^{15}N]nitrite (100 μM) in 5 ml of PBS, pH 7.0, at 37 °C. *E*, heart (1 g of tissue) and [^{15}N]nitrite (100 μM) in 5 ml of PBS, pH 7.0, at 37 °C. *F*, liver (1 g of tissue) and nitrite (100 μM) in 5 ml of PBS, pH 7.0, at 37 °C. The reactions were performed in the reaction vessel with NO continuously purged using argon from the reaction vessel into a trap vessel containing 5 ml of 2 mM $(MGD)_2$-Fe^{2+}. The samples were taken from the trap vessel after 30 min, and the spectra of the $(MGD)_2$-Fe^{2+}-^{15}NO adducts formed are shown.

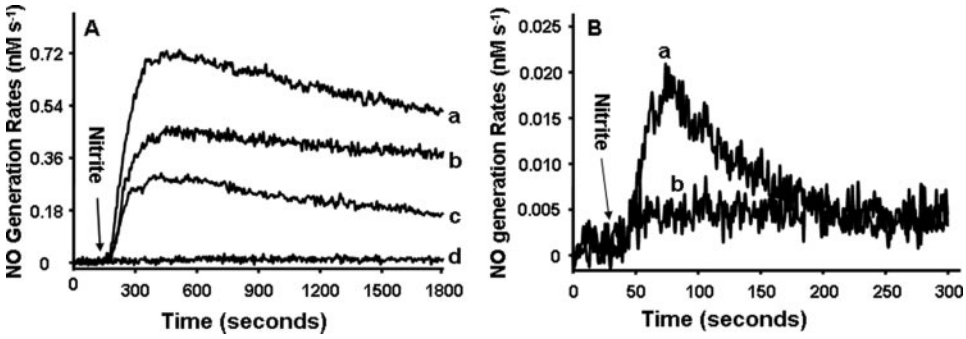

FIGURE 2. **Measurement of the rate of NO generation from nitrite in liver, heart, aorta, and blood, under anaerobic conditions.** *A*, measurements were performed using a chemiluminescence NO analyzer under anaerobic conditions at 37 °C in 5 ml of PBS, pH 7.0. *Trace a* shows the rate of NO generation by liver (1 g of tissue), and nitrite (100 μM). *Trace b* shows the rate of NO generation by heart (1 g of tissue) and nitrite (100 μM). *Trace c* shows the rate of NO generation by aorta (1 g of tissue) and nitrite (100 μM). *Trace d* shows the rate of NO generation by 5 ml of blood and nitrite (100 μM). *B*, *trace a* shows the rate of NO generation from 5 ml of blood pretreated with CO (purging with CO gas for 2 min), in the presence of nitrite (500 μM). *Trace b* shows the rate of NO generation from 5 ml of blood and nitrite (500 μM).

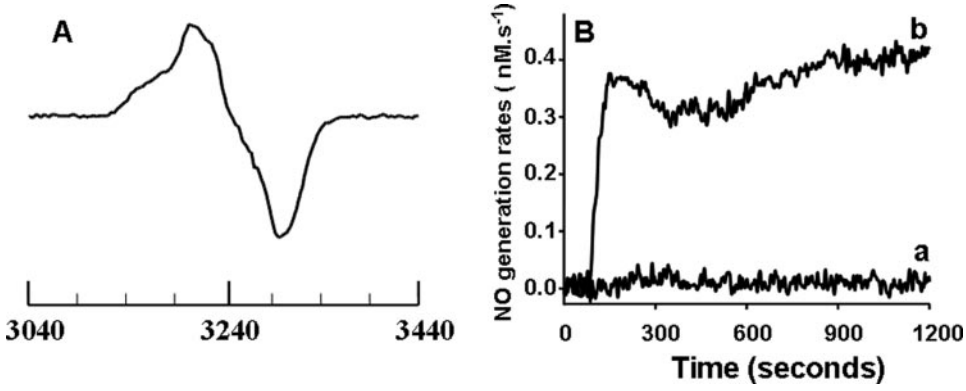

FIGURE 3. **Hemoglobin reaction with nitrite.** *A* shows the electron paramagnetic resonance spectrum of nitrosylhemoglobin formed from nitrite-mediated hemoglobin oxidation under anaerobic conditions. 200 μM deoxyHb and 200 μM nitrite were mixed under anaerobic conditions for 15 min and frozen in liquid nitrogen. EPR measurements were performed using a Bruker EMX spectrometer with HS resonator operating at X-band. The spectrum was recorded at 77 K with a microwave frequency of 9.165 GHz using 20 mw of microwave power and modulation amplitude of 3.98 G. *B* shows the rates of NO generation from a hemoglobin (200 μM) reaction with 200 μM nitrite (*trace a*) or 10 mM nitrite (*trace b*) measured by chemiluminescence NO analyzer under anaerobic conditions as described in the legend to Fig. 2.

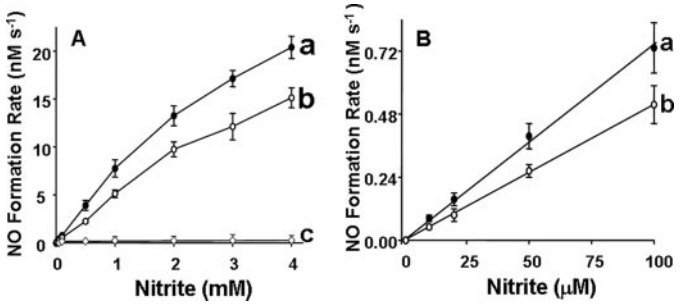

FIGURE 4. **Kinetics of NO generation as a function of nitrite concentration in liver, heart, and blood.** The maximum rates of nitrite production were measured by chemiluminescence NO analyzer under anaerobic conditions as described in the legend to Fig. 2. *A* shows the nitrite concentration dependence of the rate of NO generation over a large concentration range from liver (*line a*, 1 g of tissue) or heart (*line b*, 1 g of tissue) in 5 ml of PBS or from 5 ml of blood *(line c*), all at 37 °C. *B* shows an expanded graph for the lower concentration range of physiological or pathophysiological levels of nitrite (0.01–0.1 mM). For liver (*line a*) and heart (*line b*), linear dependence of NO generation rate *versus* nitrite concentration was observed, with linear regression $\gamma^2 > 0.99$.

To further characterize hemoglobin-mediated nitrite reduction and quantitate NO release in this process, low temperature EPR studies of isolated hemoglobin were performed. Deoxyhemoglobin (200 μM) and nitrite (200 μM) were mixed under anaerobic conditions for 15 min and then frozen in liquid nitrogen. The formation of nitrosylhemoglobin was observed by EPR spectroscopy at 77 K (Fig. 3). However, with purging no NO release was detected by EPR spin trapping, or chemiluminescence NO analyzer, under either anaerobic conditions or different oxygen tensions (purging with argon, air, or 2, 5, or 10% oxygen; data not shown). Similarly with NO electrode (17), no NO release was detected from deoxyhemoglobin (200 μM) and nitrite (200 μM). Even with higher nitrite concentrations of 1 mM, no significant NO release was detected. Only with the presence of large excess amounts of nitrite (10 mM) was NO release observed from hemoglobin (200 μM) (Fig. 3*B*, *trace b*). In contrast to reports that deoxyhemoglobin can reduce nitrite to NO and that erythrocytes are the major source of nitrite reduction in blood (3–8), we observed no release of NO from blood unless there was a large excess of nitrite.

Kinetics of Nitrite-dependent NO Generation—To further characterize the mechanism and magnitude of nitrite-dependent NO formation, kinetic studies of the effects of nitrite concentration on the magnitude of NO generation were performed. The rate of NO formation derived from nitrite reduction was measured under anaerobic conditions using the NO analyzer. Following the addition of nitrite (0.01 mM-4 mM), prominent generation of NO was detected from 1 g of minced liver (Fig. 4, *A*, *line a*, and *B*, *line a*) or heart (Fig. 4, *A*, *line b*, and *B*, *line b*) in 5 ml of PBS (pH 7.0). The rate of NO formation increased linearly with the increase in nitrite concentration at physiological or pathophysiological levels (10 μM–100 μM). In contrast to this, the NO generation measured in blood was over 50-fold lower (Fig. 4*A*, *line c*).

Effects of Oxygen on NO Generation from Nitrite—In mammalian organs under normoxic conditions, the O_2 concentration ranges from 10 to <0.5%, with values of ~14% in arterial blood and <4% in the myocardium (36). During mild hypoxia, myocardial O_2 levels drop to ~1–2% or lower (13, 37, 38). To determine whether nitrite can be reduced to form NO in tissues and to ascertain the effects of oxygen tension in this process, NO chemiluminescence studies were performed. NO formation from rat liver, heart, and blood was measured with continuous purging of air or 10, 5, or 2% oxygen, corresponding to oxygen concentrations in solution at 37 °C of 214, 102, 51, and

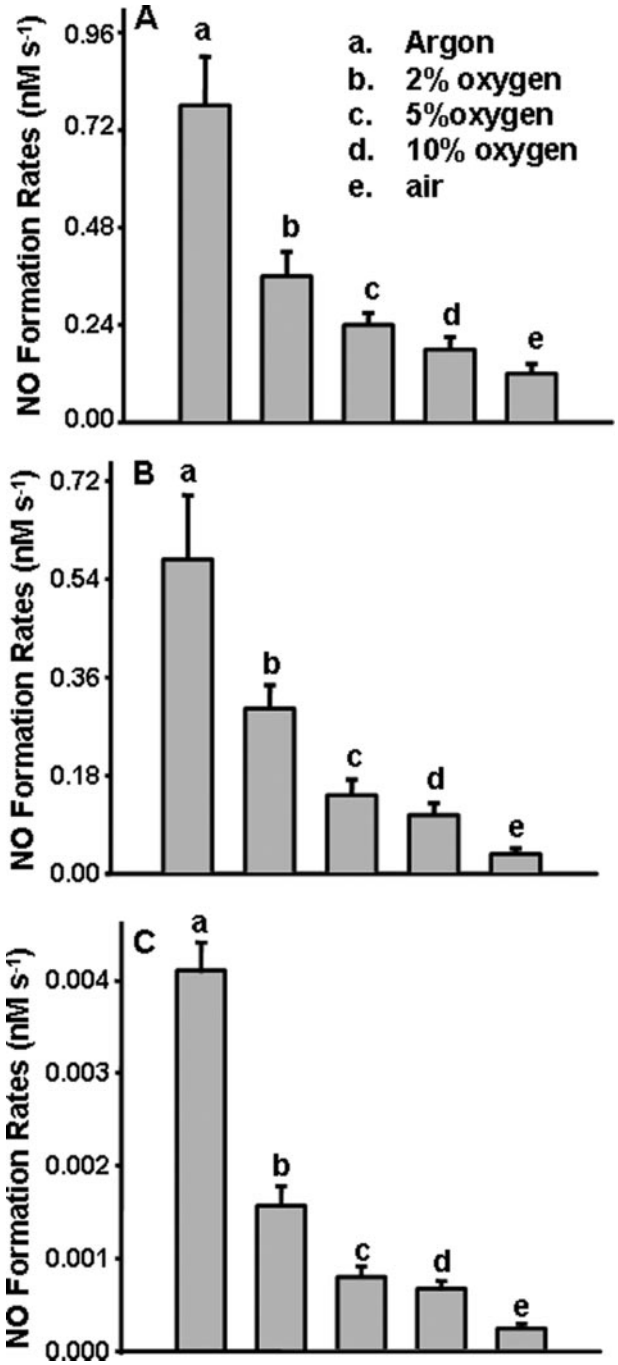

FIGURE 5. **NO generation from nitrite in tissues or blood at different oxygen tensions.** *A* shows the NO formation rates from liver (1 g of tissue) with nitrite (100 μM) and N^G-methyl-L-arginine (5 mM) in 5 ml of PBS. *B* shows the NO formation rates from heart (1 g of tissue) with nitrite (100 μM) and N^G-methyl-L-arginine (5 mM) in 5 ml of PBS. *C* shows the NO formation rates from 5 ml of blood with nitrite (500 μM) and N^G-methyl-L-arginine (5 mM). The oxygen concentrations of 214, 102, 51, 20, and 0 μM, respectively, were achieved with purging of the sample solution at 37 °C with 21, 10, 5, or 2% oxygen or argon.

20 μM, respectively. The NOS inhibitor N^G-methyl-L-arginine (5 mM) was added to prevent any possible NO generation from L-arginine. It was observed that the rate of NO generation decreased with the increase of pO_2 either in the liver (Fig. 5*A*), heart (Fig. 5*B*), or blood (Fig. 5*C*). Again, NO generation from blood was much less than that produced in tissues even in the presence of a five times higher amount of nitrite in blood than in tissues. Thus, NO generation from nitrite can be a source of NO in tissues under conditions of tissue normoxia, and it is further increased with mild hypoxia.

TABLE 1
Effect of pH on NO generation from nitrite reduction

	NO generation rate			
	pH 5.0	pH 6.0	pH 7.0	pH 8.0
	$nM\ s^{-1}$ [a]			
Liver + nitrite (20 μM)	2.71 ± 0.21	1.60 ± 0.19	0.14 ± 0.02	0.08 ± 0.01
Heart + Nitrite (20 μM)	2.59 ± 0.19	1.46 ± 0.16	0.11 ± 0.02	0.07 ± 0.01

[a] Maximal NO generation rates were measured by chemiluminescence NO analyzer as described in Fig. 2. The measurements were performed with 1 g of liver or heart in the presence of nitrite (20 μM) under anaerobic conditions at 37 °C. The values shown are the means ± S.D.

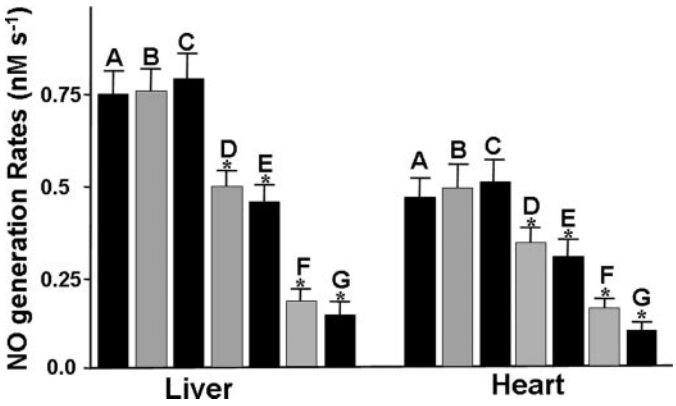

FIGURE 6. **Effect of enzymatic inhibitors on NO formation from nitrite.** *Left bars*, liver (1 g of tissue) in 5 ml of PBS at 37 °C. *Right bars*, heart (1 g of tissue) in 5 ml of PBS at 37 °C. *Bars A*, with nitrite (100 μM); *bars B*, with nitrite (100 μM) and rotenone (5 μM); *bars C*, with nitrite (100 μM) and clotrimazole (10 μM); *bars D*, with nitrite (100 μM) and oxypurinol (100 μM); *bars E*, with nitrite (100 μM) and raloxifene (50 nM); *bars F*, with nitrite (100 μM), oxypurinol (100 μM), and raloxifene (50 nM); *bars G*, with 1 g of tissues preheated at 95 °C for 5 min followed by incubation with nitrite (100 μM) in 5 ml of PBS, under anaerobic conditions at 37 °C. The values shown are the means ± S.E. *, defines significant inhibition, $p < 0.05$, compared with their respective control bars (*A*) for either liver or heart tissue measurements.

Effects of pH on Nitrite-dependent 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. To assess NO generation from nitrite under different physiological or pathological conditions and to further characterize the mechanism of nitrite reduction, experiments were performed to measure the effect of pH on the magnitude of NO generation from nitrite in liver and heart. As shown in Table 1, it was observed that under acidic conditions increased NO generation occurs. In contrast, under alkaline conditions, prominent inhibition was seen.

Specific Inhibitor Studies—To investigate the contribution of XO, AO, cytochrome P-450, and mitochondrial Complex I to the NO generation from nitrite, studies were performed using specific inhibitors. Either the XO-specific inhibitor oxypurinol (100 μM), AO-specific inhibitor raloxifene chloride (50 nM), CP inhibitor clotrimazole (1 μM), or complex I inhibitor rotenone (10 μM) was added and incubated with rat liver or heart. After 5 min of incubation, nitrite was added, and subsequent NO generation rates were detected using the chemiluminescence NO analyzer. The presence of rotenone or clotrimazole did not significantly inhibit NO generation from nitrite (Fig. 6, *B* and *C*).

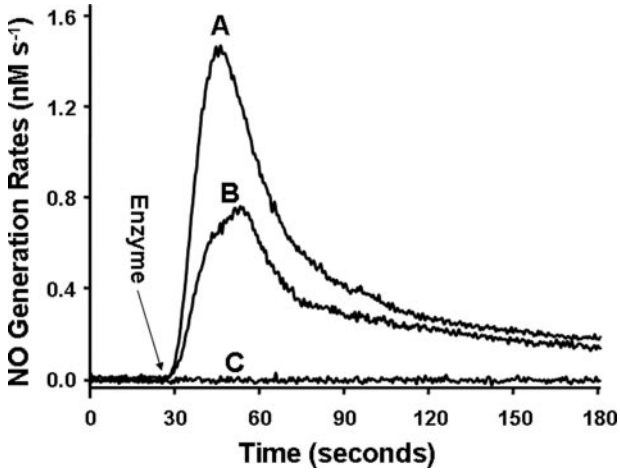

FIGURE 7. **Measurement of NO generation from isolated enzyme-mediated nitrite reduction.** Measurements were performed using a chemiluminescence NO analyzer under anaerobic conditions at 37 °C in 5 ml of PBS. The *arrow* shows the time at which AO, XO, or ferrous hemoglobin was added. NO generations are shown from nitrite (100 μM), NADH (500 μM), and AO (0.01 mg/ml) (*trace A*); nitrite (100 μM), NADH (500 μM), and XO (0.04 mg/ml) (*trace B*); or nitrite (100 μM), and ferrous hemoglobin (5 mg/ml) (*trace C*).

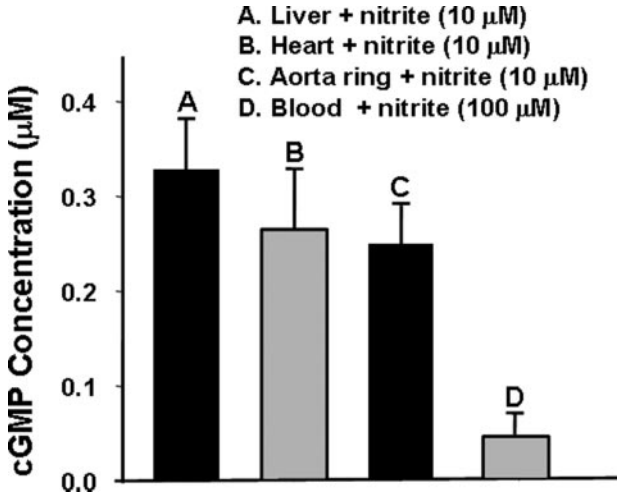

FIGURE 8. **Soluble guanylyl cyclase activation as a measure of nitrite-mediated NO formation.** sGC activation was determined from the measurements of cGMP formation. The reactions were performed in PBS (1 ml) with EDTA (5 mM), $MgCl_2$ (2 mM), sGC (10 ng), and GTP (1 mM). *A*, nitrite (10 μM) and liver (0.2 g of tissue) in reaction buffer; *B*, nitrite (10 μM); and heart (0.2 g of tissue) in reaction buffer; *C*, nitrite (10 μM) and aorta (0.2 g of tissue) in reaction buffer; *D*, nitrite (100 μM) with 10 ng of sGC, 2 mM $MgCl_2$, and 1 mM GTP in 1 ml of blood was incubated for 10 min under anaerobic conditions.

In contrast, the XO inhibitor oxypurinol decreased NO generation rate in liver from 0.75 ± 0.04 to 0.49 ± 0.03 nM/s with ~34% inhibition and decreased NO generation rate in the heart from 0.47 ± 0.04 to 0.33 ± 0.03 nM/s with ~30% inhibition. In the presence of the AO inhibitor raloxifene NO generation rate in liver decreased to 0.46 ± 0.04 nM/s with ~38% inhibition, and NO generation rate in heart decreased to 0.28 ± 0.03 nM/s with ~35% inhibition (Fig. 6, *D* or *E*). When the AO inhibitor raloxifene and XO inhibitor oxypurinol were both administered, NO generation decreased to 0.18 ± 0.02 nM/s with ~76% inhibition in liver and decreased to 0.16 ± 0.02 nM/s in heart with ~66% inhibition (Fig. 6*F*). Thus, the molybdenum enzymes XO and AO were the major enzymes that catalyzed NO generation from nitrite. When the tissue was denatured at 95 °C for 5 min, still ~15–20% of the NO generation remained, and this is may be due to nonenzymatic processes of nitrite reduction (Fig. 6*G*).

Isolated Enzyme Measurements of Nitrite Reduction to NO—From the use of specific inhibitors, XO and AO appear to play a critical role in nitrite reduction to NO in tissues. To further confirm the existence of the XO and AO-catalyzed NO generation, further studies on purified AO or XO were performed to investigate the magnitude and mechanism of their nitrite reduction. Comparative measurements of the rate of NO formation derived from AO, XO, or ferrous hemoglobin-mediated nitrite reduction were performed under anaerobic conditions using the NO analyzer. Prior to the addition of AO, no detectable NO generation was seen from nitrite (100 μM) in the presence of NADH (500 μM). However, after the addition of AO (0.01 mg/ml) or XO (0.04 mg/ml), prominent NO generation was triggered (Fig. 7, *A* and *B*). However, no NO signal was detected with the addition of even large amounts of ferrous hemoglobin (5 mg/ml) (Fig. 7*C*). These results further confirm that the molybdenum enzymes AO and XO function as potent nitrite reductases, whereas hemoglobin does not.

Effect of Nitrite Reduction on sGC Activity—To determine the effect of nitrite reduction on sGC activation, enzyme-linked immunoassays were performed to measure cGMP formation. After incubation of nitrite (10 μM) with 0.2 g of minced rat liver, heart, or aorta in the reaction buffer (10 ng of sGC, 5 mM EDTA, 2 mM $MgCl_2$, and 1 mM GTP in 1 ml of PBS) under anaerobic conditions for 10 min, measurements of the formation of the sGC product cGMP were performed in the presence and absence of nitrite. Comparative studies in blood to those in tissues were performed by incubation of nitrite (100 μM, 10-fold higher levels) with 10 ng of sGC, 2 mM $MgCl_2$, and 1 mM GTP in 1 ml of blood. Increased cGMP was detected, suggesting that sGC was activated by nitrite in either rat liver (Fig. 8*A*), heart (Fig. 8*B*), or aorta (Fig. 8*C*). However, even with 10-fold higher levels of nitrite, cGMP production was much lower in blood than that seen from the tissues (Fig. 8*D*). In the presence of nitrite, a much larger increase of cGMP production was detected in liver (0.33 ± 0.05 μM), heart (0.26 ± 0.06 μM), and aorta (0.25 ± 0.04) than that detected in blood (0.06 ± 0.02 μM). With 100 μM nitrite in the reaction buffer in the absence of tissue or with 100 μM nitrite in blood, no significant cGMP formation was detected. These results confirm that nitrite reduction and NO generation primarily takes place in the tissues not in blood.

DISCUSSION

In recent years, numerous studies have demonstrated that nitrite can be an important source rather than just a product of NO (2–8, 13, 15, 17–19, 24, 25, 39, 40). To characterize the magnitude and mechanism of NO formation from nitrite in tissues and blood, we performed a series of studies using EPR spectroscopy, chemiluminescence NO analyzer, and immunoassay to measure the magnitude of NO generation and the activation of sGC. It was observed that the addition of nitrite (100 μM) triggered a large amount of NO production from rat liver (~0.7 nM/s), heart (~0.5 nM/s), and aorta (~0.3 nM/s) but no detectable production from blood (Figs. 1, *D–F*, and 2*A*). Even

with 5-fold higher concentration of nitrite, only a trace amount of NO was released from blood with a rate of ~0.005 nM/s (Figs. 1, *B* and *C*, and 2*B*).

Hemoglobin-mediated nitrite reduction under anaerobic conditions was first reported in 1981 (41).

$$H^+ + NO_2^- \xrightarrow{k_1} HONO$$

$$Hb + HONO \xrightarrow{k_2} MetHb + NO + OH^-$$

$$Hb + NO \xrightarrow{k_3} Hb - NO$$

REACTIONS 1–3

Recent studies have suggested a critical function for hemoglobin and the erythrocyte in nitrite reduction and subsequent NO release in the vascular compartment eliciting vasodilation (3–8). However, controversy remains regarding the mechanism and magnitude by which NO escapes trapping by hemoglobin (deoxy or oxy) in the red blood cells (9).

In the current study, direct monitoring of NO release by EPR spectroscopy and chemiluminescence NO analyzer techniques demonstrated that nitrite can be a source of NO in the blood and tissues. However, in contrast to the large amounts of NO formed in tissues by nitrite reduction, only a much smaller amount of NO production was detected in blood even in the presence of five times higher nitrite (Figs. 1, *A* and *B*, and 2*B*). Carbon monoxide pretreatment of the blood increased subsequent NO formation and release, suggesting that hemoglobin is not the source of NO formation in blood and acts as a potent NO scavenger (Figs. 1*B* and 2*B*). Using isolated hemoglobin instead of blood, our EPR spectroscopy studies revealed the formation of nitrosylhemoglobin from the reaction of deoxyhemoglobin with nitrite (Fig. 3), but no NO release was detected even with high nitrite concentrations well above physiological levels, 2–20 μM). Only with nitrite levels almost 1000-fold above the physiological range and well above the hemoglobin concentration could NO release be detected. These data and the effects of CO in enhancing NO generation suggest that NO formation in blood occurs from hemoglobin-independent processes. In fact, hemoglobin appears to play a negative role by trapping NO production in the blood instead of functioning as an important NO-generating enzyme. Thus, erythrocytes are not the major source of NO generated and released from nitrite reduction.

Furthermore, we observe over a range of different oxygen tensions that NO generation rates are much faster in tissues compared with those in blood. Even with 5-fold higher nitrite levels the rate of NO production was 100–200-fold lower in blood than in tissues (Fig. 5). Thus, nitrite reduction in tissues rather than in blood appears to play the major role in NO generation.

Rather than acting as a source of NO generation, our results clearly showed that hemoglobin acts as a trap under physiological conditions where the levels of nitrite are less than the levels of hemoglobin. However, under conditions where nitrite levels exceed those of the heme centers of hemoglobin, nitrite can be reduced to NO and effectively released (42, 43). With excess nitrite all ferrous-hemoglobin is converted to methemoglobin, and NO dissociates from the weak association with the ferric heme as seen when purging with argon (equation 2). However, under physiological or even pathological conditions, nitrite concentrations are far less than those of hemoglobin inside red blood cells. Therefore, inside red blood cells, it is unlikely that significant amounts of NO can escape trapping by hemoglobin and mediate vasodilation (9, 44).

NO can be formed by the simple process of nitrite disproportionation, which is significantly accelerated under the acidic conditions that occur during ischemia (2, 14).

$$2H^+ + 3NO_2^- \xrightarrow{k_4} 2NO + NO_3^- + H_2O$$

REACTION 4

Analysis of the reactions of nitrite disproportionation shows that at physiological or pathological concentration of nitrite (less than 100 μM), the net reaction is second order in nitrite (14). But in the current study, the measured kinetics of nitrite-dependent NO formation showed that NO generation from tissues linearly increased ($r^2 > 99\%$) over the range of physiological or pathological concentrations of nitrite (10–100 μM nitrite) (Fig. 4*B*). Thus, the main source of NO generation comes from nitrite reduction rather than disproportionation.

$$e^- + 2H^+ + NO_2^- \xrightarrow{k_5} NO + H_2O$$

REACTION 5

The calculated rates of nitrite-dependent NO generation also support the hypothesis that NO generation comes mainly from nitrite reduction rather than disproportionation. Substitution of the pH 7 and nitrite 10 μM concentration values that occur in normal tissues predicts that the rate of NO production from nitrite disproportionation would be ~0.05 pM/s (14). However, a much faster NO generation rate from nitrite was detected in the tissues. According to our data, with 10 μM nitrite, the NO generation rate is 73.2 or 46.8 pM/s in liver and heart (Fig. 4).

NO generation from nitrite reduction occurs continuously in the tissues and blood under normal physiological conditions. 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). Our data showed that tissue NO generation from nitrite increased about 12-fold as the pH decreased from 7.0 to 6.0 (Table 1). Thus, the NO formation rate *in vivo* under ischemic conditions with pH of 6.0, from 10 μM nitrite, would be predicted to be ~880 pM/s in liver and ~560 pM/s heart, respectively, approaching that of the maximal tissue NO production from constitutive NOS of ~1 nM/s (45). Thus, acidosis greatly facilitates nitrite-mediated NO formation and release in these tissues. This NO production from

TABLE 2
Correlation of tissue and blood XOR, AO, and Hb concentrations with nitrite-mediated NO generation

	Liver[a]	Heart[a]	Blood
XOR	76 μg/g ww	7.2 μg/g ww	trace amount
AO	60 μg/g ww	5.1 μg/g ww	trace amount
Hb	NA[b]	NA	~0.158 g/ml
NO generation[c]	0.75	0.47	0.005

[a] The units shown for XOR and AO are μg of enzyme/g of tissue wet weight (ww).
[b] NA, not applicable.
[c] The units shown are nM s^{-1}. 100 μM nitrite in tissues, 500 μM nitrite in blood.

nitrite could serve as an alternative source of NO under ischemic conditions in which NO production from NOS is impaired.

We observe that NO generation from nitrite reduction occurs under aerobic conditions as well as under anaerobic conditions. However, oxygen tension greatly modulated nitrite-dependent NO generation with decreases in oxygen levels, greatly enhancing this NO generation process (Fig. 5). The oxygen-dependent decrease of NO release can be explained by a decrease in reducing substrates or increase in NO trapping by superoxide formed under aerobic conditions.

Several alternative pathways for NO generation from nitrite reduction have been observed. Previous isolated enzyme studies have shown that ubiquinone/cytochrome bc1 complex of mitochondrial electron chain and CP of liver microsomes are able to reduce nitrite with NO formation (24–28). Studies with specific inhibitors revealed that this pathway only plays a minor role in the nitrite reduction process in the tissues (Fig. 6, *B* and *C*). For the molybdenum enzymes XO or AO, specific inhibitors showed prominent inhibition of nitrite reduction in the tissues with approximately 30–40% decrease in NO formation rate for each of these enzymes, respectively, and >65–70% decrease with inhibition of both (Fig. 6, *D* and *E*). This reveals that AO and XO provide the major nitrite reductase activity in these tissues under hypoxic conditions and play a critical role in nitrite-derived NO formation. Of note, the NO generation rate increased linearly with physiological or pathological concentration of nitrite (Fig. 4*B*), thus the K_m value of nitrite for the enzyme is much higher than the physiological or pathological concentrations of nitrite studied. Following heat denaturation of the proteins in the tissues, there still remained ~15–20% of the NO formation, suggesting the presence of nonenzymatic nitrite reduction and NO formation (Fig. 6*G*).

Our isolated enzyme measurements of nitrite reduction to NO confirmed that AO and XO are both effective nitrite reductases with NADH as electron donor (Fig. 7, *A* and *B*). Of note, the observed rate of NO generation decreased over time. This may be caused by feedback inhibition of NO on XO and AO (46, 47) or inactivation of these enzymes in the process of gas purging. We have previously shown for XO that xanthine is also a highly effective substrate for nitrite reduction (17). Both xanthine oxidoreductase (XOR) and AO are present in the liver but are also broadly distributed in other tissues, such as lung, blood vessels, heart, and kidney (34, 48–51).

We and others have previously reported the tissue activity of XOR and AO (33, 34, 52, 53). From these reports, the tissue concentrations of these enzymes can be estimated and correlated with the nitrite-mediated NO generation observed in these tissues as shown in Table 2. Of note, it has been previously demonstrated that not only are XO and AO present in these tissues, but under ischemic conditions high levels of their substrates occur including xanthine, which increases to 10–100 μM levels in the heart, and NADH, which rises to 0.5–1 mM levels (54). In the liver, these substrates are also present (55), and their levels rise during ischemia but to a lesser extent for a given ischemic duration. According to the enzymatic studies in Fig. 7, and levels of XO and AO in Table 2, under anaerobic conditions with 0.5 mM NADH as reducing substrate, AO-mediated NO production from 100 μM nitrite would be estimated to be about 0.75 nM/s in heart, whereas that from XO in heart would be 0.14 nM/s. However, for XOR its substrate xanthine is also present at levels of >10 μM, so based on prior reports XOR-mediated NO production from 100 μM nitrite with xanthine because substrate would be estimated to be in the range of 0.7–1.4 nM/s (17). These estimated NO generation rates are higher than the NO generation detected in heart tissue of 0.47 nM/s, and this is likely because of NO scavenging in the tissues by heme centers and other reactions as well as possible inhibition of these enzymes by NO, peroxynitrite, or other factors. In liver based on the higher AO and XOR levels, even higher NO production from nitrite would be expected, and this was observed, although a greater proportion of tissue NO scavenging or enzyme inhibition appears to occur. Under ischemic conditions the accumulation of high substrate levels primes these enzymes to support increased NO production from nitrite. Although the levels of these enzymes are below μM and the hemoglobin concentration in the blood is ~2.5 mM, the rate of NO production from nitrite in tissues is over 100-fold higher than that in blood (Table 2).

Hemoglobin-mediated NO generation does not depend simply on its concentration. It is well known that hemoglobin is an extremely effective NO scavenger (9, 10). With the administration of nitrite, NO can be generated by dissociation from the MetHb-NO complex, but simultaneously NO can be consumed by ferrous-hemoglobin. According to Reactions 2 and 3, the steady state NO concentration in the reaction mixture depends on the ratio of hemoglobin and MetHb-NO, not on the total concentration of hemoglobin. Our results verified that hemoglobin traps more NO than it releases, unless the nitrite concentration rises well above that of the hemoglobin (Fig. 3*B*).

Overall, our studies demonstrate that the tissues rather than blood erythrocytes or hemoglobin play critical roles in hypoxic NO production from nitrite. We observe that this nitrite-derived NO is primarily generated from nitrite reduction by the molybdenum enzymes XOR and AO. Furthermore, the process of NO generation from nitrite reduction in tissues is regulated by pH, oxygen tension, nitrite, and reducing substrate concentrations, as is characteristic of these enzymes. This nitrite-derived NO production from XOR and AO in tissues could serve as an alternative source of NO under ischemic conditions in which NO production from NOS is impaired.

Acknowledgments—We thank Dr. Xiaoping Liu and Dr. Alexandre Samouilov for helpful comments.

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