

Nitrite reductase activity of myoglobin regulates respiration and cellular viability in myocardial ischemia-reperfusion injury

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The nitrite anion is reduced to nitric oxide (NO^{*}) as oxygen tension decreases. Whereas this pathway modulates hypoxic NO^{*} signaling and mitochondrial respiration and limits myocardial infarction in mammalian species, the pathways to nitrite bioactivation remain uncertain. Studies suggest that hemoglobin and myoglobin may subserve a fundamental physiological function as hypoxia dependent nitrite reductases. Using myoglobin wild-type (+/+) and knockout (-/-) mice, we here test the central role of myoglobin as a functional nitrite reductase that regulates hypoxic NO^{*} generation, controls cellular respiration, and therefore confirms a cytoprotective response to cardiac ischemia-reperfusion (I/R) injury. We find that myoglobin is responsible for nitrite-dependent NO^{*} generation and cardiomyocyte protein iron-nitrosylation. Nitrite reduction to NO^{*} by myoglobin dynamically inhibits cellular respiration and limits reactive oxygen species generation and mitochondrial enzyme oxidative inactivation after I/R injury. In isolated myoglobin^{+/+} but not in myoglobin^{-/-} hearts, nitrite treatment resulted in an improved recovery of post-ischemic left ventricular developed pressure of 29%. *In vivo* administration of nitrite reduced myocardial infarction by 61% in myoglobin^{+/+} mice, whereas in myoglobin^{-/-} mice nitrite had no protective effects. These data support an emerging paradigm that myoglobin and the heme globin family subserve a critical function as an intrinsic nitrite reductase that regulates responses to cellular hypoxia and reoxygenation. myoglobin knockout mice

Nitrite represents an endocrine bioavailable storage pool of nitric oxide (NO^{*}) that is bioactivated along the physiological oxygen gradient to regulate a number of vascular and cellular responses (1, 2). Nitrite is present at concentrations of 0.3–1.0 μ M in plasma and 1–20 μ M in tissue (3–5), and has been proposed to participate in hypoxic vasodilation and signaling (6–9). *In vivo*, nitrite may be converted to NO^{*} by several mechanisms including acidic disproportionation (10), and enzymatic conversion via xanthine-oxidoreductase (XOR) (11), mitochondrial enzymes (12), deoxyhemoglobin (6, 9, 13), and deoxymyoglobin (deoxyMb) (14, 15).

A cytoprotective role for low pharmacological doses of nitrite in the setting of myocardial, liver, kidney, and brain ischemia-reperfusion (I/R) injury has recently been demonstrated independently by a number of research groups (11, 16–18). The exact mechanisms behind the cytoprotective effects of exogenous nitrite, however, are still a matter of debate. Whereas XOR has been proposed as being responsible for the conversion of nitrite to NO^{*} in the heart by Webb *et al.* (11), Zweier *et al.* (19), and recently Baker *et al.* (20), no such effect was observed by our groups (14, 15). We have recently demonstrated that a decrease in myocardial oxygen tension drives the conversion of oxymyoglobin to deoxyMb, which reacts with nitrite to generate NO^{*}, regulating cardiac energetics and function (14, 15). Whether deoxyMb or XOR is responsible for nitrite reduction to NO^{*} in the ischemic heart to mediate cytoprotection remains unclear. Using myoglobin knock-

out (-/-) mice for definitive experiments, we therefore tested the hypothesis that exogenous nitrite exerts cytoprotective effects via a nitrite-reductase function of myoglobin, which via NO^{*} generation and dynamic inhibition of mitochondrial electron transfer, limits the generation of reactive oxygen species (ROS) in the reperfused myocardium.

Results

Mouse Hearts Generate NO^{*} from Myoglobin-Dependent Nitrite Reduction. NO^{*} can be generated in the heart under ischemic conditions by direct reduction or disproportionation of endogenous (10) and exogenous nitrite (11, 16, 21). These pathways display increasing NO^{*} formation depending on the increasing severity of hypoxia and acidosis (22). To evaluate the mechanism of the conversion of exogenous nitrite and a time-dependent generation of NO^{*} during I/R we examined nitrite treated intact myoglobin^{+/+} and myoglobin^{-/-} mouse hearts subjected to an *in vitro* model of I/R injury by measuring liberated NO^{*} and nitrosylated heme.

The gas space around the heart demonstrated different time-dependent production of NO^{*} in myoglobin^{+/+} and myoglobin^{-/-} hearts (Fig. 1A). During the first 15 min of ischemia, the rate of NO^{*} production showed similar values, but after 30 min of ischemia, levels increased in myoglobin^{+/+} hearts from 15 \pm 3 ppb to 25 \pm 1 ppb ($P < 0.05$; $n = 3$; Fig. 1B), whereas in myoglobin^{-/-} hearts, the generation decreased from 16 \pm 2 ppb to 12 \pm 2 ppb ($P < 0.05$; $n = 3$; Fig. 1B). The same effect was observed in heart homogenates from myoglobin^{+/+} and myoglobin^{-/-} animals treated with nitrite (1 mM). Continuous monitoring of NO^{*} generation in these homogenates showed that NO^{*} production by myoglobin^{-/-} animals was decreased by \approx 50% in comparison to myoglobin^{+/+} animals ($P < 0.05$; $n = 4$; Fig. 1C and D).

In analogy to what has been reported for hemoglobin, NO^{*} generated from the reduction of nitrite by deoxyMb can be captured by the remaining deoxyMb as iron-nitrosylated myoglobin (MbNO), which can serve as an indirect marker of NO formation (23). Therefore we performed an isotope tracer experiment detecting Mb[¹⁵N]NO formation in myoglobin^{+/+} hearts perfused with buffer and 50 μ M of isotopically labeled [¹⁵N]nitrite, respectively, 5 min before 30 min of global ischemia by EPR spectroscopy (Fig. 2A). When hearts were perfused with buffer, a pattern was

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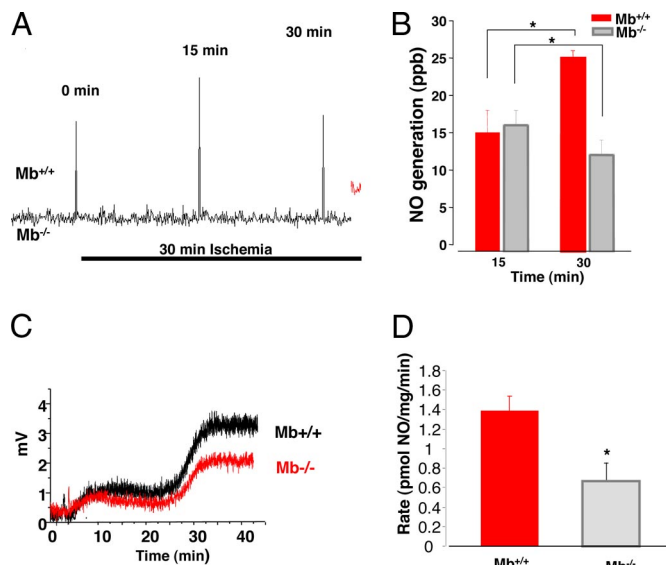


Fig. 1. NO* production from the *ex vivo* retrograde perfused heart during ischemia by using the NO* collection heart chamber. (A) Representative time courses of NO* production from nitrite during 30 min ischemia in myoglobin wild-type and knockout hearts measured by chemiluminescence every 15 min. (B) NO* generation in myoglobin^{+/+} and myoglobin^{-/-} hearts after 15 and 30 min of ischemia. Application of nitrite (100 μ M) showed a time-dependent increase of NO* production in myoglobin^{+/+} hearts from 15 ± 3 ppb to 25 ± 1 ppb ($P < 0.05$; $n = 3$), whereas in myoglobin^{-/-} hearts, a decrease from 16 ± 2 ppb to 12 ± 2 ppb was observed ($P < 0.05$; $n = 3$). (C) Representative traces of NO* generation by heart homogenates from myoglobin^{+/+} and myoglobin^{-/-} mice treated with nitrite (1 mM). (D) Quantitation of nitrite generation from several curves similar to C ($P < 0.05$; $n = 4$). *, $P < 0.05$.

observed that was similar to that previously reported for ischemic hearts (24), exhibiting the signals suggested to arise from a mitochondrial ubiquinone radical, a ROO* species and a radical with nitrogen coupling (two of the typical signals, $g = 2.008$ and $g = 2.038$, are marked in the spectrum) as well as from an iron-sulfur protein with a Fe₂S₂ cluster ($g = 1.94$) (Fig. 2B Upper). The difference in the relative signal intensities compared to the published spectra arises from saturation effects due to the high microwave power (53 mW) used to minimize the contribution of these radicals to the spectra. In addition, significant differences in the relative signal intensities were also observed comparing spectra from different samples prepared by using the same protocol. Using 50 μ M [¹⁵N]nitrite, the very pronounced characteristic Mb[¹⁵N]NO signal (21) with a doublet hyperfine splitting, arising from the ¹⁵N nuclear spin of 1/2, appeared (Fig. 2B Lower).

A series of hearts preloaded with 50 μ M [¹⁵N]nitrite were subjected to control perfusion, 15 and 30 min of ischemia, and 30 min of ischemia followed by reperfusion of 5 and 15 min duration. The NO-heme levels in myoglobin^{+/+} hearts rose from 0.4 μ M before ischemia beyond 4.1 μ M after 15 min of ischemia to 13.8 μ M to the end of ischemia. After 5 min of reperfusion the NO-heme concentration reached the highest level with 21.8 μ M. Upon reperfusion, the concentration declined to 1.4 μ M within the first 15 min ($P < 0.05$; $n = 3$; Fig. 2C). In myoglobin^{-/-} hearts, increases in the NO-heme levels could only be detected after 30 min and reached levels of 2.9 μ M (Fig. 2C; $P < 0.05$; $n = 3$). The corresponding EPR spectra confirmed the substantial myoglobin-mediated formation of NO* (Fig. 2D).

Nitrite-Dependent Regulation of Mitochondrial Respiration Requires Myoglobin. To determine whether myoglobin-dependent nitrite reduction regulates mitochondrial function in the ischemic heart, homogenates from the myoglobin^{+/+} and myoglobin^{-/-} mice were

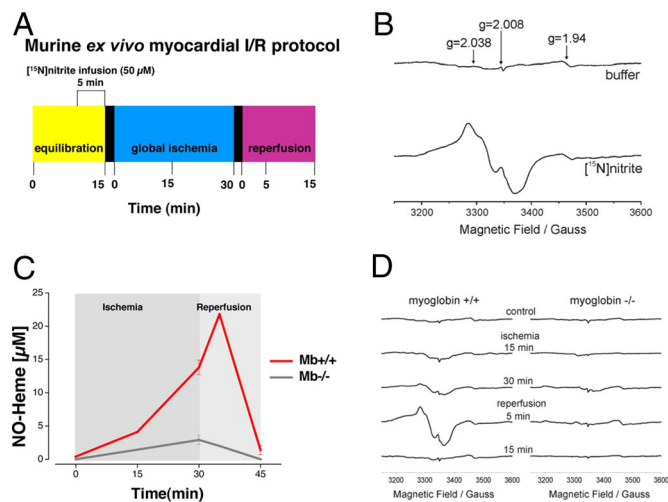


Fig. 2. Nitrite-induced formation of NO-heme complexes during ischemia and reperfusion. (A) Experimental protocol. (B Upper) EPR spectrum of the mitochondrial ubiquinone radical signal formed in a postischemic reperfused myoglobin wild-type (^{+/+}) heart loaded with Krebs-Henseleit buffer. (B Lower) EPR spectrum of nitrosylated myoglobin formed in a postischemic reperfused myoglobin^{+/+} heart loaded with 50 μ M [¹⁵N]nitrite. (C) Graph of the time courses of cardiac NO-heme levels in hearts loaded with 50 μ M [¹⁵N]nitrite before, during, and after ischemia ($n = 3$). The levels of NO-heme complexes formed in myoglobin^{+/+} hearts were quite high after 30 min of ischemia and 5 min of reperfusion compared with the concentrations of NO-heme in myoglobin^{-/-} hearts. (D) EPR spectra of the Mb[¹⁵N]NO complexes formed in nitrite-treated myoglobin^{+/+} and myoglobin^{-/-} hearts (50 μ M) before, during, and after global ischemia. The highest level of MbNO formation was observed 5 min after reperfusion.

stimulated to respire in the presence of increasing concentrations of nitrite (0–25 μ M). Once the mitochondria consumed all of the oxygen in the experimental sealed chamber, the chamber was opened to allow oxygen back in. However, even with oxygen diffusion into the chamber, the electrode trace remained at zero due to the rapid rate of oxygen consumption by the mitochondria in comparison to the detection time of the oxygen electrode. The oxygen trace remained at zero percent oxygen until the respiratory rate of the tissue decreased due to exhaustion of substrate or inhibition with cyanide (50 μ M) (Fig. 3A and B). In the myoglobin^{+/+} mouse, nitrite treatment resulted in a concentration-dependent increase in the extent of respiratory inhibition, as evidenced by a shorter time to oxygen accumulation with increasing concentrations of nitrite (Fig. 3A). In myoglobin^{-/-} mice the effect of nitrite was decreased significantly (Fig. 3B), with 25 μ M nitrite inhibiting respiration by $73 \pm 7\%$ in the myoglobin^{+/+} homogenate, but only $28 \pm 9\%$ in the myoglobin^{-/-} homogenate ($P < 0.01$; $n = 5$; Fig. 3C), demonstrating that myoglobin plays an integral role in nitrite-dependent regulation of mitochondrial respiration in the heart.

Myoglobin-Dependent Nitrite Reduction Limits Reperfusion ROS Generation and Aconitase Inactivation. A previously characterized effect of NO* on mitochondria is the inhibition of complex I which reduces reperfusion reactive oxygen species generation and ameliorates oxidative inactivation of complex II-IV and aconitase (25). To provide further evidence of myoglobin-dependent generation of NO* during I/R, we analyzed the cardiac tissue NO* content using reductive chemiluminescence analysis of tissue NO-modified proteins after 30 min of local ischemia and 5 min of reperfusion. The evaluation demonstrated an increase in NO-heme level in myoglobin^{+/+} hearts by 70% (10 ± 1 nM vs. 17 ± 3 nM; $P < 0.05$; $n = 5$; Fig. 4A), whereas in myoglobin^{-/-} hearts no change was observed (3 ± 1 nM vs. 3 ± 1 nM; $n = 5$; P values not significant; Fig. 4A). These data supported EPR analysis and were confirmed by the

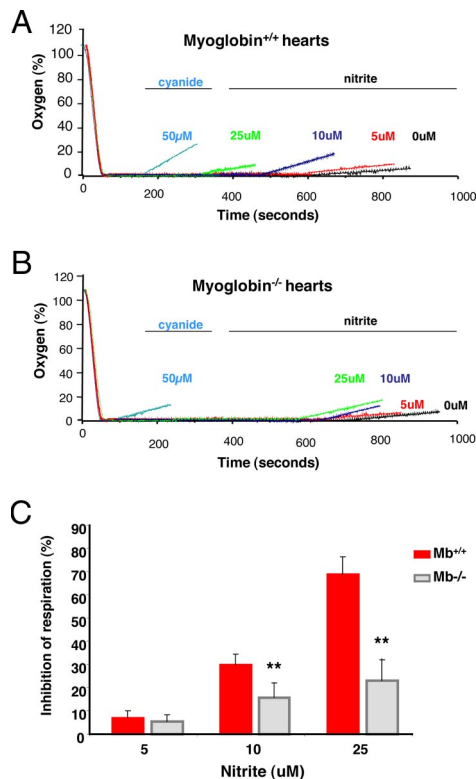


Fig. 3. Myoglobin is required for nitrite dependent inhibition of mitochondrial respiration. Representative oxygen electrode traces for myoglobin wild-type (A) and knockout (B) heart homogenates with either no treatment (0 μM) or treatment with cyanide (50 μM), or nitrite (5–25 μM). (C) Quantitation of several traces similar to A and B, where cyanide is 100% inhibition and 0 μM is 0% inhibition. ($P < 0.01$ vs. wild type; $n = 5$). **, $P < 0.01$.

results of measurement of the soluble guanylyl cyclase product cGMP after 15 min of reperfusion related to the concentration before ischemia. The basal cGMP levels did not differ between the groups (P values not significant; $n = 3$; Fig. 4B). In myoglobin^{+/+} hearts we observed increased levels from 9 ± 6 fmol/mg tissue to 86 ± 17 fmol/mg tissue ($P < 0.05$; $n = 3$; Fig. 4B), whereas myoglobin^{-/-} hearts displayed values below the detectable level ($n = 3$; Fig. 4B).

Because nitrite has previously been shown to modulate mitochondrial ROS generation through the inhibition of complex I and IV of the mitochondria, an effect now shown to be myoglobin dependent, we next examined the role of myoglobin-mediated NO[•] formation from nitrite on ROS generation following I/R injury. In myoglobin^{+/+} hearts H₂O₂ production was attenuated by nitrite treatment after 30 min of ischemia and 5 min of reperfusion from 2.5 ± 0.1 nmol/mg tissue to 1.9 ± 0.1 nmol/mg tissue ($P < 0.05$; $n = 3$; Fig. 4C). Myoglobin^{-/-} hearts showed a lower level of H₂O₂ in comparison to myoglobin^{+/+} hearts at baseline ($P < 0.05$; $n = 3$). Nitrite treatment increased H₂O₂ production (1.8 ± 0.1 vs. 2.2 ± 0.1 nmol/mg tissue; $P < 0.05$; $n = 3$; Fig. 4C).

As evidence of oxidative protein damage, the activity of the mitochondrial enzyme aconitase, which is highly susceptible to oxidative damage, was measured. Consistent with a nitrite-dependent decrease in ROS production in myoglobin^{+/+} hearts, we observed a nitrite-dependent increase in aconitase activity from 1.4 ± 0.07 to 3.8 ± 0.6 munits/mg tissue in this group ($P < 0.05$; $n = 3$; Fig. 4D). Myoglobin^{-/-} hearts displayed a higher basal aconitase activity compared to myoglobin^{+/+} hearts ($P \leq 0.05$; $n = 3$). These hearts treated with nitrite sustained more oxidative damage (3.0 ± 0.4 to 0.9 ± 0.04 mU/mg tissue; $P < 0.01$; $n = 3$; Fig. 4D). Myoglobin, therefore, converts nitrite from an enhancer to an

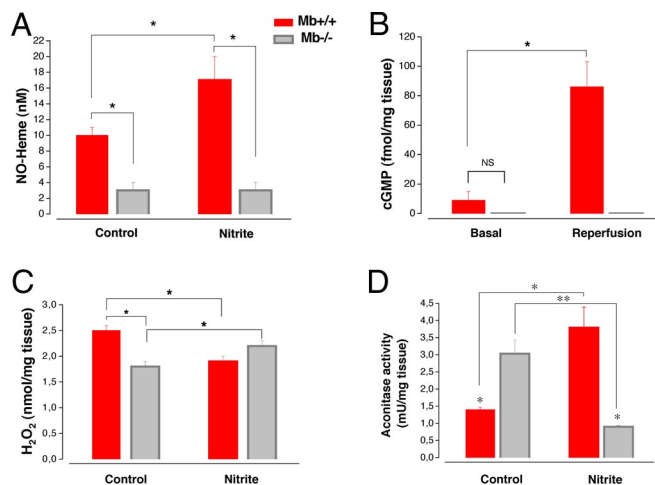


Fig. 4. Myoglobin-dependent nitrite-induced decrease of oxidative damage in myoglobin wild-type hearts compared to knockout hearts after ex vivo ischemia-reperfusion injury. (A) Cardiac NO-heme levels in myoglobin^{+/+} and myoglobin^{-/-} hearts after 30 min of local ischemia and 5 min of reperfusion ($n = 5$). Untreated myoglobin^{+/+} and myoglobin^{-/-} hearts exhibited differences in NO-heme concentration ($P < 0.05$). Nitrite (0.5 μM) increased NO-heme in presence of myoglobin by 70% ($P < 0.05$). Myoglobin^{-/-} hearts did not differ between control and nitrite treatment (0%, P values not significant). (B) cGMP in nitrite-treated (50 μM) myoglobin^{+/+} and myoglobin^{-/-} hearts before ischemia and after 30 min of global ischemia and 15 min of reperfusion ($n = 3$). A 9.5-fold increase was observed in myoglobin^{+/+} hearts, whereas myoglobin^{-/-} hearts displayed values below the detectable level. (C) In myoglobin^{+/+} hearts, nitrite (0.5 μM) decreased the H₂O₂ production by 24% ($P < 0.05$), whereas in myoglobin^{-/-} hearts, nitrite increased the H₂O₂ generation by 22% ($P < 0.05$). (D) The aconitase level in myoglobin^{+/+} hearts increased after nitrite treatment (0.5 μM) by 171%, whereas myoglobin^{-/-} hearts exhibited a decreasing aconitase level of 70% ($P < 0.01$). *, $P < 0.05$; **, $P < 0.01$.

inhibitor of ROS generation, maintaining integrity of mitochondrial proteins.

Myoglobin-Mediated Formation of NO[•] Attenuates Myocardial Infarction and Improves Left Ventricular Function. To elucidate the role of myoglobin in I/R injury after nitrite treatment, isolated perfused hearts were subjected to coronary artery occlusion and reperfusion. The primary study statement was infarct size normalized to the area at risk (AAR). [For the experimental protocol see [supporting information \(SI\) Fig. S1A](#).] The AAR and the infarct size were determined by using Blue evans dye and 2,3,5-triphenyltetrazolium chloride (TTC) staining of heart sections. As shown in Fig. S1B, the myocardial infarct size per AAR under basal conditions did not differ between myoglobin^{+/+} and myoglobin^{-/-} hearts ($45 \pm 4\%$ both; $n = 6$; P values not significant). However, nitrite administration 5 min before occlusion of the left coronary artery and during ischemia considerably reduced myocardial infarct size relative to the AAR in myoglobin^{+/+} hearts by 36% ($29 \pm 3\%$; $P \leq 0.01$; $n = 6$; Fig. S1B). In contrast, nitrite had no effect on the myocardial infarct size in myoglobin-deficient hearts ($47 \pm 3\%$; P values not significant; Fig. S1B). The size of the AAR per left ventricle (LV) were similar within the myoglobin^{+/+} ($52 \pm 2\%$ vs. $45 \pm 3\%$; P values not significant) and the myoglobin^{-/-} ($42 \pm 2\%$ vs. $43 \pm 3\%$; P values not significant) heart groups and between the groups (P values not significant; Fig. S1C).

To assess whether the effect of myoglobin reduction activity is of hemodynamic functional significance, the left ventricular developed pressure (LVdP) and the coronary flow during 30 min of reperfusion were evaluated and referred to the preischemic values. Myoglobin^{-/-} hearts showed a better LVdP during ischemia and recuperation after ischemia than myoglobin^{+/+} hearts (Fig. S1D–F). This may be secondary to changes in tissue vascularity (26).

mice. This is consistent with the attenuated ROS formation accompanied by a decrease of oxidative inactivation of aconitase in myoglobin^{+/+} hearts treated with nitrite observed in the present study. The important role of myoglobin is reflected in the fact that in myoglobin-deficient mice, the nitrite treatment led to a significant increase in ROS generation, resulting in increased protein damage. This nitrite reductase activity of myoglobin switches nitrite from an enhancer to an inhibitor of ROS generation. This myoglobin-dependent impact of nitrite on ROS generation could explain the U-shaped efficacy profile of nitrite demonstrated by Duranski *et al.* (16) and suggests a dependence of the physiological capacity of myoglobin.

Various previous studies reported the reduction of nitrite by deoxyhemoglobin, which leads to vasodilation, generation of cGMP and inhibition of mitochondrial respiration (6, 7). Here we show that nitrite exerts no cytoprotective effects in myoglobin^{-/-} mice, whereas in myoglobin^{+/+} mice, nitrite reduced the myocardial infarct size by 61%. These data suggest that the protective effects of nitrite in I/R injury are myoglobin dependent but independent of hemoglobin.

Several factors that govern the nitrite-dependent nitrosation and transient inhibition of complex I can be envisioned, considering the fact that NO^{*} will not directly nitrosate reduced thiols. The required one-electron oxidation may occur in the presence of an electron acceptor such as NAD⁺. Nitrous acid, formed in the inner mitochondrial membrane space and potentially at complexes I, III, IV and V, directly nitrosates reduced thiols. The reaction of NO^{*} with ^{*}NO₂ yields N₂O₃, which would directly nitrosate complex I as well. ^{*}NO₂ possibly mediated a one-electron oxidation of thiols with subsequent reaction of thyl radicals with NO^{*}.

The role of myoglobin in I/R is not unambiguously solved. Several studies suggest that the reactions between myoglobin and hydrogen peroxide are a key determinant of oxidative damage in the ischemic and then reoxygenated heart (35, 36). These reactions result in generation of ferryl myoglobin (MbFe^{IV} = O) and the globin radical (MbFe^{IV} = O) (35, 37). Both species are strong oxidants, which may induce lipid and protein peroxidation. The heme to protein cross-linked form of myoglobin, generated from interaction of the ferryl heme with the radical, represents the more cytotoxic form than ferryl myoglobin and oxidizes free and membrane-bound lipids (38). Irrespective of this discussion, our current data show that myoglobin, via reduction of nitrite to NO^{*}, attenuates the infarct size after I/R both *ex vivo* and *in vivo*. It has been reported that NO^{*} may act as an antioxidant, inhibiting ferryl myoglobin induced oxidative damage and globin radical catalyzed oxidation reactions (39). However, in the setup of nitrite perfusion, NO^{*} generated by nitrite reduction may reduce ferryl myoglobin to the ferric state (MbFe^{III}) and inhibit the peroxidative activity of ferryl myoglobin. The reaction between ferryl myoglobin and NO^{*} proceeds via the rapid formation of an intermediate, which then decays to MbFe^{III} and nitrite. The rate constant was determined to be $(17.9 \pm 0.5) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (pH 7.5, 20°C). Nitrite might also reduce ferryl myoglobin; the rate of reaction is significantly lower (39), but experiments with hemoglobin under acidic conditions suggest a physiological relevance during I/R (40). An additional nitrite/myoglobin shunt for protection could be the reaction of the ferryl myoglobin radical with nitrite; the product of this reaction, nitrogen dioxide, may be able to oxidize the thiols at complex I.

MbFe^{III} is regulated by an associated reductase (41) that maintains the ferrous state (MbFe^{II}) and, therefore, constitutes the basis for additional nitrite reduction. On the other hand, this reaction restores the ability of MbFe^{III} to inactivate hydrogen peroxide. Thus, the balance between the hydrogen peroxide dependent generation of ferryl myoglobin and the NO^{*} mediated reduction of ferryl myoglobin to MbFe^{III} may decide whether the peroxidase activity of myoglobin may be protective or deleterious. In principle, this scheme could also explain why myoglobin is cardioprotective in isolated, NO^{*} generating hearts in I/R experiments, whereas it may

be cytotoxic in isolated cardiomyocyte cell lines with a limited capacity to release NO^{*} (36).

In conclusion, our findings give unequivocal evidence that myoglobin serves a critical function as an intrinsic nitrite reductase that regulates through the formation of NO^{*} mitochondrial respiration and cardiac contractility during I/R. These studies support the emerging paradigm that the heme globins—myoglobin, hemoglobin, neuroglobin, and cytoglobin—subserve a critical function as nitrite reductases that regulate hypoxic NO^{*} signaling.

Materials and Methods

Chemicals and Reagents. All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany), unless otherwise indicated. In all experiments, mice were perfused with a modified Krebs-Henseleit buffer (KHB) (pH 7.4) equilibrated with 5% CO₂/95% O₂.

Animals. Myoglobin knockout mice were backcrossed for 32 generations onto the NMRI (Naval Medical Research Institute) background (26). Male myoglobin^{-/-} and myoglobin^{+/+} mice used in the present studies were at 14 ± 3 weeks of age. Body weight ranged from 30 to 45 g, and heart weight ranged from 180 to 300 mg, with no significant differences between the two groups. Mice were kept on a standard rodent chow before experimental use. All animals were treated according to the 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe Treaty Series No. 123).

Measurement of NO^{*} Generation by the Mouse Heart During the *ex Vivo* Retrograde Perfusion. Mice were anesthetized by i.p. injection of urethane (1.2 mg/kg), and anticoagulated with 1000 IE heparin i.p. 10 min before the experimental protocol, and then killed by cervical dislocation. Hearts were excised, and the aortas were cannulated and perfused retrograde with KHB at a constant pressure of 100 mm Hg by using a specially adapted sealable 20 ml heart chamber. The chamber comprised a lid to produce a reversible seal around the cannulated aorta, an effluent port, building a liquid seal, and a direct connection to the NO^{*} analyzer (Eco Physics, Germany). After 15 min of equilibration, a global no-flow ischemia was induced for 30 min. The infusion of 100 μM deoxygenated sodium nitrite was initiated 5 min before global ischemia for a period of 35 min. The nitrite solution and the organ chamber were deoxygenated with argon. The concentration of NO^{*} released by the heart was measured with chemiluminescence by opening the connection to the NO^{*} analyzer every 15 min after starting the global no-flow ischemia. NO^{*} synthase activity was blocked in all animals by i.p. injection of L-N (5)-(1-iminoethyl)-ornithine (100 mg/kg) before and during the experimental procedure.

Measurement of NO^{*} Generation by Heart Homogenates. Hearts were excised from anesthetized mice and homogenized in a buffer containing sucrose (250 mM), Tris (10 mM), and EDTA (1 mM) (pH 7.4). NO^{*} generation from the tissue homogenate (3 mg/ml) treated with nitrite (1 mM) was measured in a vessel in which the suspension and head space were purged with helium, and the vessel was connected in line to a NO^{*} chemiluminescence analyzer.

Myocardial I/R *ex Vivo* Protocols. Hearts were excised, and the aortas were cannulated and perfused retrogradely with KHB at a constant pressure of 100 mm Hg by using the isolated heart apparatus. Heart rate was controlled by right atrial pacing (600 beats min⁻¹).

Biochemical Analysis. For measurements of NO-heme, nitrosylated myoglobin and cGMP generation in cardiac tissue, the isolated hearts were initially perfused for 15 min to enable stabilization of contractile function and then subjected to given durations of global ischemia or ischemia followed by reperfusion. A side-arm in the perfusion line allowed direct infusion of 50 μM sodium nitrite-¹⁵N just proximal to the heart 5 min before ischemia (21).

Myocardial Infarct Size and Biochemical Analysis. To analyze myocardial infarct size, ROS generation, aconitase activity, and NO-heme, the hearts were equilibrated for 20 min, and the left main coronary artery (LCA) was completely ligated proximally with 7–0 silk suture mounted on a tapered needle (Serag-Wiessner, Naila, Germany). The coronary occlusion was maintained for 30 min, followed by removal of the suture and reperfusion for 180 min. The application of 32 nmol (0.5 μM) sodium nitrite was initiated 5 min before LCA occlusion for a period of 35 min. For biochemical analyses reperfusion was stopped after 5 min.

Determination of Nitrosylated Myoglobin via EPR Spectroscopy. Isolated hearts were snap frozen in liquid nitrogen, fractured to 1 to 2-mm pieces, and placed into an EPR quartz capillary (3-mm inner diameter) to a sufficient height to fill the critical volume of the EPR cavity. Measurements were performed on a Bruker Elexsys E580 EPR spectrometer operating at X-band, equipped with a Super High Sensitivity Probehead (V2.0). EPR spectra were recorded at 77K. The EPR spectra were obtained as an average of 20 scans with a sweep time of 83 s with a time constant of 40.96 ms, a modulation amplitude of 4.00 G, modulation frequency of 100 kHz, and 53.2 mW of microwave power at a frequency of 9.404 GHz. The scan range was 500 Gauss, centered at 3352.8 Gauss ($g = 2.0040$).

NO[•] Analysis of Cardiac Tissue. Isolated hearts were snap frozen in liquid nitrogen, weighed, and homogenized immediately in ice-cold sodium chloride with a Schuett homogenizer semi automatic glass-on-glass-homogenizer and kept on ice. NO-heme was determined by injection of aliquots of tissue homogenates into a 0.05 M ferricyanide solution to achieve 1-electron oxidation and quantify the liberated NO[•] by using gas-phase-chemiluminescence (5).

Measurement of Tissue Respiration and Inhibition. Whole hearts were homogenized in a buffer consisting of sucrose (250 mM), Tris (10 mM) and EGTA (1 mM) (pH 7.4). Protein concentration was measured by using the Pierce protein assay kit against a standard curve of BSA. Homogenates (2 mg/ml) were suspended in a sealed, stirred chamber fitted with a Clark-type oxygen electrode (Instech Corp.) and stimulated to respire by the addition of succinate (15 mM) and *p*-trifluoromethoxy carbonyl cyanide phenyl hydrazine (FCCP 20 μ M). The extent of mitochondrial inhibition of respiration was determined by measuring time to oxygen reaccumulation after removal of chamber lid, using inhibition by cyanide as 100% and exhaustion of substrate as 0% inhibition as previously described (14).

cGMP in cardiac tissue was determined by enzyme immunoassay using the cGMP EIA kit (GE Healthcare, Buckinghamshire, U.K.).

ROS generation in cardiac tissue was assayed spectrophotometrically by monitoring the oxidation of amplex red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to the product resofurin at 560 nm using the Amplex Red Hydrogen Peroxide/Peroxidase assay kit (Invitrogen, Karlsruhe, Germany).

Aconitase activity in cardiac tissue was measured by spectrophotometrically monitoring the concomitant formation of NADPH from NADP⁺ by the increase in

absorbance at 340 nm using the Bioxytech Aconitase-340 kit (Oxis Research, Portland, OR).

Hemodynamic Parameters. See *SI Text*.

Myocardial Area at Risk and Infarct Size. After reperfusion, the LCA was re-ligated in the same location as before. Evans blue dye (1 ml of a 1% solution) was injected into the aorta and coronary arteries for delineation of the ischemic AAR from the nonischemic zone. The tissue was wrapped in a clear food wrap and stored for one hour in a -20°C freezer. The heart was then serially sectioned perpendicularly to the long axis in 1-mm slices, and each slice was weighed. The sections were incubated in 1% TTC for 5 min at 37°C for demarcation of the viable and nonviable myocardium within the risk zone (34). The areas of infarction, AAR, and nonischemic left ventricle (LV) were assessed with computer-assisted planimetry by an observer blinded to sample identity. The size of the myocardial infarction was expressed as a percentage of the AAR as described (34).

Myocardial I/R in Vivo Protocol. Mice were anesthetized by i.p. injection of ketamine (45 mg/kg) and xylazine (Rompun 10 mg/kg). They were orally intubated and ventilated at a tidal volume of 2.1–2.5 ml and a respiratory rate of 122 breaths per minute. The mice were supplemented with 100% oxygen via a rodent ventilator side port. The chest was opened through a midline sternotomy and the LCA was ligated described above. The application of 48 nmol sodium nitrite (50 μ l) into the left ventricular cavity occurred 5 min before reperfusion (16). After 24 h of reperfusion the mice were anesthetized by i.p. injection of urethane (1.2 mg/kg), and anticoagulated with 1000 IE heparin i.p. 10 min before the experimental protocol, and then killed by cervical dislocation. Hearts were excised and reperfused with KHB for 5 min.

Statistical Analysis. The results are presented as mean \pm SEM. Data were analyzed by ANOVA with post hoc Bonferroni analysis with Origin 7G SR 2 (Northampton, MA) software. A value of $P < 0.05$ was considered to be statistically significant.

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