Nitrite Reductase Function of Deoxymyoglobin
Oxygen Sensor and Regulator of Cardiac Energetics and Function

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Abstract—Although the primary function of myoglobin (Mb) has been considered to be cellular oxygen storage and supply, recent studies have suggested to classify Mb as a multifunctional allosteric enzyme. In the heart, Mb acts as a potent scavenger of nitric oxide (NO) and contributes to the attenuation of oxidative damage. Here we report that a dynamic cycle exists in which a decrease in tissue oxygen tension drives the conversion of Mb from being an NO scavenger in normoxia to an NO producer in hypoxia. The NO generated by reaction of deoxygenated Mb with nitrite is functionally relevant and leads to a downregulation of cardiac energy status, which was not observed in mice lacking Mb. As a consequence, myocardial oxygen consumption is reduced and cardiac contractility is dampened in wild-type mice. We propose that this pathway represents a novel homeostatic mechanism by which a mismatch between oxygen supply and demand in muscle is translated into the fractional increase of deoxygenated Mb exhibiting enhanced nitrite reductase activity. Thus, Mb may act as an oxygen sensor which through NO can adjust muscle energetics to limited oxygen supply. (Circ. Res. 2007;100:1749-1754.)

Key Words: nitrite ■ hypoxia ■ myoglobin ■ cardiac function

Myoglobin (Mb) is an important intracellular oxygen binding hemeprotein and one of the most widely studied proteins. The first pioneering review on Mb was published as early as 1939, in which Millikan concluded that “muscle hemoglobin” acts primarily as a short term oxygen store, tiding the muscle over from one contraction to the next. In the past decade several additional biological functions were ascribed to Mb and its molecular relative hemoglobin (Hb). Experiments with transgenic mice deficient in Mb have shown that Mb is an important scavenger of nitric oxide (NO) under normoxia, which also protects the heart against reactive oxygen species. Very recently, it has been reported that Hb body concentration of Mb and Hb are similar, a putative role of Mb participates in NO metabolism not only by oxidative inactivation, but also by the nonenzymatic NO formation from nitrite by

devolved Mb acting as an NO scavenger under normoxic conditions may function as a relevant source of NO under hypoxia and (2) what functional consequences this Mb-derived NO may have on muscle function and energy metabolism.

Materials and Methods

Reaction of Mb With Nitrite
Horse Mb from Sigma was suspended in phosphate buffer saline (PBS) to a final concentration of 200 µmol/L, according to the myocardial concentration of myoglobin. Mb was deoxgenated by argon and oxygen saturation was measured spectrophotometrically. Nitrite was added to deoxygenated Mb and nitrosylated myoglobin (nitrosylMb) as a "diosmier of NO formation" was measured by gas-phase-chemiluminescence using ferricyanide solution. Further, hearts from mice were homogenated, deoxygenated, spiked with nitrite, and formation of NO was measured directly using gas-phase chemiluminescence. To characterize the impact of xanthine-oxidoreductase and cytochrome on formation of NO, experiments with inhibitors were performed. Xanthine-oxidoreductase was inhibited by incubation of homogenate with 100 µmol/L allopurinol to block the molybdenum-site and 200 µmol/L diphenyldiethylenone to block the flavin adenine dinucleotide-(FAD)-site. The electron-transfer from ubiquinol to bc1-complex in the mitochondrial respiratory chain was blocked by incubation with 300 µmol/L myoxalin.

NO Analysis of Cardiac Tissue
Tissue nitroso species (the sum of S-nitrosothiols and the mercury-stable NO-adducts N-nitrosamines, iron-nitrosyls) and nitrite were determined applying group-specific reductive denitrosation by triiodine with subsequent detection of the NO liberated by gas-phase-chemiluminescence. Nitrate was quantified after enzymatic reduction to nitrite by nitrate reductase using flow-injection analysis based on the Griess reaction. NO-heme was determined by parallel injection of replicate aliquots of tissue homogenates into a 0.05 mol/L ferricyanide solution to achieve 1-electron oxidation and quantify the liberated NO using gas-phase-chemiluminescence.

Animals and Langendorff Heart Perfusion
All experiments were approved by the local ethic committee. NO synthase activity was blocked in all animals by pretreatment with...
L-N(5)-(1-iminoethyl)-ornithine (L-NIO). Preparation and perfusion of murine hearts of myo-<sup>−/−</sup> and wt animals were performed essentially as described. Until excision of the heart, animals received standard rodent chow. For NMR measurements, hearts were placed inside a 10-mm NMR tube, immersed in perfusion buffer (containing in mmol/L: NaCl 116, KCl 4.6, MgSO<sub>4</sub> 24.9, CaCl<sub>2</sub> 2.5, KH<sub>2</sub>PO<sub>4</sub> 1.2, glucose 8.3, and EDTA 0.5), and transferred into a heated (37°C) 10-mm H<sub>2</sub>OP dual probe inside the spectrometer. Perfusion pressure, coronary flow, and left ventricular developed pressure (LVDP) were measured continuously. Signals were recorded with a sampling rate of 1000 Hz using a PC with dedicated software (Chart, AD Instruments). Arterial and venous pO<sub>2</sub> were measured simultaneously with implantable oxygen microsensors based on 140-μm optical silica fiber (Pensens) as previously described. All hearts were initially perfused at constant coronary pressure with Krebs-Henseleit buffer equilibrated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. After heart function had stabilized inside the magnet, cardiac pacing (500 bpm) was initiated and continued throughout. Left ventricular end-diastolic pressure was set to 5 mm Hg. Thirty minutes after the onset of cardiac pacing, coronary perfusion was fixed to the steady flow at which the hearts had stabilized. After the switch to constant flow, baseline data were recorded. Perfusion was then turned to buffer gassed at 50% O<sub>2</sub>/45% N<sub>2</sub>/5% CO<sub>2</sub> (Figure 1) to induce partial deoxygenation of Mb and a normoxic buffer (95% O<sub>2</sub>/5% CO<sub>2</sub>) as control, respectively. Subsequently, solutions with increasing concentrations of nitrite (0.1; 1; 10; 100 μmol/L) were infused stepwise, and in each section of the protocol hemodynamic data as well as NMR spectra were acquired.

**31P NMR Spectroscopy**

Spectra were recorded on a Bruker DRX 400 WB NMR spectrometer, operating at frequencies of 400 MHz for 1H and 161.97 MHz for 31P. Shimming was done on the free induction decay of the water signal. A line width at half height of 15 Hz could be routinely obtained. Transients (n=240) were accumulated with a 75-degree flip angle, a repetition time of 1 second, a spectral width of 5682 Hz, a data size of 240, and 10-Hz line broadening (4 minutes of signal accumulation). Chemical shifts were referenced to the phosphocreatine (PCr) resonance at −2.52 ppm. After baseline correction relative peak areas were obtained by integration and converted to concentrations as described before. Calculated values for the free energy of adenosine-triphosphate (ATP) hydrolysis (ΔG<sub>ATP</sub>) were derived from established relations.

**Results**

In a first step we determined whether free NO radicals can be formed from the reaction of nitrite and Mb in vitro. For this purpose nitrite was injected into an aqueous solution of Mb and the formed NO was analyzed by gas-phase-chemiluminescence. At 100% deoxygenated Mb and 10 μmol/L nitrite the rate of NO production was determined to be 1.5±0.2 pmol/L/s. In contrast, in the absence of Mb or when Mb was fully oxygenated there was no detectable formation of NO. Similar to the findings reported for Hb these experiments show that the NO generated can escape from autoscavenging by the remaining heme group. This becomes possible because the reaction with nitrite converts Mb to its ferric form (metMb, MbFe<sup>III</sup>), which exhibits only limited NO scavenging properties.

To characterize the total amount of NO formed by reaction with Mb, consecutive reactions of NO have to be considered. In analogy to what has been reported for Hb, NO released from Mb can be captured by the remaining deoxygenated Mb (deoxyMb; MbFe<sup>III</sup>) as nitrosylated Mb (nitrosyl-Mb; MbFe<sup>II</sup>-NO), which therefore can serve as an index of NO-formation. Using the same gas-phase-chemiluminescence approach described above, but adding ferricyanide to liberate the Mb-bound NO, we found a substantial Mb-mediated formation of NO at physiologically relevant cytosolic levels of pO<sub>2</sub> and nitrite (Figure 2A and 2B). The release of NO into the gas phase increased with the deoxygenation level of Mb and the concentration of nitrite.

We next analyzed the release of NO using murine myocardial tissue homogenates (Figure 2C through 2E). The basal rate of NO formation from deoxygenated tissue homogenates in the presence of 100 μmol/L nitrite was 2.5±0.3 nmol/g/s. In comparison, in homogenates from hearts of Mb-deficient (myo-<sup>−/−</sup>) mice the formation of NO was decreased by 60% as compared with wt controls (Figure 2E). Addition of Mb (200 μmol/L final concentration) to myo-<sup>−/−</sup> samples increased the formation of NO to levels of wt mice (Figure 2E). In contrast, inhibition of potential alternative nitrite-dependent NO producers, such as xanthine-oxidoreductase and the flavin-adenine dinucleotide-(FAD)-site by allopurinol and diphenyliodonium, respectively, did not reduce NO release (Figure 2D). Similarly, inhibition of the electron-transfer from ubiquinol to the bc<sub>1</sub>-complex in the mitochondrial respiratory chain by myxothiazol did not significantly reduce the formation of NO (Figure 2C).

The transport of intracoronarily applied nitrite into the myocardium has not been studied so far. To define the amounts of nitrite needed to increase endogenous levels, in a separate series of experiments we analyzed myocardial tissue content of nitrite. To differentiate between endogenous nitrite formation and the exogenously applied nitrite, NOS activity was inhibited with L-NIO (5 times every 30 minutes before excision of hearts). Isolated perfused mouse hearts were then subjected to mild hypoxia (50% buffer O<sub>2</sub>) which increases the fraction of deoxyMb to 50% and is associated with a cellular pO<sub>2</sub> of less than 4 mm Hg. This was followed by replenishment of the nitrite pool during perfusion with increasing nitrite concentrations (0.1, 1, 10, 100 μmol/L). Baseline cardiac nitrite levels were determined to be 2.96±0.42 μmol/L, which were significantly reduced to 1.14±0.06 μmol/L by application of L-NIO (Figure 3A; n=4, P<0.05). Perfusion with buffer containing 0.1 μmol/L and 1 μmol/L nitrite did not restore the initial cardiac nitrite levels (1.15±0.13 μmol/L for 0.1 μmol/L nitrite, P=n.s., and 1.59±0.36 μmol/L for 1 μmol/L nitrite, P=n.s.). Repleni-
A B

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

![Graph E](image5.png)

Figure 2. A and B, DeoxyMb converts nitrite to NO leading to the subsequent formation of nitrosyl-Mb. A, Nitrosyl-Mb (MbNO) formation is dependent on oxygen saturation. Deoxyxygenation of Mb was achieved by purging the solution with argon. Oxygen saturation of Mb was measured spectrophotometrically. Increasing levels of oxygen saturation were obtained by adapting the duration of deoxyxygenation. The dashed line marks the oxygen saturation of 50% applied in the Langendorff heart studies (Figures 4 and 5). This equals a cellular pO2 of less than 4 mm Hg as shown before.20 After deoxyxygenation, nitrite (10 μmol/L) was added. Increasing oxygenation of Mb is associated with decreasing formation of NO and thus nitrosyl-Mb. B, Concentration-dependent formation of nitrosyl-Mb. Nitrite (1 and 10 μmol/L, respectively) was added to deoxygenated Mb solutions. The area shaded emphasizes the concentration range relevant for cardiomyocytes. C through E, Cardiac Mb converts nitrite. Heart-homogenates of wild-type and myo⁺/⁻ mice (300 μg protein) were added to a solution of deoxygenated nitrite (100 μmol/L at pH 5 and 37°C), and the subsequent formation of NO was measured by gas phase chemiluminescence. Where required, inhibitors were added 30 minutes before addition to nitrite-solution. Inhibition of xanthine-oxidoreductase by incubation of homogenate with 100 μmol/L allopurinol to block the molybdenum-site and 200 μmol/L diphenyliodonium (dpi) to block the flavin-adenine dinucleotide (FAD)-site (C) and inhibition of the electron-transfer from ubiquinol to bc₁-complex in the mitochondrial respiratory chain by incubation with 100 μmol/L myxothiazol (D) did not influence conversion of nitrite to NO in comparison to untreated wt samples. E, Conversion of nitrite to NO was reduced by 60% in myo⁺/⁻ as compared with wt mice. Replenishment of Mb in the myo⁺/⁻ homogenate restored formation of NO to the levels of wt mice. Data represent mean±SEM of n=6 with *P<0.05.

The impact of the depleted tissue nitrite levels was only achieved by perfusion with concentrations ≥10 μmol/L nitrite (Figure 3A). Buffer containing 10 μmol/L nitrite led to cardiac nitrite levels of 2.54±0.75 μmol/L, being close to basal values, whereas 100 μmol/L nitrite increased tissue levels up to 2.5-fold above basal. Additional analysis of cardiac tissue NO content after nitrite application during hypoxia revealed a significant intracellular increase in nitroso-species and NO-heme products only at the level of 10 and 100 μmol/L nitrite, respectively (Figure 3A). No increase in NO-heme and nitroso-species was seen in myocardial tissue after stepwise increasing extracellular nitrite concentrations (0.1, 1, 10, 100 μmol/L), hemodynamic analysis combined with 31P NMR spectroscopy was performed in hearts from wt mice with myo⁺/⁻ mice serving as appropriate controls. No changes were observed in both groups during perfusion with 0.1 and 1 μmol/L nitrite. However, at extracellular concentrations ≥10 μmol/L nitrite dose-dependently increased myocardial inorganic phosphate (Pi) and decreased phosphocreatine (PCr) levels in wt hearts (Figure 4), thereby reducing the free energy of ATP hydrolysis (ΔG_Hydrolysis). The impairment of the energy status was accompanied by a drop in left ventricular developed pressure and an increase of the coronary venous pO₂ in wt mice. Because hearts were perfused in the constant flow mode, an increase in coronary venous pO₂ reflects a reduced myocardial oxygen extraction and consumption (Figure 5). Under the same conditions no changes in energetic or functional parameters were observed in hearts of myo⁺/⁻ mice (Figures 4 and 5).
indicating the observed differences to be specific for deoxyMb. This conclusion is further supported by experiments in which perfusion of wt and myo⁻/⁻ hearts with normoxic medium (equilibration with 95% O₂ and 100 μmol/L nitrite did not alter cardiac contractility (LVDP: 104±11 versus 103±13 mm Hg for WT and 102±12 versus 104±11 mm Hg for myo⁻/⁻), oxygen consumption (MVO₂: 12±1 versus 12±1 μmol/min/g for WT and 12±1 versus 12±1 μmol/min/g for myo⁻/⁻), and cardiac high energy phosphates (ΔGₜₚ: −61±0.3 versus −61±0.3 kJ/mol for WT and −61±0.2 versus −61±0.2 kJ/mol for myo⁻/⁻).

Figure 3. Cardiac tissue levels of NO-heme, nitroso, and nitrite after intracoronary application of nitrite. A, Under hypoxia, baseline cardiac levels of NO-heme, nitroso-species, nitrite, and nitrate of wt-mice are depicted left. Application of L-NIO significantly reduced tissue nitrite content. Perfusion with media containing 0.1, 1, 10, and 100 μmol/L nitrite, respectively, increased intracellular NO-heme, nitroso-species, and nitrite levels. Only 10 and 100 μmol/L nitrite replenished the reduced intracellular levels of nitrite. Data represent mean±SD of n=4 with *P<0.05. B, Under basal conditions, cardiac NO-heme and nitroso levels did not differ between wt and myo⁻/⁻ mice, whereas treatment with nitrite (100 μmol/L) significantly increased NO-heme and nitroso species in wt compared with myo⁻/⁻ hearts (C). Data represent mean±SD of n=6 with *P<0.05.

Discussion
The results of the present study demonstrate that under hypoxic conditions Mb transforms nitrite to NO, which then interacts in a reversible manner with myocytic cytochromes and downregulates cardiac energy status. This leads to a reduction in oxygen consumption and consecutively also of cardiac contractility. The increased inorganic phosphate levels caused by the enhanced breakdown of PCr may furthermore be an additional and important link between energetics and contractility. Together, these reactions may represent a crucial endogenous protecting mechanism for the heart. It is well known that cardiac contractile function and energy metabolism are actively downregulated, when coronary blood supply is critically reduced and this “perfusion-contraction matching” is a unique feature of the heart. On acute coronary artery inflow reduction, contractile function of the ischemic region is rapidly decreased and is associated with a decrease in oxygen consumption. This dampens the fall in high energy phosphates and over time even can restore myocardial energy balance. The mechanisms underlying this adaptive response, termed short-term hibernation, remained largely unclear until now. On nitrite infusion we observe a scenario which strongly resembles the characteristics described for acute hibernation: a decrease in PCr, a concomitant increase in Pₐ, and a reduction of the available driving force for all energy-consuming processes (ΔGₜₚ). Furthermore, we provide evidence for a reduction of ATP utilization (decrease in left ventricular developed pressure) and ATP synthesis (decrease in myocardial oxygen consumption) during nitrite infusion. Obviously, a new steady state for ATP is reached and ATP levels are maintained at lower steady state levels of PCr. Therefore, the Pₐ-dependent nonenzymatic formation of NO by reaction of Mb with nitrite may represent an important causal factor of short-term hibernation. Although the presented experiments were performed under hypoxic perfusion conditions which cause Mb to be deoxygenated by about 50%, low-flow ischemia certainly can further lower tissue pO₂ thereby further augmenting the ability of deoxy-myoglobin to form NO from nitrite. It is noteworthy that Martin et al. recently provided evidence for a NO synthase–independent NO formation during myocardial ischemia, which can be easily explained on the basis of the present work.

Published experimental studies support our findings that under hypoxic conditions myoglobin may react with nitrite to form NO, and that this reaction may play a crucial role in the regulation of physiological functions. Similar to the present work, Shiva et al have most recently demonstrated that in isolated cardiomyocytes the nitrite reductase activity of deoxymyoglobin releases NO in proximity to mitochondria and regulates respiration through cytochrome c oxidase. Furthermore, different groups have shown that the application of low doses of nitrite prevents ischemia/reperfusion-injury in the Langendorff heart model as well as in the liver and heart of mice. The xanthine-oxidoreductase dependent reduction of nitrite to NO and a deoxyhemoglobin- and myoglobin-mediated nitrite reduction to NO have been proposed as poen-
tial mechanisms, but we (Figure 2C and 2D) and others were not able to show a significant role for xanthine-oxidoreductase in reducing nitrite.

Our data may be criticized that rather high extracellular concentrations of nitrite (10 to 100 μmol/L) were required to elicit the biological response. However, it is the intracellular concentration of nitrite which is of critical importance for the reaction with deoxyMb. Pretreatment of animals with the NOS inhibitor NIO decreased cytosolic nitrite by approximately 70%, and perfusion with concentrations 10 μmol/L nitrite was required to replenish the myocytic levels to the range of untreated controls. Obviously, comparatively high extracellular nitrite concentrations have to be applied under our experimental conditions to mimic the in vivo conditions with unrestricted activity of NOS and unlimited availability of its substrate arginine which was deliberately not supplemented with the perfusion buffer. Together our data suggest that the effect of nitrite on cardiac function occurred at physiological cytosolic nitrite concentrations.

NO formation by deoxyMb may not only be relevant for the heart, but it also could contribute to hypoxic vasodilation described for the human circulation. As already pointed out above, the total body amounts of Hb and Mb are similar so that the ability of both proteins to act as nitrite-reductase might have been involved in the vasodilation of the exercising muscle previously reported. Using quadriceps maximum isometric voluntary torque and measuring PCr and deoxyMb by interleaved 1H and 31P NMR spectroscopy, the fraction of deoxyMb was found to increase up to 70%, whereas PCr reversibly decreased to 20% of control. Given this significant deoxygenation of Mb in human exercising muscle, this most likely has profoundly increased the Mb-mediated formation of NO (Figure 2). Because of the low diffusion distances between Mb and mitochondria, this NO may be critically involved in the observed inhibition of oxidative phosphorylation, which is known to be extremely NO sensitive. This mechanism may therefore play an important role in limiting muscle oxygen consumption and thus the exercise capacity of skeletal muscle.

In summary this study describes a novel homeostatic mechanism by which a mismatch between oxygen supply and demand is translated into the fractional increase of deoxyMb exhibiting enhanced nitrite reductase activity. DeoxyMb may act as an important oxygen sensor through which NO can regulate muscle energetics and function. This appears to be functionally important in the infarcted heart, during acute myocardial hibernation, and intense muscle exercise.

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Figure 5. Nitrite dampens myocardial function during hypoxia through Mb. Hypoxia was achieved by equilibrating the perfusion buffer with a gas mixture of 50%O2/45% N2/5% CO2. A, Representative registration of coronary venous PO2 during application of increasing concentrations of nitrite to perfused wt (red) and myo (blue) hearts. B, Quantitative analysis of coronary venous PO2 shows a dose-dependent increase in wt hearts, whereas no changes were observed in myo hearts. Nitrite dose-dependently reduced myocardial oxygen consumption (C) and LVDP (D) under hypoxia in wt mice (black bars) compared with myo mice (white bars). No such effects of nitrite were seen under normoxic conditions. Data represent mean±SD of n=6 with *P<0.05.

Disclosures

None.

References

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