Deoxymyoglobin Is a Nitrite Reductase That Generates Nitric Oxide and Regulates Mitochondrial Respiration

Sruti Shiva,* Zhi Huang,* Rozalina Grubina, Junhui Sun, Lorna A. Ringwood, Peter H. MacArthur, Xiuli Xu, Elizabeth Murphy, Victor M. Darley-Usmar, Mark T. Gladwin

Abstract—Previous studies have revealed a novel interaction between deoxyhemoglobin and nitrite to generate nitric oxide (NO) in blood. It has been proposed that nitrite acts as an endocrine reservoir of NO and contributes to hypoxic vasodilation and signaling. Here, we characterize the nitrite reductase activity of deoxymyoglobin, which reduces nitrite approximately 36 times faster than deoxyhemoglobin because of its lower heme redox potential. We hypothesize that physiologically this reaction releases NO in proximity to mitochondria and regulates respiration through cytochrome c oxidase. Spectrophotometric and chemiluminescent measurements show that the deoxymyoglobin-nitrite reaction produces NO in a second order reaction that is dependent on deoxymyoglobin, nitrite and proton concentration, with a bimolecular rate constant of 12.4 mol/L·s⁻¹ (pH 7.4, 37°C). Because the IC₅₀ for NO-dependent inhibition of mitochondrial respiration is approximately 100 mol/L at physiological oxygen tensions (5 to 10 μmol/L); we tested whether the myoglobin-dependent reduction of nitrite could inhibit respiration. Indeed, the addition of deoxymyoglobin and nitrite to isolated rat heart and liver mitochondria resulted in the inhibition of respiration, while myoglobin or nitrite alone had no effect. The addition of nitrite to rat heart homogenate containing both myoglobin and mitochondria resulted in NO generation and inhibition of respiration; these effects were blocked by myoglobin oxidation with ferricyanide but not by the xanthine oxidoreductase inhibitor allopurinol. These data expand on the paradigm that heme-globins conserve and generate NO via nitrite reduction along physiological oxygen gradients, and further demonstrate that NO generation from nitrite reduction can escape heme autocapture to regulate NO-dependent signaling. (Circ Res. 2007;100:654-661.)

Key Words: nitrite ■ myoglobin ■ mitochondria

Recent studies have established a role for nitrite as an endocrine storage pool of NO that can be bioactivated along the physiological oxygen gradient to mediate a number of responses, including hypoxic vasodilation,1–4 cytoprotection following ischemia/reperfusion,5,6 and NO-dependent and independent signaling.7 Several mechanisms for nitrite conversion to NO have been described including enzymatic reduction by xanthine oxidoreductase,8 the mitochondria9 and NO synthase (NOS),10 as well as nonenzymatic acidic disproportionation.11,12 We have described nitrite-dependent vasodilation in vitro1–2 and in vivo1 associated with nitrite reduction by deoxymyoglobin, consistent with a reaction first characterized by Brooks13 and Doyle:14

(1) Nitrite + deoxyHb + H⁺ → NO + metHb + OH⁻ (average k=0.35 mol/L·s⁻¹)

Nitrite is reduced to NO by deoxyhemoglobin, oxidizing hemoglobin in the process. This reaction possesses both pH and oxygen sensor chemistry because of its requirement for deoxygenated hemoglobin and a proton.15 NO generated from the reaction binds to unreacted hemoglobin to form iron-nitrosyl hemoglobin by the following reaction:

(2) NO + deoxyHb→HbNO (k=6×10⁷ M⁻¹s⁻¹)

Furthermore, this reaction is regulated by the allosteric structural conformation of hemoglobin, such that the maximal rate of nitrite reduction occurs at the transition of R (oxy) to T (deoxy) state, around the P₅₀ of hemoglobin.15,16 This is reflected in the fact that the bimolecular rate constant varies over the reaction time as the allosteric conformation of the hemoglobin molecule changes, such that R state hemoglobin has a bimolecular rate constant of 6 mol/L⁻¹ s⁻¹ whereas the constant for T state is 0.12 mol/L⁻¹ s⁻¹, giving the reaction an average bimolecular rate constant of 0.35 mol/L⁻¹ s⁻¹ (pH 7.4, 25°C).15 Mechanically, the lower heme redox potential in the R-state oxygenated conformation of hemoglobin is associated with an equilibrium distribution of heme electrons that thermodynamically favor the reduction of nitrite. Notably,
myoglobin possesses a significantly lower heme redox potential than R-state hemoglobin and we found that deoxymyoglobin reduced nitrite and generated NO at a faster rate than deoxyhemoglobin, in a similar manner to what has been described for the reduction of S-nitrosogluthathione by deoxyhemoglobin and myoglobin.\textsuperscript{24,15,17}

NO regulates a number of hypoxic signaling responses including mitochondrial respiration\textsuperscript{18–20} and biogenesis,\textsuperscript{21} expression of hypoxia inducible factor -1,\textsuperscript{22,23} and angiogenesis.\textsuperscript{24} Although myoglobin is thought to limit NO bioavailability in tissues because of its rapid reaction with NO, in hypoxic tissue myoglobin-dependent nitrite reduction may provide a mechanism by which NO is generated to regulate these responses when NO becomes oxygen limited. We sought to determine whether the paradigm of heme-based nitrite reduction could be extended beyond hemoglobin to myoglobin in tissue. Here, we test the hypothesis that nitrite reduction could be extended beyond hemoglobin to myoglobin.\textsuperscript{15,17}

Materials and Methods

Hemoglobin and Myoglobin

Human hemoglobin was prepared as previously described.\textsuperscript{15} Oxygen saturation and concentration of hemoglobin and horse myoglobin were measured by visible absorption spectroscopy.

Determination of the Product Yields of the Nitrite-Myoglobin Reaction

The kinetics of the nitrite-deoxyheme reaction and the concentration of each heme species was monitored spectrophotometrically and analyzed by least-squares deconvolution using standard reference spectra as previously described.\textsuperscript{15}

Animals

Mitochondria and tissue were isolated from male Sprague-Dawley rats 250 to 300 g in weight which were treated in compliance with the guidelines of the animal care and use committee of NIH.

Mitochondrial Isolation and Respiration

Mitochondria were isolated by differential centrifugation as previously described.\textsuperscript{19} And protein concentration determined using the Bradford Assay. To measure respiration, protein was suspended in respiration buffer [KCl (120 mmol/L); sucrose (25 mmol/L); HEPES (10 mmol/L); EGTA (1 mmol/L); KH2PO4 (1 mmol/L); MgCl2 (5 mmol/L)] in a stirred, sealed chamber fit with a Clark type oxygen electrode (Instech Corp, Plymouth, Mass) connected to a data recording device (DATAQ systems, Akron, Ohio).

Isolation and Respiration of Cardiomyocytes

Cardiomyocytes were isolated from rats as previously described.\textsuperscript{25} To measure respiration, myocytes were suspended in media 199 at 37°C we observed an increase in the bimolecular rate constant of 6 mol/L\textsuperscript{-1}s\textsuperscript{-1} (25°C, pH 7.4).\textsuperscript{15} To further characterize this reaction, we monitored the disappearance of deoxymyoglobin as well as the formation of total metmyoglobin (the sum of metmyoglobin) and iron-nitrosyl-metmyoglobin over the time course of the reaction (Figure 1A). Whereas examination of equations 1 and 2 would suggest that 1 nitrite reacting with 1 deoxymyoglobin would produce 1 metmyoglobin and iron-nitrosyl-metmyoglobin, work by other investigators suggests that the reaction would produce more metmyoglobin than iron-nitrosyl myoglobin, and that this nonstoichiometric product yield occurs secondary to NO-metmyoglobin formation.\textsuperscript{26} This is important because the reactions described in equations 1 and 2 would generate only NO whereas NO-metmyoglobin could generate NO\textsuperscript{-}ferrous myoglobin, which could nitrosate thiols. We examined these reactions and found that the product yields are actually stoichiometric (Figure 1A and B) with 1 nitrite reacting with 1 deoxymyoglobin to produce 1 metmyoglobin and NO (equation 1), which subsequently binds to one deoxymyoglobin to form one iron-nitrosyl-metmyoglobin (equation 2). Whereas we saw a slightly higher yield of metmyoglobin when low concentrations of nitrite were used, this was because of the autooxidation of deoxymyoglobin. We observed that 2% of deoxymyoglobin was oxidized to metmyoglobin over 1000 seconds in the absence of nitrite.

To examine the stoichiometry of the reaction we performed least-squares deconvolution over the reaction time and included standard spectra for deoxymyoglobin, iron-nitrosyl-metmyoglobin, and iron-nitroso-metmyoglobin (nitrite bound to metmyoglobin). As shown in Figure 1B, virtually no NO-metmyoglobin forms during the reaction, but rather nitrite-metmyoglobin forms. We therefore included nitrite-metmyoglobin and NO-metmyoglobin reference spectra in our analysis and compared the \(\chi^2\) values over time. The inclusion of nitrite-metmyoglobin resulted in significantly lower residuals than NO-metmyoglobin (Figure 1C). These data suggest that NO is a radical, rather than NO\textsuperscript{-}ferrous myoglobin, is the primary species generated and that no stable NO-metmyoglobin intermediate forms.

As expected, with an increase in temperature from 25\(^\circ\) to 37\(^\circ\)C we observed an increase in the bimolecular rate constant (from 6 to 12 mol/L\textsuperscript{-1}s\textsuperscript{-1}) (data not shown). Further kinetic analysis of the reaction showed expected increases in
the initial reaction rate when either nitrite (0 to 5 mmol/L) or deoxymyoglobin concentration was increased (Figure 1D and E). The bimolecular rate constants calculated from each of the conditions in Figure 1D and E were in agreement with one another and averaged 12.83 ± 0.157 and 12.86 ± 0.429 mol/L⁻¹s⁻¹ respectively.

Deoxymyoglobin dependent nitrite reduction requires a proton and hence should be regulated by pH, with reaction rate increasing by 10 fold with each decrease in pH unit. Indeed, as pH was lowered the initial reaction rate of deoxymyoglobin (29 μmol/L) and nitrite (0.5 mmol/L), increased from 0.065 μmol/L/s at pH 7.8 to 1.30 μmol/L/s at pH 6.5 (Figure 1F).

Deoxymyoglobin-Dependent Nitrite Reduction Generates NO Gas
We compared the formation of NO from the reaction of deoxymyoglobin and deoxyhemoglobin (35 μmol/L) with nitrite (5 mmol/L) (Figure 2A). Because of their different reaction rates (Figure 2A, inset), the NO generation profiles for hemoglobin and myoglobin differ. NO generation from myoglobin occurs quickly as a large burst; whereas hemoglobin-dependent NO generation is much slower and peaks at the T-to-R allosteric transition.

In this experiment, supraphysiological nitrite concentrations were used to measure NO gas accumulation in the head space over a short period of time. However, the calculated rate of NO generated for physiological concentrations of myoglobin (25 μmol/L) and nitrite (20 μmol/L) is 3.5 nM/second at pH 7.4 and 37°C. This rate of conversion of nitrite to NO should produce sufficient concentrations of NO to inhibit mitochondrial respiration within ten to twenty seconds at neutral pH and only a few seconds at pH 6.4.

Deoxymyoglobin Dependent Reduction of Nitrite Regulates Mitochondrial Respiration
Nitric oxide is a well characterized modulator of mitochondrial function, with physiologic concentrations regulating respiration, biogenesis, cytochrome c release and reactive oxygen species production. The best characterized effect of NO on mitochondria is the binding of NO to cytochrome c oxidase to inhibit respiration. This reversible inhibition is more potent as oxygen concentration decreases (IC₅₀ = 140 nmol/L at 5 to 10 μmol/L O₂) and is thought to limit hypoxic tissue damage by the extension of tissue oxygen gradients.

We hypothesized that NO produced by the myoglobin-dependent reduction of nitrite could inhibit mitochondrial respiration. However, because myoglobin must be at least partially deoxygenated (which occurs below its P₅₀ of 3.1 μmol/L oxygen), we developed a method to measure
inhibition of respiration at low oxygen concentration. Using a Clark-type oxygen electrode in a sealed chamber, mitochondria (2 mg/mL) were stimulated to respire by the addition of succinate (15 mmol/L) and ADP (1 mmol/L). Once the chamber was made anoxic by allowing the mitochondria to consume the oxygen in the chamber, the chamber lid was removed to allow diffusion of air back into the buffer. However, even with the diffusion of air into the stirred chamber, the chamber remained anoxic because of the constant rate of oxygen consumption by the mitochondria exceeding the rate of oxygen diffusion into the chamber. The chamber remained at zero oxygen tension until the respiratory rate decreased either because of the exhaustion of substrate (in control mitochondria) or the addition of a respiratory inhibitor such as cyanide (20 μmol/L) or NO (10 μmol/L Papa NONOate) (Figure 2B). In this model, the time from the equilibration of mitochondria with air to the time at which an increase in the oxygen concentration in the chamber was detected by the electrode was used as a measure of the extent of mitochondrial inhibition. With complete inhibition of respiration in the presence of cyanide (20 μmol/L), the time from equilibration to increase in the respiratory trace was 42±8 seconds, whereas in the absence of an inhibitor, this time was 182±23 seconds. Using the time to inhibition in the presence (100% inhibition) and absence (0% inhibition) of cyanide, inhibition in other conditions was expressed as a percentage of total inhibition (extent of inhibition). For example, in the presence of the NO donor PapaNONOate (10 μmol/L), the extent of inhibition is 81±7% consistent with partial inhibition by NO. It is important to note that in this system the time to inhibition reflects the time necessary for oxygen diffusion into the buffer and detection by the oxygen electrode and does not represent instantaneous inhibition.

To determine whether deoxymyoglobin dependent nitrite reduction could produce NO capable of inhibiting respiration, mitochondrial respiration was stimulated in the presence of oxygenated myoglobin (25 μmol/L). Once the chamber became anoxic and the myoglobin deoxygenated, nitrite (20 μmol/L) was added and diffusion of air into the chamber was initiated. In this case, the mitochondria were inhibited by 79±9%, whereas respiration in the presence of nitrite (20 μmol/L) (9±7%) or myoglobin (25 μmol/L) (9.8±5.5%) alone was not significantly inhibited in comparison to control mitochondria (Figure 2C and D). The addition of superoxide dismutase (50 U/mL) and catalase (50 U/mL) or the lipid soluble antioxidant butylated hydroxytoluene (100 μmol/L) did not significantly change the extent of inhibition in the presence of nitrite and myoglobin, indicating that neither oxygen nor NO consumption by superoxide or lipid peroxidation played a significant role in this system. The use of oxidized myoglobin (metmyoglobin, 20 μmol/L) instead of oxymyoglobin did not inhibit respiration, consistent with the inability of metmyoglobin to reduce nitrite (Figure 2D). Isolated heart mitochondria (2 mg/mL) were also inhibited in the presence of nitrite (20 μmol/L) and myoglobin (25 μmol/L) (83±5%) but not by myoglobin (3.5±9%) or nitrite (6.8±5%) alone (Figure 2E).

The inhibition of respiration with increasing myoglobin concentration (0 to 100 μmol/L) in the presence of nitrite (25 μmol/L) was biphasic with the greatest inhibition occurring at 25 μmol/L of myoglobin (Figure 3A). Nitric oxide binds to deoxymyoglobin with a rate of 1.7×10^7 M^-1s^-1 and to cytochrome c oxidase at a similar rate (1.0×10^7 M^-1s^-1), suggesting that an efficient competition is established between the mitochondria and myoglobin for NO. Hence, in conditions of excess myoglobin, myoglobin can successfully “outcompete” mito-
chondria for NO. The biphasic curve seen with increased myoglobin concentration is most probably because of this autocapture of NO by myoglobin. The extent of inhibition (in the presence of 25 μmol/L myoglobin) was also dependent on the concentration of nitrite (0 to 50 μmol/L) (Figure 3B and C).

Notably, at concentrations above 50 μmol/L, nitrite had myoglobin independent inhibitory effects on mitochondria, consistent with studies showing that nitrite bound to cytochrome c oxidase results in inhibition of respiration as well as the ability of mitochondria to reduce high concentrations of nitrite to NO independent of myoglobin.

Inhibition of Respiration by Deoxygenated Myoglobin, Hemoglobin, and Erythrocytes Is Determined by Their Nitrite Reductase Rates

Although hemoglobin and myoglobin reduce nitrite by the same reaction, their reduction rates are different. The rate of hemoglobin dependent nitrite reduction is regulated by the allosteric transition of hemoglobin and the bimolecular rate constant for T-state deoxyhemoglobin is 0.39 mol/L·s⁻¹. Because of its lower heme reduction potential, the rate of myoglobin-dependent nitrite reduction is 32 times faster than hemoglobin. The bimolecular rate constant of T-state deoxyhemoglobin encapsulated in erythrocytes is reduced by approximately half (0.18 mol/L·s⁻¹) in comparison to cell free hemoglobin, because of membrane effects that decrease the rate of nitrite uptake.

Comparison of the rate of mitochondrial inhibition for hemoglobin (50 μmol/L), myoglobin (50 μmol/L), and erythrocytes (0.3% hematocrit) in the presence of nitrite (20 μmol/L) showed that whereas all 3 forms of deoxyhemoglobin were capable of inhibiting mitochondrial respiration in the presence of nitrite, the extent of inhibition was significantly different. The extent of inhibition correlated with measured nitrite reduction and NO production rates from the reactions (Figure 4A and B). As expected, myoglobin was the most potent inhibitor of respiration (54±8% inhibited) followed by cell free hemoglobin (38±9%) and then erythrocytes (19±9%).

Nitrite Inhibits Mitochondrial Respiration in Heart Homogenates

Whereas we have shown that isolated myoglobin can reduce nitrite to NO, it is important to determine whether myoglobin-
dependent NO generation can occur in a physiological environment with other proteins present. To test this, NO generation was measured by chemiluminescence in rat heart homogenate (1 mg/mL) in the presence and absence of nitrite (1 mmol/L). Consistent with previous studies, when nitrite was added to the homogenate under anaerobic conditions NO (12.1 ± 2 nmol/min/mg) was detected, whereas no NO was generated in the absence of nitrite (Figure 5A). Previous studies have shown that enzymatic reduction of nitrite by xanthine oxidoreductase is responsible for a significant portion of tissue-dependent nitrite reduction. However, in these experiments, treatment of tissue with an inhibitor of xanthine oxidase, allopurinol (100 μmol/L), showed minimal change in NO production by the heart (10.3 ± 4.0 nmol/min/mg; Figure 5A and 5B). Treatment of the heart homogenate with ferricyanide (1 mmol/L) to oxidize myoglobin decreased NO production by approximately 65% (4.2 ± 1.5 nmol/min/mg; Figure 5A and 5B).

We also measured NO production from nitrite in lung homogenate, which contains minimal amounts of myoglobin. Addition of nitrite to the lung generated NO (6.2 ± 2.1 nmol/min/mg), and this rate was unchanged (5.9 ± 3.2 nmol/min/mg) by treatment with ferricyanide (5 mmol/L), consistent with the lack of myoglobin in the tissue. In contrast to the heart, allopurinol (100 μmol/L) decreased the NO production in lung homogenate by approximately 33%. These data are consistent with the predominant nitrite reductase in the heart being myoglobin, whereas in the lung, other nitrite reductases, including xanthine oxidoreductase, playing a role.

To determine whether the NO production seen by chemiluminescence could regulate mitochondrial respiration, respiration was monitored in rat heart homogenates (4 mg/mL protein) in the presence and absence of nitrite. In the presence of nitrite (40 μmol/L), the extent of inhibition was significantly greater (64 ± 3%) than in the absence of nitrite (Figure 5C and D). Whereas no significant effect was seen at nitrite concentrations below 40 μmol/L, this may be because of the insensitivity of our system which requires significant inhibition, the presence of high concentrations of myoglobin, or the loss of compartmentalization of mitochondria and myoglobin in the homogenate. Interestingly, whereas treatment of the homogenate with allopurinol (100 μmol/L) alone increased the extent of inhibition by approximately 10% in comparison to tissue alone, there was no change in the extent inhibition in the presence of nitrite and allopurinol (65 ± 4%). These data again suggest that the majority of nitrite-dependent inhibition of respiration in the tissue homogenate is independent of xanthine oxidoreductase.

Low Concentrations of Nitrite Inhibit Respiration in Cardiomyocytes

To determine the effect of membrane barriers on nitrite-dependent regulation of mitochondrial respiration, we used intact isolated cardiomyocytes which contain high concentrations of both myoglobin and mitochondria. We monitored respiration of the intact myocytes (10⁶ cell/mL) in the presence and absence of nitrite and found that nitrite inhibited respiration in a concentration-dependent manner (Figure 6A and B). Interestingly, myocytes were even more sensitive to nitrite than isolated mitochondria or heart homogenate. With nitrite concentrations as low as 2.5 μmol/L, this may be because of the insensitivity of our system which requires significant inhibition, the presence of high concentrations of myoglobin, or the loss of compartmentalization of mitochondria and myoglobin in the homogenate. Interestingly, whereas treatment of the homogenate with allopurinol (100 μmol/L) alone increased the extent of inhibition by approximately 10% in comparison to tissue alone, there was no change in the extent inhibition in the presence of nitrite and allopurinol (65 ± 4%). These data again suggest that the majority of nitrite-dependent inhibition of respiration in the tissue homogenate is independent of xanthine oxidoreductase.

Discussion

We have shown here that deoxymyoglobin is an efficient nitrite reductase with a bimolecular rate constant of 12 mol/L·s⁻¹ at 37°C that generates bioavailable NO. Nitric oxide generated from the reaction of physiological concentrations of myoglobin and nitrite during hypoxia can inhibit respiration in isolated mitochondria in a manner that is
The present study extends this paradigm to generate NO. This reaction produces vasodilation, dependent on the concentration of both nitrite and myoglobin. Furthermore, this reaction is regulated by oxygen and occurs during hypoxia when oxygen concentration falls below the p50 of myoglobin (2 to 4 mm Hg). This is particularly relevant in organs such as the heart and skeletal muscle where cells remain hypoxic physiologically. To that end, we have shown here that in heart homogenate and isolated cardiomyocytes, the addition of nitrite generates NO and inhibits mitochondrial respiration.

Several earlier studies demonstrated that deoxyhemoglobin-dependent reduction of nitrite mediates biological responses. This reaction produces vasodilation, generates cGMP, inhibits mitochondrial respiration, and generates NO. The present study extends this paradigm to myoglobin. Furthermore, the ability of myoglobin-dependent reduction of nitrite to inhibit mitochondrial respiration highlights the ability of NO to escape autocapture by vicinal heme groups. Indeed, in many of these experiments, approximately 20 μmol/L nitrite reacts with an excess of myoglobin (25 to 100 μmol/L) to inhibit respiration; despite the fact that in the time frame of these reactions much of the myoglobin remains reduced and remains capable of reaction with the formed NO. The ability of nitrite to inhibit respiration in tissue homogenate and intact cardiomyocytes is integral to this point because these data demonstrate that even in the presence of other tissue components the NO generated is able to inhibit mitochondria before being scavenged.

The physiological implications of these data should be considered in the context of hypoxic signaling within tissue. Oxygen is a requisite substrate for the production of NO by NOS (km = 100 μmol/L). We have recently shown that NO produced in normoxia reacts with the multicopper oxidase ceruloplasmin, which oxidizes the NO to nitrite. As oxygen tension decreases and oxygen becomes limiting for NO-dependent NO formation, this nitrite reservoir can be reduced to NO by reactions with deoxymyoglobin. This would ensure a constant supply of NO production along a large gradient of oxygen tensions in the tissue. This myoglobin-dependent reduction of nitrite would be further enhanced when tissue pH drops, accelerating the reaction. This pathway is likely to be physiologically relevant as oxygen concentrations within the subendocardium of the mammalian heart decrease to 4 mm Hg during ventricular systole and to 2 to 5 mm Hg in skeletal muscle during exercise or ischemic stress.

The ability for myoglobin dependent reduction of nitrite to modulate respiration has physiological implications for cellular respiration. In cardiac tissue and skeletal muscle, myoglobin is closely associated with mitochondria and acts as an oxygen reservoir for the organelle. This association has generated long-standing questions about other mechanisms of crosstalk existing between myoglobin and mitochondria. For example, some have speculated that scavenging of NO by myoglobin in the heart is a major mechanism of regulation of mitochondrial function. Here we have shown that as nitrite reductase, myoglobin may play the role of an NO generator, instead of scavenger, to regulate hypoxic mitochondrial respiration. Interestingly, the km for oxygen binding of cytochrome c oxidase (0.4 to 1 μmol/L), below which the enzyme becomes oxygen limited, is lower than the P50 of myoglobin (3.1 μmol/L). This is important for the delivery of oxygen from myoglobin’s reserve to the mitochondria, but also has implications for the nitrite-myoglobin dependent regulation of mitochondrial function. According to this paradigm, myoglobin deoxygenates and is able to reduce nitrite to NO, which can inhibit respiration mitochondria to conserve tissue oxygen, allowing diffusion of oxygen deeper into tissue (extension of oxygen gradients).

Whereas these data describe a role for myoglobin as a nitrite reductase that regulates mitochondrial respiration, they also highlight the potential for other heme-proteins, such as neuroglobin and cytoglobin, to be involved in hypoxic NO generation. These data also suggest that other NO-dependent processes may be regulated by nitrite reduction. Further investigation is needed to compare the relative efficiency of different heme proteins as nitrite reductases and characterize the physiological processes they modulate.

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**Disclosures**

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**Determination of the components and product yields of the nitrite-myoglobin reaction.**

The kinetics of the nitrite reaction with deoxyhemoglobin and deoxymyoglobin was monitored spectrophotometrically under positive helium pressure. The enzyme protocatechuate dioxygenase was used with its substrate 3,4-dihydroxybenzoic acid to facilitate deoxygenation of myoglobin and limit metmyoglobin formation for anaerobic experiments. Experiments with greater than 5% metmyoglobin at the beginning of the reaction were discarded. The concentrations of deoxyheme, metHEME, nitrite-metheme, iron-nitrosyl heme, nitrite-bound metmyoglobin, and NO-bound metmyoglobin at any time point were determined through least squares spectral deconvolution using standard reference spectra. To vary pH, deoxymyoglobin and nitrite were made in phosphate buffer adjusted to target pH values by varying proportions of monobasic and dibasic phosphate buffer.

**Measurement of hypoxic respiration**

To measure inhibition of respiration in hypoxic conditions, respiring mitochondria were allowed to consume oxygen until the chamber became anoxic and then the chamber lid was removed to allow the diffusion of air back into the chamber. The rate of mitochondrial respiration was greater than the rate of oxygen entering the chamber such that the oxygen electrode trace remained at zero while the mitochondria were respiring. Mitochondrial inhibitors or nitrite was added to the chamber prior to the removal of the lid and deviation of the oxygen trace from a zero reading signified a decrease in respiration rate. In control mitochondria, oxygen levels began to increase when substrates were exhausted and respiration could be restored by the addition of fresh substrate. All experiments were performed under conditions where substrates were not limiting.
The extent of respiratory inhibition was quantified by measuring the time from equilibration of the mitochondria with air to the time when the oxygen trace deviated from zero. This time to inhibition was expressed as a percentage of maximal inhibition, where 100% inhibition was defined as the time to inhibition in the presence of cyanide and the time to the exhaustion of substrates was used as a measure of 0% inhibition.

**Isolation of cardiomyocytes**

Adult rat cardiomyocytes were isolated from the left ventricle of male rats, which were anesthetized with pentobarbital and heparinized before the hearts were excised. Cannulated hearts were perfused with Krebs-Henseleit buffer containing 1.36 mM calcium for 5 minutes, followed by calcium-free Krebs-Henseleit buffer for 5 minutes and then Krebs-Henseleit buffer containing collagenase II (150 U/ml) for 9-12 minutes. Ventricles were removed, cut, and shaken gently at 34°C in the collagenase II solution. This digestion was repeated 4-6 times as needed. Myocytes were suspended in a medium containing Media 199 (Gibco Life Tech), Creatine (5mM), L-carnitine (2mM), Taurine (5mM), Penicillin (1000 U/ml), Streptomycin (10,000 µg/ml), Sodium Pyruvate (2.5 mM), and NaHCO₃ (26mM).