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# Oxygen Regulates Tissue Nitrite Metabolism

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## Abstract

***Aims:*** Once dismissed as an inert byproduct of nitric oxide (NO) auto-oxidation, nitrite ($NO_2^-$) is now accepted as an endocrine reservoir of NO that elicits biological responses in major organs. While it is known that tissue nitrite is derived from NO oxidation and the diet, little is known about how nitrite is metabolized by tissue, particularly at intermediate oxygen tensions. We investigated the rates and mechanisms of tissue nitrite metabolism over a range of oxygen concentrations. ***Results:*** We show that the rate of nitrite consumption differs in each organ. Further, oxygen regulates the rate and products of nitrite metabolism. In anoxia, nitrite is reduced to NO, with significant formation of iron–nitrosyl proteins and S-nitrosothiols. This hypoxic nitrite metabolism is mediated by different nitrite reductases in each tissue. In contrast, low concentrations ($\sim 3.5\,\mu M$) of oxygen increase the rate of nitrite consumption by shifting nitrite metabolism to oxidative pathways, yielding nitrate. While cytochrome $P_{450}$ and myoglobin contribute in the liver and heart, respectively, mitochondrial cytochrome *c* oxidase plays a significant role in nitrite oxidation, which is inhibited by cyanide. Using cyanide to prevent artifactual nitrite decay, we measure metabolism of oral and intraperitoneally administered nitrite in mice. ***Innovation:*** These data provide insight into the fate of nitrite in tissue, the enzymes involved in nitrite metabolism, and the role of oxygen in regulating these processes. ***Conclusion:*** We demonstrate that even at low concentrations, oxygen is a potent regulator of the rate and products of tissue nitrite metabolism. *Antioxid. Redox Signal.* 17, 951–961.

## Introduction

NITRITE IS NOW ACCEPTED as an endocrine storage form of nitric oxide (NO) that is reduced to bioactive NO by heme-containing proteins (42). In the blood, hemoglobin is the predominant nitrite reductase, which reduces nitrite in the presence of a proton to form metHb and NO:

$$\text{deoxy Hb}(Fe^{2+}) + NO_2^- + H^+ \rightarrow \text{metHb}(Fe^{3+}) + NO + OH^-$$

This reaction is regulated by the allosteric structural transition of hemoglobin and is linked to the mechanism of hypoxic vasodilation (29). While hemoglobin-dependent nitrite reductase activity has been characterized (12, 28), little is known about the mechanisms of tissue-dependent nitrite metabolism.

Submicromolar levels of nitrite mediate fundamental biological tissue responses. On a cellular level nitrite regulates mitochondrial function (38, 45, 47), cytochrome $P_{450}$ ($CYP_{450}$) activity and protein expression (11). At the organ level, nitrite stimulates angiogenesis (37), prevents vascular hyperplasia (3), increases exercise efficiency (5, 38, 39) and limits chlorine gas-induced lung injury (51). Perhaps the most robust nitrite-dependent tissue response is the prevention of ischemia/reperfusion (I/R) injury in the heart (16, 19, 23, 50), liver (19, 47), and brain (32). Further, mice with low basal levels of

### Innovation

This study demonstrates that nitrite oxidation to nitrate is rapid in tissue and significant even at low (physiological) oxygen tensions. While nitrite oxidation was previously thought to be a mechanism of nitrite detoxification, with the emergence of a physiological role for nitrite and nitrate, this idea warrants reconsideration. Given recent reports demonstrating hepatic nitrate reductase activity by XOR (31), and elucidation of the entero-salivary pathway by which nitrate can be recycled to nitrite (and NO) (41, 42), it is possible that nitrite oxidation represents a mechanism to preserve nitrite bioactivity through conversion to nitrate, which has an extended half-life. Here we define the distinct enzymatic pathways responsible for nitrite oxidation and reduction in each tissue. Further study will determine the interplay between oxidative and reductive tissue pathways and the physiological implications of nitrite oxidation.

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nitrite have exacerbated injury after I/R (48). The emerging importance of basal nitrite levels in modulating tissue responses and the potential for nitrite-based therapeutics necessitate understanding the mechanisms of tissue nitrite metabolism.

Nitrite is derived from both NO oxidation and dietary sources. Additionally, Lundberg and colleagues have described the reduction of nitrate to nitrite by xanthine oxidoreductase (XOR) (31). However, little is known about the metabolism of nitrite once it is formed. Several proteins, including XOR (17, 40, 50), myoglobin (25, 45), nitric oxide synthase (NOS) (49), $CYP_{450}$ (35), cytochrome c (7), mitochondrial complex III (36), and cytochrome c oxidase (Ccox) (13), reduce nitrite in hypoxic tissue, but the relative contribution of these enzymes between organs has not been compared. Furthermore, the metabolism of nitrite and its conversion to other species at intermediate oxygen tensions has not been explored.

Herein we investigate the rate of nitrite metabolism by different tissues over a range of oxygen tensions and find that nitrite consumption rate increases with rising oxygen concentration. We determine the products of nitrite metabolism and the enzymes responsible for hypoxic nitrite reduction as well as normoxic nitrite oxidation. Using cyanide to prevent artifactual tissue metabolism of nitrite *ex vivo*, we measure the fate of intraperitoneal and orally administered nitrite *in vivo*.

## Results

### *Nitrite is differentially metabolized in hypoxia and normoxia*

To determine whether all major organs metabolize nitrite at the same rate, rat liver, heart, brain and lung homogenates were treated with nitrite (10 $\mu M$) at 21% $O_2$. Measurement of the nitrite concentration in the homogenates over time showed that nitrite was metabolized at different rates by each tissue, with the liver and heart consuming nitrite the most rapidly ($t_{1/2} = 12.3 \pm 0.5$ and $12.1 \pm 0.3$ min) and the brain being the slowest ($t_{1/2} > 1$ h) (Fig. 1A). Notably, the rate of nitrite consumption was not significantly changed by increasing concentrations of nitrite (10–100 $\mu M$; data not shown).

We next determined the role of oxygen in regulating tissue nitrite consumption. Tissue homogenates were treated with nitrite (10 $\mu M$) at 1.5–100% oxygen and nitrite consumption measured. In all tissues, the rate of nitrite consumption was greater with increasing oxygen concentration (Figs. 1B and 1C). To confirm that hypoxic tissue was still viable and able to metabolize nitrite, nitrite was added to hypoxic (1% $O_2$) liver homogenate and its consumption measured. While only 6% of nitrite was consumed during 20 min of hypoxia, oxygenation (21% $O_2$) of the tissue immediately increased the rate of nitrite consumption (Fig. 1D). These data demonstrate that oxygen increases the rate of tissue-dependent nitrite consumption.

### *Hypoxic tissue reduces nitrite*

To determine whether nitrite was reduced in hypoxia, NO production was measured in tissue homogenates at 0%–10% $O_2$ after nitrite (1 m$M$) addition. Consistent with hypoxic nitrite reduction, NO was formed in anoxia at a rate that was greatly decreased at 1.5% oxygen and absent at 10% oxygen (Fig. 2A). The NO generated in anoxia accounted for approximately 85%

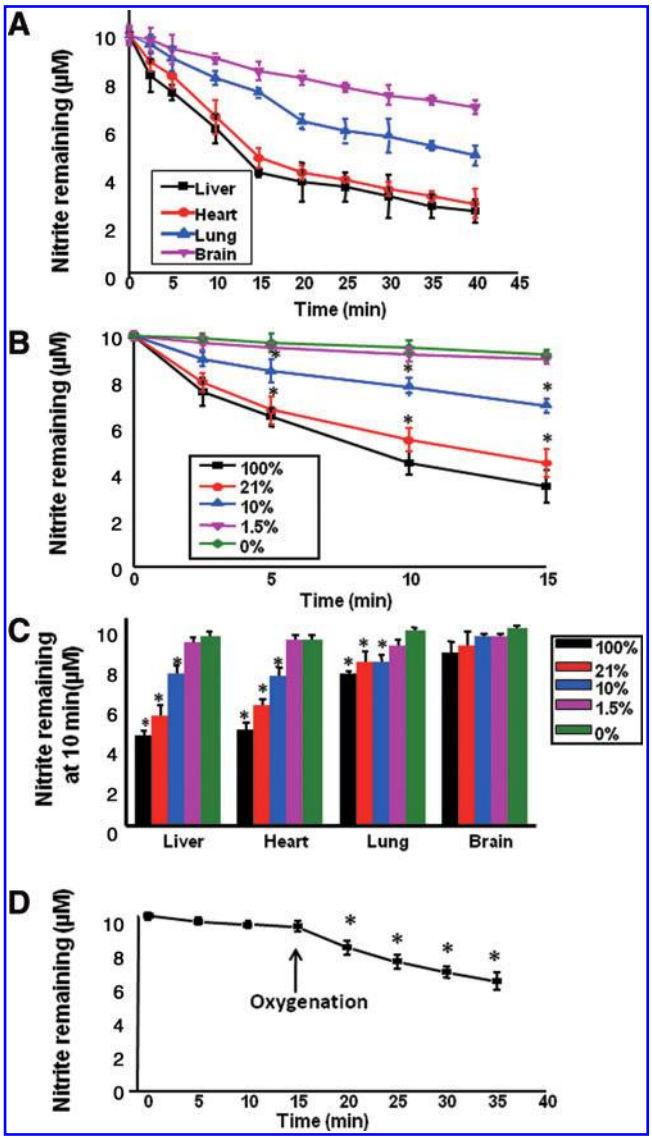


**FIG. 1. Nitrite metabolism is regulated by oxygen.** Nitrite (10 $\mu M$) was added to tissue homogenates (4 mg/ml) and the concentration remaining in the homogenate was measured over time. **(A)** Nitrite concentration remaining in tissue homogenates incubated at 21% oxygen. **(B)** Nitrite remaining in liver homogenates incubated at 0, 1.5, 10, 21, or 100% oxygen. **(C)** Nitrite concentration in tissue homogenates after 10 min of incubation at 0, 1.5, 10, 21, or 100% oxygen. $^*p < 0.01$ versus 0% oxygen. **(D)** Nitrite concentration in liver homogenate after 20 min at 0% oxygen and then 21% oxygen. $^*p < 0.01$ versus time 0; $n \geq 4$. Data are expressed as means ± SEM. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

of the nitrite consumed. We hypothesized that a greater concentration of NO was generated and scavenged by tissue heme proteins. To test this hypothesis, the concentration of iron-nitrosyl (Fe-NO) was measured. As predicted, tissue Fe-NO concentration increased, indicating that at least a fraction of the NO generated was scavenged by tissue heme (Fig. 2B). To test whether nitrite contributes to hypoxic S-nitrosation, the concentration of SNO was measured after nitrite treatment and shown to increase over time (Fig. 2C).

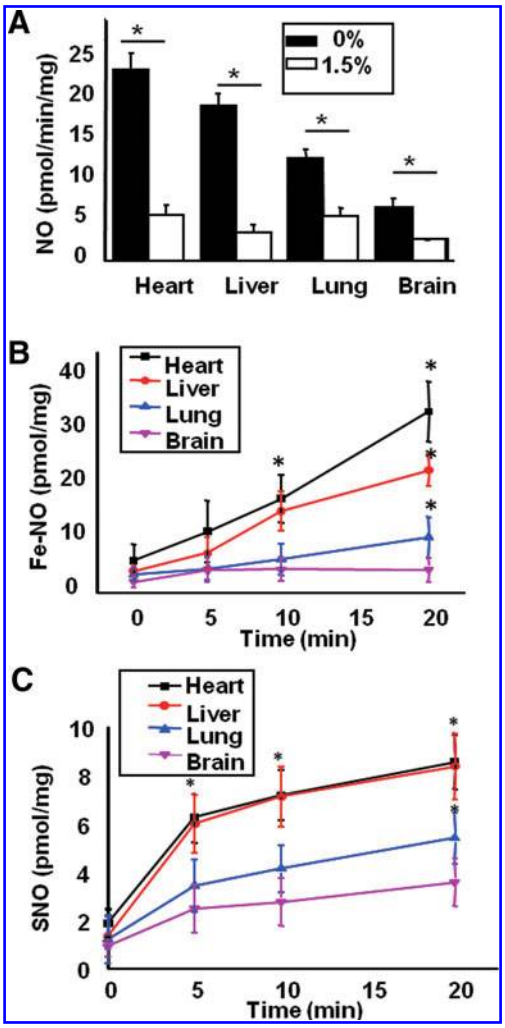


**FIG. 2. Nitrite is reduced in hypoxia. (A)** NO generation rate (average rate from time 0 until the trace reached steady state) after the addition of nitrite (1 m*M*) to liver, heart, lung, and brain homogenates (4 mg/ml) at 0% and 1.5% oxygen; $^*p < 0.001$. The concentration of **(B)** Fe-NO and **(C)** SNO generated in anoxia in the homogenates in panel A. $^*p < 0.01$ versus time = 0; $n \geq 4$. Data are expressed as means ± SEM. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

### *Different nitrite reductases exist in tissues*

To determine the relative contribution of known nitrite reductases to NO generation in different tissues, tissue homogenates were made, pre-treated with inhibitors of each nitrite reductase and then anoxic nitrite-dependent NO generation measured. Consistent with prior studies (21), liver and heart showed the greatest rate of NO generation (Fig. 3A). In the heart, oxidation of ferrous heme by potassium ferricyanide (500 $\mu M$), decreased NO generation in the tissue by 65 ± 5%. While these data are consistent with studies demonstrating that myoglobin is the major cardiac nitrite reductase in heart (25, 45), it is important to note that the metal centers of other enzymes could be oxidized by ferricyanide. Thus, specific inhibitors of XOR, mitochondria, and $CYP_{450}$ were also tested. NO generation was attenuated by 30 ± 7% in the presence of allopurinol (500 $\mu M$), an XOR inhibitor, and by 7 ± 4% after treatment with the mitochondrial complex III inhibitor myxothiazol (100 $\mu M$) (Fig. 3B). As expected, when heart tissue was treated together with ferricyanide, allopurinol, and myxothiazol, nitrite-dependent NO generation was abolished. In contrast, XOR was the predominant nitrite reductase in the lung, as allopurinol treatment inhibited 85 ± 6% of NO generation. The remainder of NO generation was inhibited by myxothiazol and ferricyanide (Fig. 3C). In the brain, oxidation of heme proteins by ferricyanide significantly inhibited nitrite reduction (Fig. 3D). L-NAME and troleandomycin, inhibitors of NOS and $CYP_{450}$, respectively, had no effect in the heart, lung, or brain. In the liver, treatment with allopurinol, myxothiazol, troleandomycin (300 $\mu M$), and L-NAME (250 $\mu M$) demonstrated that XOR, mitochondria, and $CYP_{450}$ all contributed significantly to hepatic nitrite reduction, with a smaller contribution by NOS (10 ± 4%; Fig. 3E). These data suggest that while all tissue types reduce nitrite, the mechanism of reduction differs.

To determine whether nitrite reductases were responsible for the conversion of nitrite to Fe-NO and SNO, reductase activity was completely inhibited in each tissue and the concentration of Fe-NO/SNO measured after nitrite addition. Inhibition of nitrite reduction eliminated Fe-NO formation, consistent with a lack of NO production. However, while inhibition of NO formation attenuated SNO formation, a significant concentration remained (Fig. 3F).

### *Nitrite is oxidized during normoxia*

To determine the products of normoxic nitrite metabolism, tissue homogenates were treated with nitrite at 21% $O_2$, and concentrations of NO, nitrate, SNO, and Fe-NO measured. In all tissues, the predominant product of nitrite metabolism was nitrate, which accounted for approximately 70-85% of nitrite consumed (Fig. 4A). Since physiological tissue oxygen levels range from 2% to 10% $O_2$, the products of nitrite metabolism in the liver were measured over this range. As expected, nitrate formation increased with increasing oxygen. Interestingly, while Fe-NO decreased as oxygen concentration increased, SNO levels did not change significantly between 1.5% and 21% oxygen (Figs. 4B–4D, Table 1).

### *Mechanisms of nitrite oxidation*

To determine the mechanism of nitrite oxidation, liver homogenate was fractionated and nitrite consumption measured in each fraction. Both the mitochondrial and microsomal fractions had significant rates of nitrite consumption, and their combined rate of consumption was equivalent to that of the unfractionated homogenate (Fig. 4E).

To confirm that mitochondria were partially responsible for nitrite oxidation, the mitochondrial fraction was incubated with substrate and nitrite/nitrate measured over time. As expected, nitrite was consumed and nitrate was generated (Fig. 5A). Previous studies show that nitrite can interact with Ccox. To confirm that Ccox was the active site of nitrite consumption, mitochondria were supplied with ascorbate and N,N,N,N-tetramethyl p-phenylenediamine dihydrochloride (TMPD) to donate electrons to Ccox and bypass complexes I-III. In the presence of Ccox turnover, we observed a significant increase in nitrite consumption and nitrate formation. Inhibition of Ccox with cyanide (100 $\mu M$) inhibited nitrite consumption (Fig. 5B).

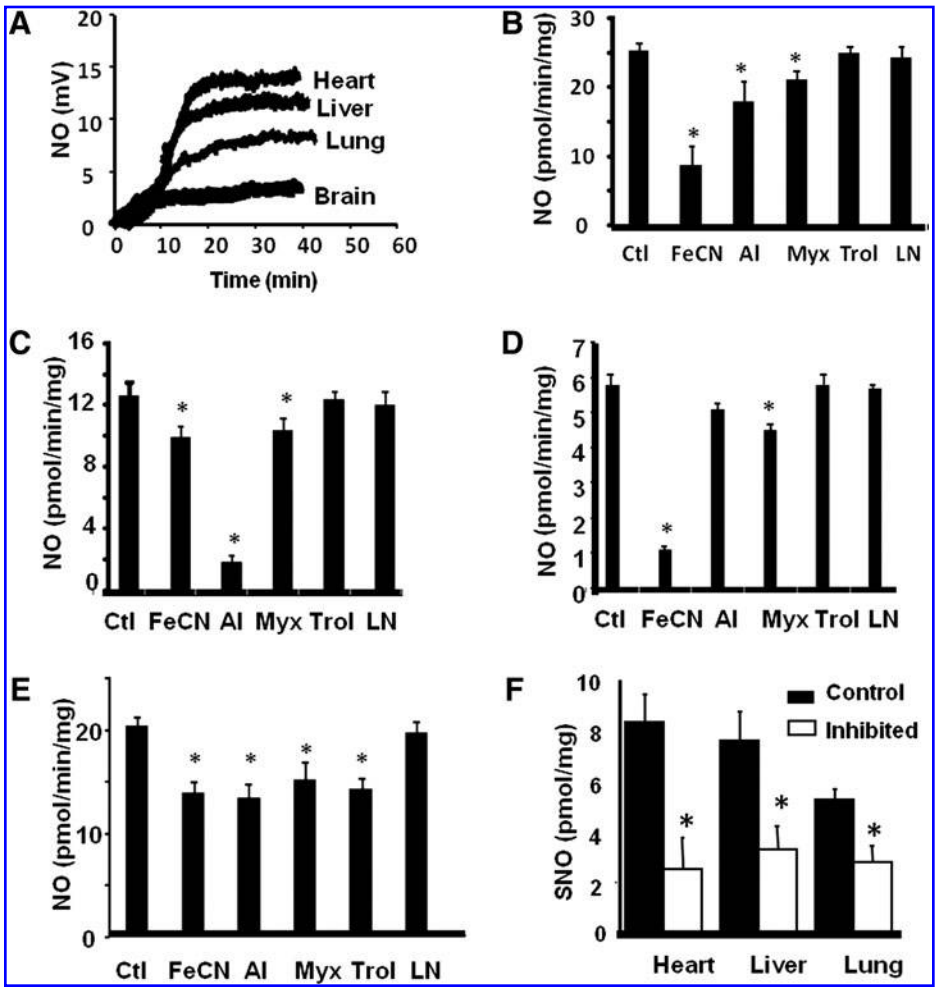


**FIG. 3. Nitrite is reduced to NO by different enzymes in each tissue.** Nitrite (1 m*M*) was added to anoxic tissue homogenates (4 mg/ml) and NO generation measured in untreated tissue or tissue pretreated with ferricyanide (FeCN; 500 $\mu M$), Allopurinol (Al; 500 $\mu M$), Myxothiazol (Myx; 100 $\mu M$), Troleandomycin (Trol; 300 $\mu M$), and L-NAME (LN: 250 $\mu M$). **(A)** Representative NO generation trace from each untreated tissue. **(B–D)** Rate of NO generation in **(B)** heart, **(C)** lung, **(D)** brain, and **(E)** liver in the absence (ctl) or presence of each inhibitor. Rates are calculated as the average rate from time 0 until the trace reached steady state. **(F)** Concentration of SNO in each tissue after 20 min of anoxic nitrite treatment in the absence (ctrl) or presence (inhibited) of complete inhibition of nitrite reductase activity by the enzymes in **B–D** above. $^*p < 0.01$ versus control; $n \geq 6$. Data are expressed as means ± SEM.

To investigate the role of the microsomal fraction, this fraction was treated with nitrite and tetrahydrobiopterin ($BH_4$; 250 $\mu M$) and nitrate production monitored over time. Nitrate formation was equivalent to nitrite consumption (Fig. 5C). A small but significant consumption was measured in the absence of $BH_4$, consistent with a necessity for enzyme turnover and with previous studies demonstrating $BH_4$-dependent nitrite oxidation in hepatocytes (34). To confirm that $CYP_{450}$ was responsible for this oxidation, the fraction was treated with troleandomycin, an inhibitor of CYP3A4, which eliminated nitrite consumption (Fig. 5D).

To determine the major nitrite oxidases in other organs, rat heart, brain, and lung homogenates were fractionated and nitrate production measured in each fraction after nitrite addition (100 $\mu M$). In the heart, the cytosolic and mitochondrial fraction both consumed nitrite. Consumption by the cytosol was consistent with oxidation by myoglobin, which is highly expressed in this compartment. However, in all other tissues examined, the mitochondrial fraction was the major nitrate generator (Fig. 6A). These data suggest that a common mechanism of normoxic nitrite metabolism, namely oxidation by Ccox, exists for the major organs.

### *Cyanide preserves tissue nitrite*

To determine whether tissue nitrite could be preserved by blocking the heme centers of Ccox and $CYP_{450}$, tissues were homogenized in cyanide (1 m*M*) and treated with nitrite (100 $\mu M$). Measurement of the nitrite consumed after 30 min showed that cyanide significantly decreased nitrite metabolism by the homogenates (Fig. 6B). Further, nitrite added to the cyanide-treated liver homogenate was stable for up to 24 h at 37°C. When separate samples were treated with nitrite and frozen, cyanide treatment prevented the significant loss of nitrite that was measured 2 days later when the samples were thawed (Fig. 6C). Prior studies have inhibited tissue nitrite metabolism using N-ethylmaleimide (NEM) to block reduced thiols and ethylenediamine tetraacetic acid (EDTA) to chelate metals and calcium. Comparison of nitrite (10 $\mu M$) consumption by liver homogenate treated with cyanide versus NEM (10 m*M*)/EDTA (1 m*M*) showed that, while NEM/EDTA significantly decreased the rate of consumption, cyanide was a more potent inhibitor of normoxic nitrite consumption (Fig. 6D).

### *Tissue nitrite levels*

To determine whether the oxygen-dependent changes in nitrite metabolism observed in tissue homogenates were relevant in intact tissue, isolated rat hearts were made normoxic (21%) or hypoxic (1%) and perfused with nitrite (50 $\mu M$). Consistent with a greater rate of nitrite metabolism in normoxia, measurement of nitrite/nitrate 5 min after perfusion showed that nitrite concentrations were ~2.5-fold higher in

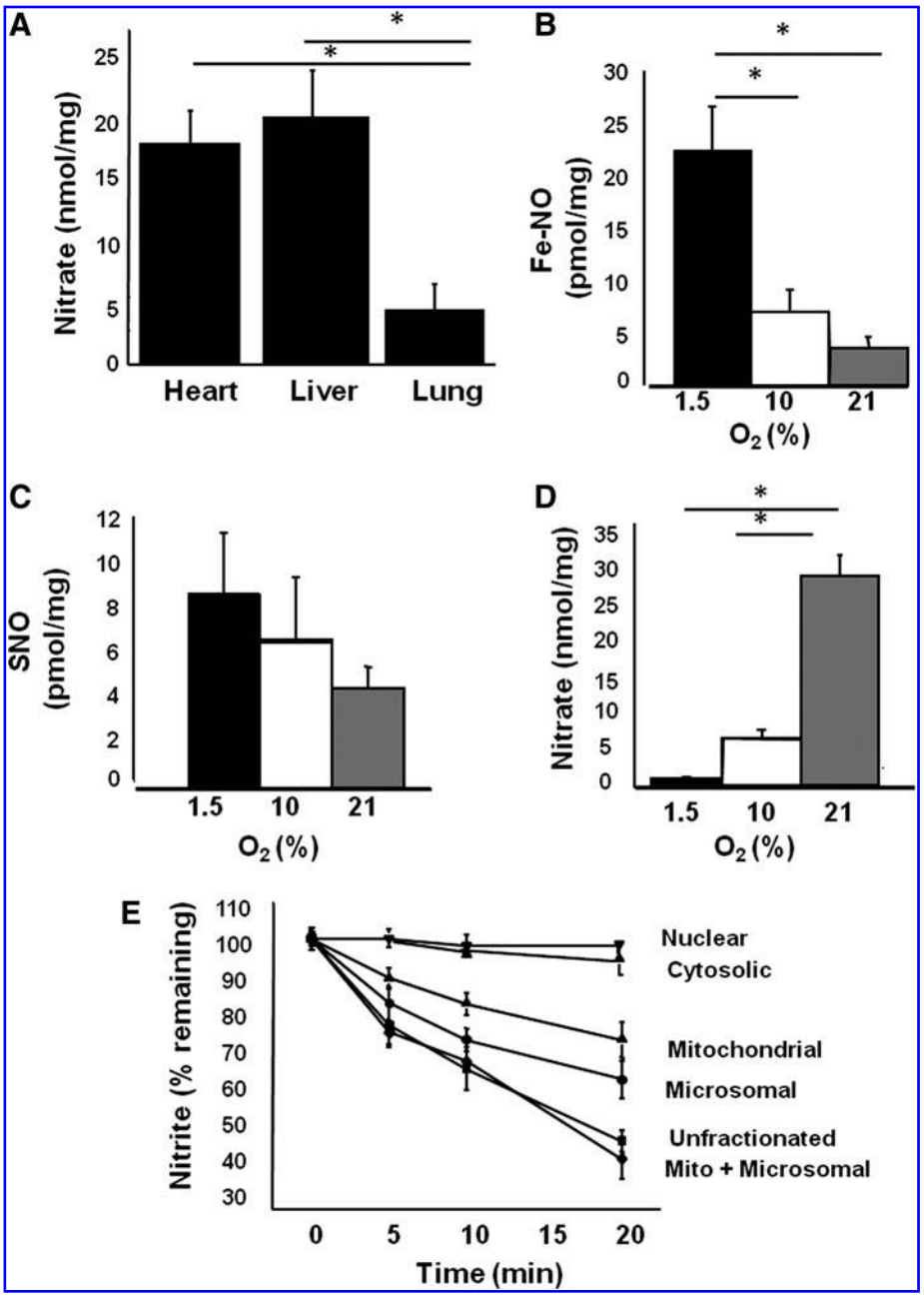


**FIG. 4. Nitrite is oxidized in normoxia. (A)** Nitrate concentration in heart, liver, and lung homogenates 20 min after nitrite addition (100 $\mu M$) at 21% oxygen. **(B)** Fe-NO, **(C)** SNO, and **(D)** nitrate concentration in liver homogenate (4 mg/ml) after nitrite addition at 1.5, 10, or 21% oxygen. **(E)** The percent of nitrite remaining over time in liver homogenate (unfractionated), the nuclear, cytosolic, microsomal fraction with $BH_4$ (100 $\mu M$), mitochondrial fraction with succinate (50 m$M$), or the mitochondrial and microsomal fraction combined. $^*p < 0.01$; $n > 5$. Data are expressed as means ± SEM.

hypoxic hearts compared to normoxic hearts, while nitrate levels were significantly greater in normoxic hearts (Fig. 7).

Basal nitrite levels in mice on a standard diet were next measured. Once removed from the mouse, the heart, liver, lung, and brain were homogenized in saline containing the detergent NP-40 (0.1%) and cyanide (1 m$M$). Among tissues, heart had the greatest basal level of nitrite (18 ± 3 pmol/mg or 0.9 ± 0.15 $\mu M$), followed by liver (9 ± 3 pmol/mg or 0.45 ± 0.15 $\mu M$), lung (7 ± 2 pmol/mg or 0.35 ± 0.1 $\mu M$), and brain (4 ± 0.5 pmol/mg or 0.2 ± 0.02 $\mu M$) (Fig. 8A).

We then administered nitrite by intraperitoneal injection (99–264 $\mu$g/kg) and determined that nitrite levels were elevated in the blood by 5 min and declined with a half life of approximately 10 min (Fig. 8B). The half life of nitrite in the liver showed a similar time course (Fig. 8C). In the heart, nitrite levels peaked later (5–10 min) with the highest dose (Fig. 8D). However, in the lung, no significant change was observed, even at the highest nitrite dose (data not shown).

The effect of oral nitrite administration on tissue nitrite levels was then measured. Supplementation of the drinking water with nitrite (1 g/L) showed significantly increased blood nitrite levels (1.0 ± 0.3 $\mu M$ versus 0.5 ± 0.15 $\mu M$ in controls). In these mice, nitrite levels in the lungs, heart, brain, and kidney approximately doubled in the first 3 days of supplementation and reached a plateau, while in the liver, nitrite levels increased up to day 7 (Figs. 8E–8F).

## Discussion

This study demonstrates that oxygen is a potent regulator of tissue nitrite consumption, regulating the rate and products of tissue nitrite metabolism. While anoxic tissue reduces

Table 1. Oxygen Regulates Nitrate Formation in Heart and Lung

| *Organ/Species* | *1.5% Oxygen* | *10% Oxygen* | *21% Oxygen* |
|---|---|---|---|
| **Heart** | | | |
| Nitrate (nmol/mg) | 3±1 | 8±3 | 18±4 |
| SNO (pmol/mg) | 7±2 | 6±2 | 6±2 |
| Fe-NO (pmol/mg) | 18±6 | 8±2 | 4±1 |
| **Lung** | | | |
| Nitrate (nmol/mg) | N/A | 1±0.5 | 4±2 |
| SNO (pmol/mg) | 1±0.5 | 2±1 | 2±0.5 |
| Fe-NO (pmol/mg) | 5±1 | 2±1 | N/A |

nitrite to NO and forms Fe-NO and SNO, oxygenation of tissue (at concentrations as low as 1.5%) results in more rapid nitrite consumption and a shift towards oxidative pathways, yielding nitrate. We demonstrate that the contribution of the enzymatic nitrite reductase systems responsible for hypoxic nitrite reduction varies among tissues. However, the mitochondrion plays a role in nitrite oxidation in most tissues. Finally, we have demonstrated a method to stabilize nitrite in normoxic tissue *ex vivo* using cyanide and have used this technique to measure the metabolism of nitrite *in vivo*.

Comparison of the nitrite metabolism rate in different tissues demonstrates that the heart and liver metabolize nitrite more rapidly than the lung and brain at any oxygen tension. Our results confirm prior studies (21) showing greatest nitrite reductase activity in the heart and liver and further demonstrate an identical trend among tissues for nitrite oxidation. Feelisch and colleagues have shown a direct relationship between tissue mitochondrial content and hypoxic nitrite reductase activity (21). The present study extends this relationship to nitrite oxidation as well, suggesting that nitrite utilization at every oxygen tension is linked to tissue metabolic activity. This is interesting, given studies demonstrating the regulation of mitochondrial function by nitrite (38, 45, 47) and the metabolism of nitrite by mitochondria (7, 9, 13). Additionally, nitrite metabolism rate directly correlates with basal nitrite levels, suggesting that tissues exposed to higher nitrite levels are more efficient at nitrite metabolism.

Perhaps expectedly, the contribution of each nitrite reductase enzyme is proportional to its relative tissue expression. In the heart, myoglobin is most highly expressed (150 nmol/g (6) versus 35 mU/g XOR (15) and 30.5 nmol/g Ccox (6)), and is the predominant nitrite reductase. In the rat lung, XOR is more highly expressed than Ccox and $CYP_{450}$ and is the major reductase. Similarly, in the liver, Ccox, $CYP_{450}$, and XOR are all highly expressed and contribute equally to nitrite reduction. This suggests that changes in protein expression may modulate the contribution of these proteins to NO generation. This is important in pathological conditions in which the expression of nitrite reductases may be altered. For example, the expression of XOR increases in heart failure (14), while Ccox expression decreases (33). This is also a consideration in studies spanning different species. Though rodents are used to decipher the role of nitrite reductases, the expression of XOR in rat heart and lung is ~100 times greater than in humans (15).

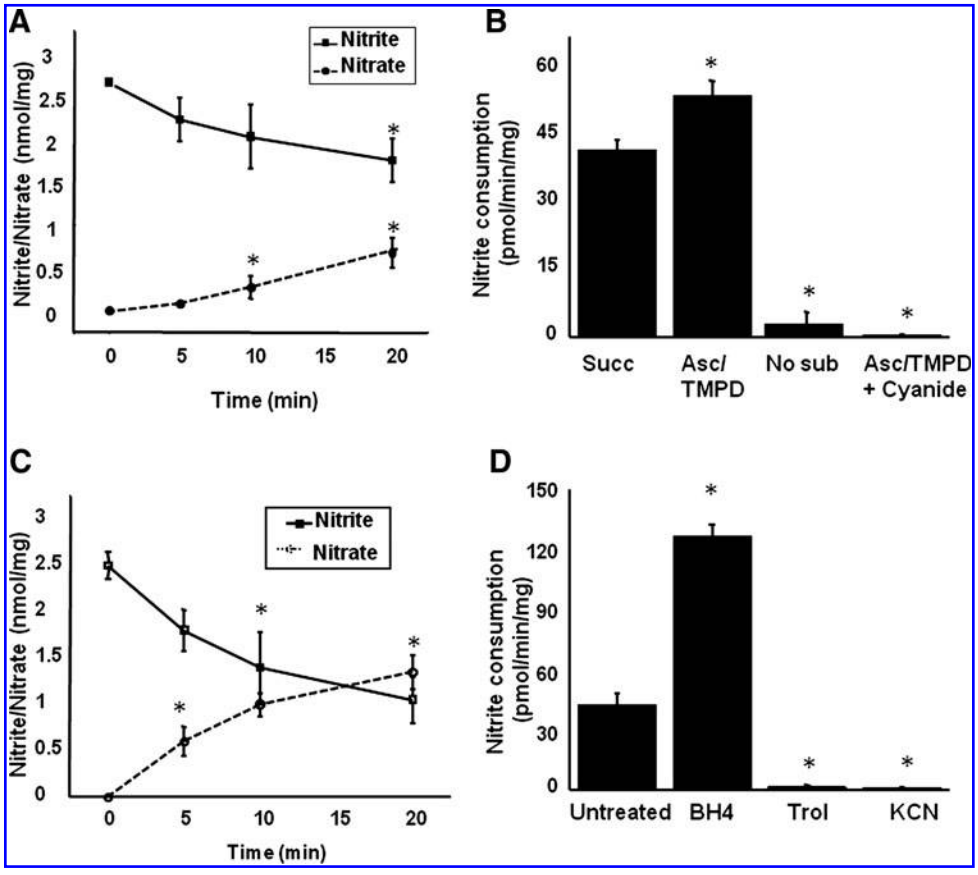


**FIG. 5. Mitochondria and $CYP_{450}$ oxidize nitrite to nitrate.** Nitrite (10 $\mu M$) was added to the **(A)** mitochondrial fraction or **(C)** microsomal fraction of liver tissue and nitrite decay (*solid line*) and nitrate formation (*dashed line*) measured over time. **(B)** The rate of nitrite consumption measured in the mitochondrial fraction in the presence or absence of succinate (500 $\mu M$), ascorbate (500 $\mu M$), and TMPD (50 $\mu M$), no substrate (no sub), cyanide (KCN; 1 m$M$). **(D)** Nitrite consumption in the microsomal fraction in the presence or absence of, tetrahydrobiopterin ($BH_4$; 100 $\mu M$), troleandomycin (Trol; 250 $\mu M$), or cyanide (KCN 1 m$M$). *$p <$ 0.01; $n > 3$. Data are means ± SEM.

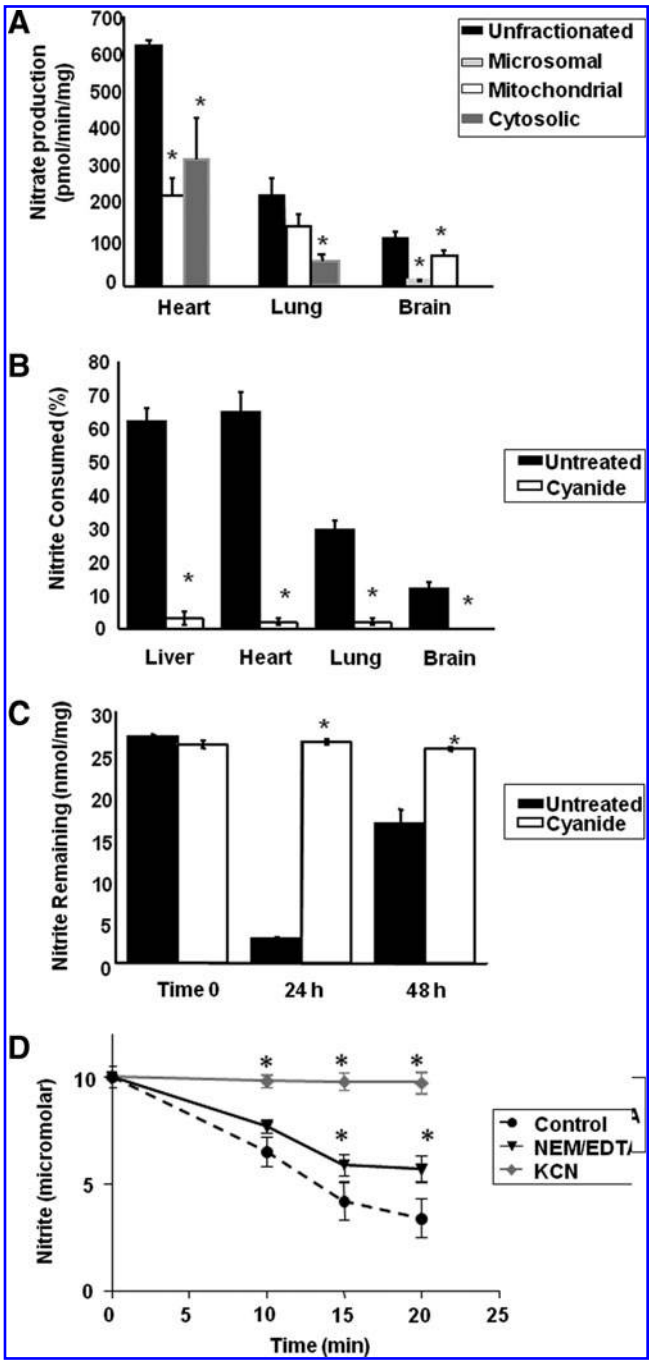


**FIG. 6. Cyanide prevents nitrite metabolism. (A)** Average rate of nitrate production by heart, lung, and brain homogenates and subcellular fractions of the same tissue calculated over the first 10 min after addition of nitrite (100 $\mu M$). **(B)** The percent of added nitrite consumed in heart, liver, and lung homogenates treated with or without cyanide (1 m$M$). **(C)** The concentration of nitrite (100 m$M$) remaining in liver homogenate, 1 day after (37°C) or after being frozen immediately and thawed 2 days later. **(D)** The consumption of nitrite (10 $\mu M$) by liver homogenate left untreated or treated with NEM (10 m$M$)/EDTA (1 m$M$) or cyanide (1 m$M$). *$p < 0.01$; $n = 3$. Data are means ± SEM.

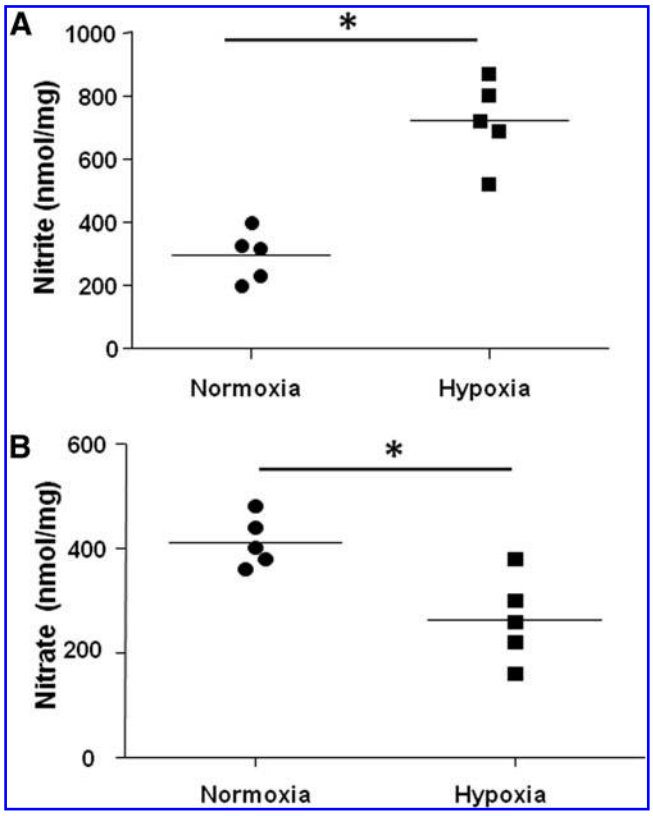


**FIG. 7. Nitrite metabolism in hypoxia and normoxia.** Isolated rat hearts were made normoxic (21%$O_2$) or hypoxic (1%$O_2$) and perfused with nitrite (50 $\mu M$) for 15 min. **(A)** Nitrite and **(B)** nitrate were measured in the tissue 5 min after perfusion. *$p < 0.01$; $n = 5$.

Notably, all the tissues examined contain significant basal concentrations of NOS, which utilizes oxygen as a substrate. Given that the $K_m$ oxygen for eNOS has been reported between 4 and 23 $\mu M$ (1), and we do not observe nitrite-dependent NO generation above ~3 $\mu M$, our data are consistent with the current paradigm that nitrite serves as a mechanism of NO formation in conditions in which NOS is oxygen limited. However, the role of oxygen in modulating the contribution of nitrite reductases also warrants further study. While the relative contribution of the nitrite reductases were measured in anoxia, this relationship may be altered as oxygen tension increases, since nitrite reduction is dependent on deoxygenation of the metal centers of each enzyme. It is likely that each enzyme becomes inhibited at different points between 0% and 1.5% (~4 $\mu M$) $O_2$, leading to the decrease in NO generation observed. For example, myoglobin reduces nitrite at oxygen concentrations lower than its p50 (3.1 $\mu M$) (29, 45). While the exact mechanism of Ccox-dependent reduction is unknown, it potentially involves the binuclear center of the enzyme ($K_m$ oxygen <1 $\mu M$) (13). In the case of XOR, nitrite is reduced at the molybdenum center of the enzyme, resulting in a direct competition between nitrite and oxygen for electrons and suggesting that submicromolar concentrations of oxygen inhibit nitrite reduction (43). Thus, it is possible that XOR and Ccox-dependent nitrite reduction is inhibited by low concentrations of oxygen and at 1.5%, only myoglobin is a functional reductase.

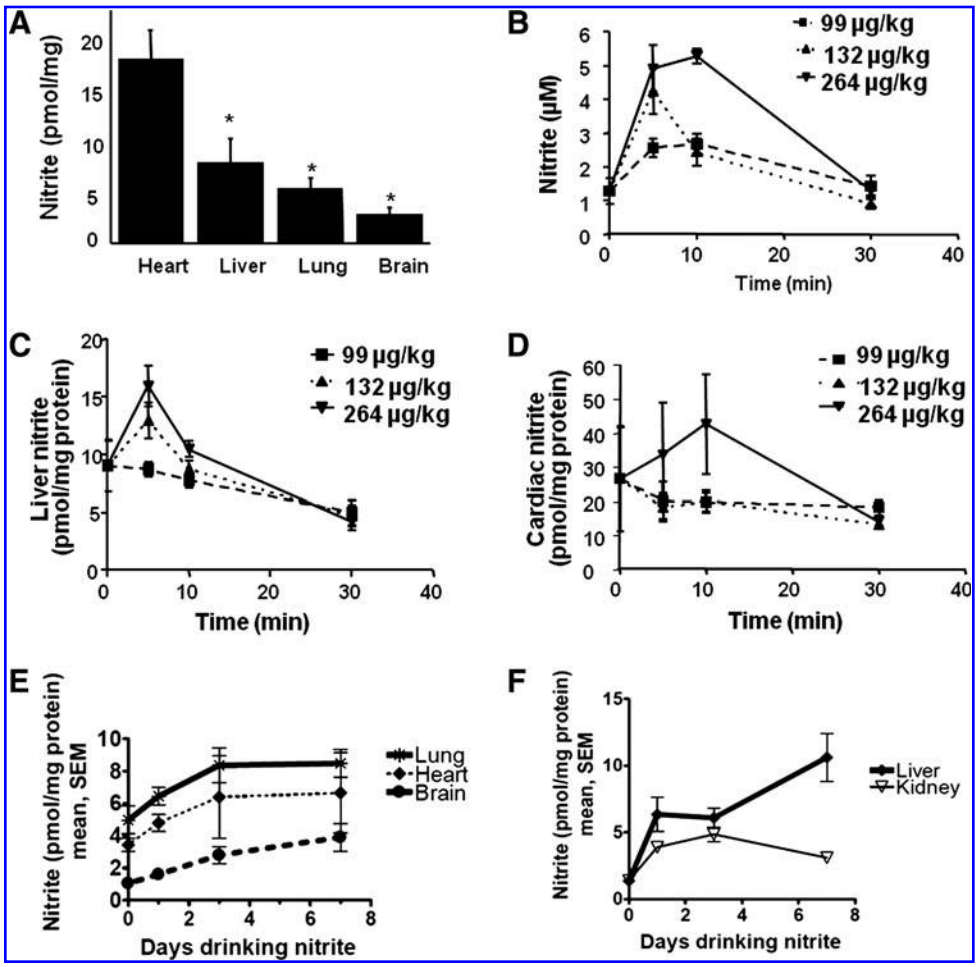


**FIG. 8. Metabolism of intraperitoneal and oral nitrite.** **(A)** Basal levels of nitrite in the organs of untreated mice. Nitrite concentration in the **(B)** whole blood, **(C)** liver, and **(D)** heart over time after the administration of 99,132, or 264 mg/kg of sodium nitrite to each mouse. The concentration of nitrite in (**E**) heart, lung, brain, (**F**) kidney and liver of mice supplemented with nitrite (1 g/L) in the drinking water for 1–7 days.

Interestingly, nitrite-dependent S-nitrosothiol formation was independent of oxygen concentration. While the chemistry underlying nitrite-dependent tissue S-nitrosation is not entirely clear, this oxygen independence is similar to what has previously been observed in studies using low levels of NO as a nitrosating agent (10). Recent studies describe chemistry by which nitrite reacts with heme proteins to produce a ferric heme-$NO_2^{\bullet}$-like intermediate. This intermediate reacts with NO, forming $N_2O_3$ as a nitrosating agent (8, 27). Alternatively, NO generated from hypoxic nitrite reduction may react with superoxide to form peroxynitrite. While these pathways depend on the reduction of nitrite to NO, our data demonstrate that SNO persists even when NO generation is inhibited, suggesting that pathways of nitrite-mediated S-nitrosation exist independent of nitrite reduction. One potential explanation for this is nitrite acidification in hypoxic tissue, which may lead to thiol nitrosation by nitrous acid.

In contrast to hypoxia, normoxic nitrite metabolism is rapid and catalyzed by Ccox, myoglobin, and $CYP_{450}$. Although it is accepted that myoglobin oxidizes nitrite (18, 30), this role for Ccox and $CYP_{450}$ is not characterized. Further, the relative contribution of these enzymes to tissue nitrite oxidation is unknown. The ability for Ccox to oxidize nitrite is consistent with prior studies in which oxygen consumption by the purified enzyme was observed in the presence of nitrite (4, 44). However, millimolar concentrations of nitrite were required for this activity (44). Here, we observed this activity with physiological concentrations of nitrite. While it is difficult to reconcile this discrepancy, it is possible that the physiological environment of the mitochondrial inner membrane amplifies the local nitrite concentration around Ccox or that in the physiological milieu, other Ccox modulators make nitrite oxidation more favorable.

Several groups have shown nitrite oxidase activity in liver homogenate and hepatocytes (26, 34). While Porterfield suggested that this activity was dependent on catalase, inhibition of catalase with aminotriazole had no effect in our study. However, consistent with studies by Lancaster (34), in which a role for microsomal mixed-function oxidases was identified, we confirmed that $CYP_{450}$ is the major nitrite oxidase in liver. Troleandomycin, an inhibitor of CYP3A4, attenuated nitrite oxidation by the microsomal fraction, suggesting that this isoform of the enzyme is responsible for nitrate formation. CYP3A4 is the most highly expressed $CYP_{450}$ in the liver and catalyzes the oxidation of the largest range of substrates. Beyond the liver, CYP3A4 is expressed in the brain, where it is responsible for psychoactive drug metabolism (2). Of note, the brain demonstrated significant microsomal nitrite oxidation. It is interesting to consider that nitrite may influence the metabolism of psychotropic drugs, especially given studies demonstrating nitrite-dependent modulation of $CYP_{450}$ activity (11, 20). Further study is required to determine the mechanism of nitrite oxidation by CYP3A4.

Notably, all three nitrite oxidase enzymes also catalyze nitrite reduction. Future studies will determine the oxygen threshold at which each enzyme shifts from reduction to oxidation. While the exact mechanisms of nitrite oxidation/reduction by $CYP_{450}$ and Ccox are unclear, putative mechanisms suggest that these reactions involve the heme centers of these enzymes (34, 44). Thus, the shift from oxidation to reduction depends on the oxygen affinity of these enzymes. Potentially, at intermediate oxygen tensions, at which these enzymes are partially deoxygenated, nitrite oxidation and reduction occur in parallel, as has recently been reported for the hemoglobin-nitrite reaction (24). Consistent with this, we show that at 1.5% oxygen, nitrite is metabolized to both NO and nitrate.

While tissue nitrite levels are now measured by a number of laboratories, *ex vivo* tissue metabolism of nitrite is not routinely considered. We demonstrate that significant nitrite consumption can occur in isolated organs and that cyanide can be used to prevent this metabolism. Using cyanide, we demonstrate that intraperitoneal administration of nitrite increases nitrite levels most significantly in the liver and heart, where nitrite uptake is rapid. In these studies, changes in nitrate were undetectable due to the high levels of basal nitrate present *in vivo* and low concentrations of nitrite administered. However, the rapid metabolism of nitrite in the tissue suggests that oxidation is at least partially responsible.

## Materials and Methods

### *Chemicals*

All reagents were obtained from Sigma-Aldrich.

### *Animals*

Male Sprague-Dawley rats (250 g) and C57/Bl6 mice (20–30 g) were used in accordance with the Animal Care and Use Committee of the University of Pittsburgh. Mice were on oral nitrite supplementation and rats were on a low nitrite/nitrate diet (TD99366 Harlan Tekland) prior to studies. See details in Supplementary Methods (supplementary data are available online at www.liebertonline.com/ars).

### *Isolated heart perfusion*

Rat hearts were isolated and perfused on the Langendorff system, as previously described (46). See Supplementary Methods.

### *Tissue homogenization and deoxygenation*

Organs were removed from the anesthetized animal after 10 min of saline perfusion (21% $O_2$). Tissues were chopped and rinsed twice with saline to remove erythrocytes and to decrease nitrate concentration. The tissue was then homogenized and suspended in Krebs-Henseleit (KH) buffer at a concentration of 4 mg/ml (measured with BCA protein assay kit; Pierce). Homogenates were deoxygenated by passing premade purchased gas mixtures (0%–21% $O_2$, 5% $CO_2$ balanced with $N_2$; Matheson Gas, Pittsburgh, PA) over the homogenate in a closed chamber. Tissue homogenates had undetectable levels of NO production or SNO/Fe-NO prior to nitrite treatment. In experiments in which homogenates were frozen (Fig. 6C), samples were flash frozen in liquid nitrogen to prevent artifactual S-nitrosation.

### *Measurement of NOx by chemiluminescence*

All species were measured after oxidation or reduction chemistry in a vessel connected inline to a Nitric Oxide Analyzer (Sievers). Nitrite and SNO were measured by triiodide-based reduction (22), nitrate measured in vanadium chloride (22). Fe-NO concentration was measured by oxidation in potassium ferricyanide (0.1 *M*) (22). NO generation was measured in PBS (45). The rate of NO production was calculated as the average rate over the period of time 0 until the trace reached steady state. See Supplementary Methods for details on chemiluminescence.

### *Tissue fractionation*

Tissue was homogenized in a buffer consisting of 250 m*M* sucrose, 10 m*M* Tris, 1 m*M* EGTA (pH 7.4) and centrifuged at 10,000 *g* to pellet the mitochondrial fraction. The microsomal fraction was obtained by ultracentrifugation of the post-mitochondrial supernatant at 100,000 *g*. The cytosolic fraction was the cellular fraction devoid of mitochondria, microsomes, and nuclear material.

### *Statistics*

All values are means ± SEM of at least three independent experiments. Single comparisons were tested for significance using a two-tailed Student's *t*-test. ANOVA followed by the Bonferroni post hoc test was used for multiple comparisons.

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## Author Disclosure Statement

No competing financial interests exist.

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**Abbreviations Used**

Ccox = cytochrome *c* oxidase
CYP450 = cytochrome $P_{450}$
metHb = methemoglobin
NO = nitric oxide
NOS = nitric oxide synthase
SNO = S-nitrosothiols
XOR = xanthine oxidoreductase

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