Nitrite Regulates Hypoxic Vasodilation via Myoglobin-Dependent Nitric Oxide Generation

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Background—Hypoxic vasodilation is a physiological response to low oxygen tension that increases blood supply to match metabolic demands. Although this response has been characterized for >100 years, the underlying hypoxic sensing and effector signaling mechanisms remain uncertain. We have shown that deoxygenated myoglobin in the heart can reduce nitrite to nitric oxide (NO·) and thereby contribute to cardiomyocyte NO· signaling during ischemia. On the basis of recent observations that myoglobin is expressed in the vasculature of hypoxia-tolerant fish, we hypothesized that endogenous nitrite may contribute to physiological hypoxic vasodilation via reactions with vascular myoglobin to form NO·.

Methods and Results—We show in the present study that myoglobin is expressed in vascular smooth muscle and contributes significantly to nitrite-dependent hypoxic vasodilation in vivo and ex vivo. The generation of NO· from nitrite reduction by deoxygenated myoglobin activates canonical soluble guanylate cyclase/cGMP signaling pathways. In vivo and ex vivo vasodilation responses, the reduction of nitrite to NO·, and the subsequent signal transduction mechanisms were all significantly impaired in mice without myoglobin. Hypoxic vasodilation studies in myoglobin and endothelial and inducible NO synthase knockout models suggest that only myoglobin contributes to systemic hypoxic vasodilatory responses in mice.

Conclusions—Endogenous nitrite is a physiological effector of hypoxic vasodilation. Its reduction to NO· via the heme globin myoglobin enhances blood flow and matches O2 supply to increased metabolic demands under hypoxic conditions. (Circulation. 2012;126:325-334.)

Key Words: hypoxic vasodilation ▪ myoglobin ▪ nitrite

Hypoxic vasodilation occurs as an adaptive response to a developing imbalance between demand and supply for O2. This mechanism ensures an adequate increase of local blood flow to fulfill the need for delivery of O2 to metabolically active tissue. This response is critical for exercising muscle, for adaptation to high altitude, and for the regulation of perfusion during embryonic development.1 Hypoxic vasodilation occurs in conduit2–6 and resistance-size arteries.6–9 Although this response has been characterized in the coronary circulation since 1879, the hypoxic sensor and the coupled vasodilatory effector of this response remain elusive. Various effectors for this response have been suggested, ranging from hydrogen ion concentration10 to local mediators such as adenosine and ATP-sensitive potassium channels, as well as prostacyclin.11,12 Nitric oxide (NO·) is known to contribute to the mechanism of hypoxic vasodilation via the downstream activation of an extended signaling pathway, which culminates in the decrease of intracellular [Ca2+] in smooth muscle cells and relaxation of vascular tone.13–15 The origin of NO· under hypoxia, however, has not yet been formally identified, although the source of NO· contributing to normoxic vasodilation is widely believed to be endothelial NO synthase (eNOS).16 However, NO· formation by eNOS requires O2, with a Km estimated at 25 to 100 µmol/L O2, suggesting that under hypoxic conditions, NO· formation by eNOS would decrease rather than increase. Moreover, eNOS does not possess an intrinsic mechanism for increased NO· production in response to hypoxia, suggesting that NOS-independent NO· formation pathways are more likely to determine hypoxic responses.

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The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.111.087155/-/DC1.

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Circulation is available at http://circ.ahajournals.org

DOI: 10.1161/CIRCULATIONAHA.111.087155
High concentrations of the inorganic anion nitrite have been known to be vasoactive for many years. Indeed, millimolar to high micromolar pharmacological concentrations of exogenous nitrite have been demonstrated to relax preconstricted isolated vessels. Unexpectedly, it has been shown more recently that nitrite functions as a more potent vasodilator under mild hypoxic or acidic conditions and in the human circulation. Moreover, hypoxia effectively enhances the effects of exogenously administered nitrite. The pioneering study from Cosby and coworkers revealed a striking effect of nitrite infusions on systemic blood flow in normal human volunteers at near physiological nitrite concentrations. The vasodilation was inversely correlated with hemoglobin O2 saturation and directly correlated with the formation of NO-modified hemoglobin (iron-nitrosyl hemoglobin and, to a lesser extent, S-nitrosated hemoglobin). These results were recapitulated in aortic ring preparations, in which the addition of nitrite to deoxyhemoglobin and deoxygenated erythrocytes resulted in vessel relaxation. Furthermore, reactions between nitrite and hemoglobin in isolated aortic rings along physiological hemoglobin fractional O2 saturations exhibited a distinct interaction: Nitrite-dependent vasodilation is inhibited at high hemoglobin O2 fractional saturation, whereas vasodilation is promoted when hemoglobin unloads to 50% saturation. Taken together, these findings and the ability of hemoglobin to reduce nitrite to NO in vitro, as shown by Brooks and Doyle et al., suggested a role of hemoglobin and of the heme globin family more generally in exogenous nitrite-mediated hypoxic vasodilation.

After these investigations of hemoglobin, our groups have shown that myoglobin and neuroglobin also react with and reduce nitrite to NO, suggesting a generalizable biological role for heme globins in hypoxic NO signaling. Importantly, myoglobin reduces nitrite to NO 60 times faster than deoxyhemoglobin as a result of its low heme redox potential. Although the biochemical reaction mechanisms between nitrite and these heme globins have been thoroughly characterized, the NO produced via these reactions will bind to excess deoxyhemoglobin or deoxyxymyoglobin to form an iron-nitrosyl complex, effectively limiting NO signaling. For this reason, the notion that heme globins can signal via nitrite reduction has remained controversial. Whereas other enzymes, such as eNOS and xanthine oxidoreductase (XOR), have been shown to reduce nitrite to NO at low O2 tensions and pH values, the inhibition of both eNOS and XOR does not block nitrite-dependent vasodilation in the human circulation.

The availability of a viable myoglobin-deficient (Mb−/−) mouse model allows for more definitive testing of these still controversial candidate pathways. Using this model, we have shown that myoglobin is required for hypoxic and ischemic nitrite signaling in the heart. The reduction of exogenous nitrite by cardiac myoglobin mediates cardioprotection during ischemia and reperfusion. Myoglobin-dependent nitrite reduction to NO regulates O2 consumption under moderate hypoxia, and this limits the generation of reactive oxygen species under ischemia. Despite the widely held belief that myoglobin is expressed only in oxidative striated and cardiac myocytes, it is now known to be localized in a wide variety of tissues, including smooth muscles, with as yet unidentified physiological functions. It is therefore tempting to speculate that nitrite-deoxymyoglobin interactions occur in the vasculature and contribute to hypoxic vasodilation. Over a decade, studies using particularly low concentrations of nitrite infusions in humans have shown vasodilatory effects for the naturally occurring anion nitrite. In addition, dietary approaches using nitrate to elevate circulating nitrite levels are emerging as a potential treatment regimen for high blood pressure. Although these studies have provided increasing evidence that the reduction of exogenous nitrite may be an essential trigger mechanism for vasodilation under hypoxic conditions, the role of endogenous nitrite in the regulation of hypoxic vasodilation and the resulting impact on blood pressure remain uncertain. Taking advantage of the Mb−/− mouse, in the present study we demonstrate that endogenous nitrite is activated by myoglobin in vascular tissue and that this cross talk is relevant under physiological conditions.

**Methods**

**Assessment of In Vivo Hemodynamics**

Mice were anesthetized by intraperitoneal injection of ketamine (45 mg/kg) and xylazine (Rompun, 10 mg/kg). A tracheal tube was inserted, and mechanical ventilation was initiated according to body weight (volume-controlled ventilation; Inspira, Harvard Apparatus, March-Hugstetten, Germany). Ventilation was controlled by end-tidal capnography (Hugo Sachs, March-Hugstetten, Germany). Isoflurane 1.2 vol% was added as anesthetist to O2 and nitrogen. A pressure-volume catheter (Millar Instruments, Seechem-Ober Beerbach, Germany) was inserted via the right carotid artery into the thoracic aorta, and heparin (70 IU/kg) was injected intraperitoneally. Heart rate and systolic and diastolic blood pressures were recorded beat to beat.

To assess basal hypoxic vasodilation, hypoxia was induced by ventilation with 10% O2/90% N2, and the effects on hemodynamics were recorded continuously without the administration of exogenous nitrite. We measured heart functions through a pressure-volume catheter placed in the left ventricle (1.4F, Millar Instruments).

To investigate the effects on blood pressure of increased nitrite levels by adding exogenous nitrite under steady state hypoxia, we first injected the NOS inhibitor L-NIO (100 mg/kg IP) to inhibit endogenous NO synthesis via NOS and unmask the genuine effects of exogenous nitrite. After 30 minutes, we injected nitrite (1.67 or 16.7 μmol/kg) intravenously and measured hemodynamics for the following 20 minutes. For the hypoxic experiments, the ventilation gas mixture was changed to 10% O2/90% N2 after the injection of NOS inhibitor. Hypoxic ventilation was conducted for 30 minutes to establish steady state conditions before nitrite was injected.

Final blood gas composition was determined (Siemens Blood Gas Analyzer 865, Eschborn, Germany) in all in vivo experiments (steady state hypoxia with exogenous nitrite and basal hypoxic vasodilation), and only those animals with a PO2 of 36±6 mm Hg (SD) and with normal CO2 and pH were included.

**Supplemental Methodology**

For a detailed explanation of the methods applied to determine myoglobin gene regulation, to demonstrate myoglobin mRNA and protein in situ, to investigate vascular myoglobin nitrite-reductase activity, to analyze cGMP formation, to measure nitrite-derived NO and nitrite, and as well as for aortic ring bioassay and the assessment of downstream signaling pathways, please see the Methods section of the online-only Data Supplement.

**Statistical Analysis**

Descriptive statistics such as mean±SD or mean±SEM were used to summarize continuous variables. The data were analyzed by Student...
Results

Myoglobin Is Expressed in Vascular Smooth Muscle and Reduces Nitrite to NO∗

The expression of myoglobin transcript in mouse aortic tissue was confirmed by 2 methods. First, we used reverse transcription polymerase chain reaction, checked by sequencing, to demonstrate the presence of myoglobin mRNA transcripts in mouse vascular tissue (data not shown). Second, we used RNA in situ hybridization of an antisense riboprobe directed against the murine myoglobin transcript to identify the smooth muscle cells of the media as the source of myoglobin mRNA (Figure 1A). The corresponding sense riboprobe was inactive (Figure I of the online-only Data Supplement), and no signal was obtained in Mb−/− mice (Figure 1B). The presence of myoglobin protein was confirmed with the use of both immunohistochemistry, with the use of a custom-made polyclonal antibody directed against the C-terminus of mouse myoglobin (Figure 1C and 1D; negative controls are shown in Figure I in the online-only Data Supplement), and Western blotting (Figure 1E). Figure 1C through 1E shows that aortas from Mb+/− mice contain myoglobin (namely, in all smooth muscle cells); vessels from Mb−/− mice did not exhibit a signal.

We then examined whether vascular tissue can generate NO∗ from nitrite under hypoxia and whether this was dependent on the presence of deoxymyoglobin. Homogenates of mouse aortas were added to a phosphate-buffered saline solution (pH 5.5) containing nitrite (100 µmol/L), final) under anaerobic conditions, and the sustained release of NO∗ was monitored by headspace gas-phase chemiluminescence. Figure 1F shows that the rate of NO∗ release from aortas of Mb−/− mice was substantially lower than for Mb+/− mice. Figure 1G shows that the inhibition of XOR, by simultaneous addition of its inhibitors allopurinol (100 µmol/L) and diphenyldionium (10 µmol/L) and inhibition of the electron transfer from ubiquinol to bc1 complex in the mitochondrial respiratory chain by incubation with 300 µmol/L myxothiazol, had no effect in Mb+/− and Mb−/− tissue, consistent with a negligible contribution in previous observations.26,33,34 Pre-incubation with 50 mmol/L ferricyanide to oxidize all cellular heme proteins significantly reduced the rate of NO∗ generation in Mb+/−. By contrast, no significant NO∗ release from nitrite from aortic tissues was detected under normoxia (data not shown). Figure 1H shows that addition of 20 µmol/L hemoglobin did not significantly affect the rate of NO∗ generation in both Mb+/− and Mb−/− tissue. However, we observed a tendency to greater rates of NO∗ release in the Mb−/− group, and therefore a statistical analysis of the Mb−/− approaches was conducted. This revealed a small but significant increase of nitrite reduction in Mb−/− tissue after incubation with hemoglobin, which is consistent with our previously reported observations under the same experimental conditions.27

Nitrite-Induced Hypoxic Vasodilation Depends on NO/Soluble Guanylate Cyclase/cGMP Signaling Pathway

The in vivo differences between Mb+/− and Mb−/− mice were consistent with significantly higher levels of nitrosо species in aortic tissue relating to higher rates of NO∗ production in Mb+/− mice (Figure 3A; comparable normoxic values and N-nitrosoamine levels in Figure IIA through IID in the online-only Data Supplement). The smaller sample size of these approaches, P values should not be overinterpreted. However, this was further evidenced by higher plasma cGMP levels and in aortic tissue of Mb−/− mice (Figure 3B; relevant normoxic baseline values are in Figure III in the online-only Data Supplement). These cGMP levels were quantified by competitive enzyme immunoassay in excised thoracic aortas incubated under hypoxia with 10 µmol/L nitrite for 10 minutes.36 This suggests that nitrite-dependent formation of NO∗ via deoxymyoglobin significantly elevates the cellular signaling molecule cGMP. Using an ex vivo bioassay with phenylephrine-constricted Mb+/− and Mb−/− aortic rings, we then assessed the effect of the NO∗ scavenger cPTIO and the soluble guanylate cyclase (sGC) inhibitor ODQ on vasorelaxation under 10 µmol/L nitrite. In Mb+/− vessels, both inhibitors significantly but not totally inhibited the nitrite-dependent vasodilatory response (Figure 3C). To quantify the production of NO∗, we used electron paramagnetic resonance spectroscopy on single excised aortas that were preincubated with 100 µmol/L nitrite under hypoxia (1% O2, pH 7.4). A spin trap was generated anoxically from ferrous sulfate and diethylthiocarbamate [0.2 mmol/L Fe-(DETC)2 and added to trap was generated anoxically from ferrous sulfate and diethylthiocarbamate [0.2 mmol/L Fe-(DETC)2 and added to trap was generated anoxically from ferrous sulfate and diethylthiocarbamate [0.2 mmol/L Fe-(DETC)2 and added to
Figure 1. Cytolocalization and myoglobin (Mb)-dependent nitrite reduction. A and B, Cytolocalization of myoglobin transcripts in the aorta of wild-type (A, Mb$^{+/+}$) and myoglobin-deficient (B, Mb$^{-/-}$) mice by RNA in situ hybridization. With the use of the antisense myoglobin riboprobe, smooth muscle cells exhibited a focal cytoplasmic signal (arrows), whereas the endothelium was negative. For both A and B, Papanicolaou hematoxylin counterstain was used. C and D, Immunohistology, with the use of an antibody directed against the mouse myoglobin protein. Whereas the Mb$^{-/-}$ aorta contains myoglobin in all smooth muscle cells (C), there is no evidence of myoglobin expression inMb$^{-/-}$ mice (D). E, Presence of myoglobin protein was confirmed with Western blotting. Myoglobin protein and Mb$^{+/+}$ hearts served as positive controls. F through H, Nitrite-reductase activity of aortas from Mb$^{+/+}$ and Mb$^{-/-}$ mice. F, Representative tracings show a decreased nitric oxide (NO) formation in Mb$^{-/-}$ compared with Mb$^{+/+}$ aortic tissue. G, Quantitative analysis reveals a significant difference between Mb$^{+/+}$ and Mb$^{-/-}$ mice (mean±SD; *P<0.05 comparing Mb$^{+/+}$ and Mb$^{-/-}$ mice; n=6–7). Inhibition of xanthine oxidoreductase (allopurinol+ diphenyliodonium [DPI]) or blocking of mitochondrial respiratory chain (myxothiazol) did not significantly change NO release in either Mb$^{+/+}$ or Mb$^{-/-}$ mice, whereas preincubation with ferricyanide to oxidize all cellular heme proteins significantly decreased NO generation in Mb$^{+/+}$ mice (mean±SD; #P<0.05 compared with untreated control). H, Addition of 20 μmol/L hemoglobin (Hb) did not significantly change the rate of NO formation; control experiments used methemoglobin (metHb). A statistical analysis of the Mb$^{-/-}$ approaches (red columns) revealed a small but significant increase of nitrite reduction in Mb$^{-/-}$ tissue after incubation with hemoglobin.
Hypoxic Vasodilation Is Independent of Enzymatic NO’ Production With Normal Responses in eNOS−/− and iNOS−/− Mice

NO’ could be generated by O2-dependent eNOS or inducible NOS (iNOS) rather than by myoglobin. To explore the involvement of eNOS/iNOS in the initiation of in vivo hypoxic vasodilation, we used eNOS−/− and iNOS−/− mice. eNOS−/− mice and, to a lesser extent, iNOS−/− mice displayed somewhat higher vascular pressures than the corresponding wild-type mice (genetic background: C57BL/6), but despite this, both displayed pronounced hypoxic vasodilation responses that in relative terms were indistinguishable from those of their respective wild-type controls (Figure 3F and Figure III of the online-only Data Supplement). This is incompatible with a role for eNOS and iNOS in hypoxic vasodilation.

Effects of Escalating Nitrite Doses on Hypoxic Vasodilation In Vivo Depend on the Presence of Myoglobin

To further demonstrate the role of nitrite as the principal source of NO’, we assessed the in vivo myoglobin-dependent effects on blood pressure under steady state hypoxia using different exogenous nitrite doses. Pharmacological concentrations of exogenous nitrite have been demonstrated to relax preconstricted isolated arteries.5,17,18 In our experimental protocol, endogenous NO’ synthesis was inhibited pharmacologically with the use of L-NIO,4 and mice were exposed for 30 minutes to hypoxia (ventilation with 10% O2 to reach a final PaO2 of 30 mm Hg). Exogenous nitrite was then administered intravenously, and hemodynamics were monitored (schema in Figure 4A). Nitrite at 16.7 μmol/kg caused a significantly greater reduction in blood pressure in Mb+/+ than in Mb−/− mice (Figure 4B), and the same but smaller effects were evident at 1.67 μmol/kg nitrite, which is a physiological dose (Figure 4C; absolute values and effects of exogenous nitrite under normoxia as controls are shown in Figures IV and V of the online-only Data Supplement).

If NO’ was generated from applied nitrite via vascular myoglobin, then we anticipated the formation of iron-nitrosylated myoglobin products30,37 in aortic extracts, an indirect marker of nitrite reduction. Indeed, after hypoxic incubation of aortas with the isotope [15N]-labeled nitrite, we detected the Mb[15N]NO product using electron paramagnetic resonance spectroscopy in Mb+/+ but not Mb−/− aortas (Figure 5A). The electron paramagnetic resonance spectrum was identical to that obtained for an authentic Mb[15N]NO
solution, which directly links the nitrite-dependent production of NO to the myoglobin protein itself and not to other heme proteins in smooth muscle, including cytoglobin, which should occur in \( \text{Mb}^-/- \) mice. Other potential nitrite reduction mechanisms could also be negated by using ex vivo aortic ring preparations under hypoxic conditions. Thus, Figure 5B shows that neither the specific inhibition of XOR or aldehyde oxidase nor the absence of eNOS by removal of the endothelium affected the nitrite-induced (10 \( \mu \text{mol/L} \)) vasodilation or the impact of myoglobin.

Nitrite-Driven Hypoxic Vasodilation via Vascular Myoglobin Is Not Related to Changes in Cardiac Output

Finally, hypoxic vasodilation might be linked to mechanisms of cardiac origin, either through reduced cardiac output or by
transfer of cardiac NO<sup>+</sup> to vascular smooth muscles. The former is unlikely because we found sustained or even enhanced heart functions during hypoxic challenge using a pressure-volume catheter placed in the left ventricle (Figure 6A and Figure VI in the online-only Data Supplement), perhaps because of a decrease in afterload. In addition, Figure 6B shows that the circulating NO<sup>+</sup> pool represented by nitrite and nitroso compounds in the plasma was identical in both species under hypoxia and was unaffected by the absence of myoglobin, which argues against any influence on vasodilation of NO<sup>+</sup> generated by the heart.

Figure 4. Nitrite-evoked vasodilation under hypoxia is dose dependent and reduced under myoglobin deficiency. A, Experimental design and relative effects of 16.7 (B) and 1.67 μmol/kg (C) exogenous nitrite on hemodynamics under hypoxic ventilation. The relative decrease in systolic blood pressure (Psys) and diastolic blood pressure (Pdia) in wild-type (Mb<sup>+/+</sup>) was significantly higher than in myoglobin-deficient (Mb<sup>−/−</sup>) mice (mean±SEM; n=5; *P<0.05).

Figure 5. Formation of nitrosyl-myoglobin (MbNO) as an indirect marker for the formation of NO<sup>+</sup> and independence of other nitrite-reductases. A, Exogenously applied nitrite was converted to NO<sup>+</sup>, and this nitrosylated myoglobin. Incubation of [15N]-labeled nitrite led to the formation of Mb[15N]NO as detected by electron paramagnetic resonance spectroscopy (with deoxygenated myoglobin solution as authentic control). B, Nitrite reduction to vasodilatory NO<sup>+</sup> was independent of xanthine oxidoreductase (inhibited by 100 μmol/L allopurinol, 10 μmol/L diphenyliodonium [DPI]), aldehyde oxidase (50 nmol/L raloxifen), or mechanisms located in the endothelial layer (Endothel.) Compared with untreated controls, no significant decrease in vasorelaxation was detected, whereas a significant difference between wild-type (Mb<sup>+/+</sup>) and myoglobin-deficient (Mb<sup>−/−</sup>) aortic rings remained detectable (mean±SD; n=3; *P<0.05, **P<0.01).
Role of Heme Globins
Nitrite-reductase activity has been identified in a variety of different proteins ranging from XOR,46 cytochrome c oxidase,41 cytochrome c,42 to eNOS.40 Comparable activity was also described for heme globins (eg, hemoglobin and myoglobin),4,19,26 and neuroglobin has emerged recently as a novel redox-regulated nitrite-reductase.28 Because heme globin can also react with NO\(^-\) to form stable nitrosyl-heme complexes that may limit NO\(^-\) bioavailability, a role for heme globin–driven NO\(^-\) signaling has been questioned. Despite a general belief that myoglobin expression and function are limited to cardiomyocytes and striated muscle cells, recent experimental studies confirmed the existence of myoglobin in the vasculature and in a wide range of nonmuscle tissues of experimental studies confirmed the existence of myoglobin in limited to cardiomyocytes and striated muscle cells, recent general belief that myoglobin expression and function are globin–driven NO\(^-\). Establishing NO\(^-\) signaling via vascular myoglobin activates the well-established NO/sGC/cGMP signaling pathway; and (3) this occurs independently of the NOS system, other potential nitrite-reductases, and hypoxia-modified cardiac function.

Role of Nitrite
Nitrite-reductase activity has been identified in a variety of different proteins ranging from XOR,46 cytochrome c oxidase,41 cytochrome c,42 to eNOS.40 Comparable activity was also described for heme globins (eg, hemoglobin and myoglobin),4,19,26 and neuroglobin has emerged recently as a novel redox-regulated nitrite-reductase.28 Because heme globin can also react with NO\(^-\) to form stable nitrosyl-heme complexes that may limit NO\(^-\) bioavailability, a role for heme globin–driven NO\(^-\) signaling has been questioned. Despite a general belief that myoglobin expression and function are limited to cardiomyocytes and striated muscle cells, recent experimental studies confirmed the existence of myoglobin in the vasculature and in a wide range of nonmuscle tissues of the hypoxia-tolerant carp.31,43 In the present study, we confirmed the presence of myoglobin transcript in smooth muscle layers of vessels and, using electron paramagnetic resonance spectroscopy, we confirmed myoglobin protein expression according to its distinct spectrum.37 No transcript signal was evident in endothelial cells, as described recently for capillaries located in the central nervous system,31 consistent with its presence in smooth muscle cells. We also showed that ablation of myoglobin in vascular tissue led to a marked decrease in reduction of nitrite to NO\(^-\), an observation that was independently verified by chemiluminescence and by electron paramagnetic resonance spectroscopy. By contrast, we failed to detect a role for XOR-mediated nitrite reduction,36 and the contribution of acidic disproportionation77 was negligible. The local presence of myoglobin in the vessel wall and a relevant myoglobin-dependent NO\(^-\) generation implicate a role for this protein in the regulation of vascular functions under hypoxic conditions.

Recent advances in understanding the role of this heme globin in cardiac function were achieved by taking advantage of the myoglobin knockout mouse. We were therefore able to demonstrate the relevance of cardiac myoglobin in nitrite reduction along the physiological O\(_2\) gradient, thus restoring myocardial energy balance and yielding a much reduced infarct size.26,30 In the present study, we showed that genetic ablation of myoglobin impaired the hypoxia-induced vasodilation response by up to 54%, which points directly at myoglobin as a key component in the signal transduction mechanism necessary for hypoxic vasodilation. Although these observations specifically assess a role for myoglobin that accounts for more than half of the vasodilatory response, it is likely that multiple overlapping enzymatic and nonenzymatic pathways for nitrite reduction are present both in vascular tissue and in the blood compartment to allow for the graded reduction of nitrite to NO\(^-\) at different oxygen tensions along physiological gradients.24 These data therefore also highlight the potential for hemoglobin and red blood cells, neuroglobin, cytoglobin, and potentially undiscovered proteins to be involved in hypoxic nitrite reduction, accounting for the remainder of the vasodilatory response. Further investigations are needed to compare each of their relative contributions.

Role of Nitrite
Nitrite is not only the oxidation product of NO\(^-\) but also a key reservoir for NO\(^-\) in blood and cellular compartments. The largest component of the bodily nitrite provision derives from

Discussion
Although NO\(^-\) is known to be involved as a trigger in hypoxic vasodilation, the source of NO\(^-\) remains unclear. Plasma and vascular nitrite may provide an alternative source of NO\(^-\), but questions remain relating to the activator of nitrite, the exact role of heme globins, and the relevance of endogenous nitrite in the regulation of physiological functions. Our present study seeks to address these current controversies.

We here show that (1) endogenous nitrite, reduced by vascular myoglobin to bioactive NO\(^-\), is a regulator of the physiological vasodilatory response to hypoxia; (2) heme globin–related nitrite signaling via vascular myoglobin activates the well-established NO/sGC/cGMP signaling pathway; and (3) this occurs independently of the NOS system, other potential nitrite-reductases, and hypoxia-modified cardiac function.

Figure 6. Cardiac function does not contribute to the decrease in blood pressure under hypoxia. A, Cardiac functions on induction of hypoxia as measured by an indwelling left ventricular pressure-volume catheter. Either parameter shows a small increase that is incompatible with a contribution of cardiac function on hypoxic vasodilation (mean±SEM). HR indicates heart rate; SV, stroke volume. B, Wild-type (Mb\(^{+/+}\)) and myoglobin-deficient (Mb\(^{-/-}\)) mice were anesthetized and intubated tracheally. After stabilization, mice were challenged with hypoxia (10% O\(_2\)/90% N\(_2\), analogous to our in vivo protocol in Figure 2A). Chemiluminescence and high-performance liquid chromatography were used to determine the plasma levels of nitrite and nitroso compounds (RNO). The latter comprises S-nitroso compounds (RSNO) and the remainder of bound NO\(^-\) (RXNO; eg, N-nitroso compounds). No significant differences were measured between the 2 strains for either compound (mean±SEM).
endogenous generation by NOS, and a smaller percentage is derived from nutritional sources.\textsuperscript{44} Nitrite is present in the blood in nanomolar concentrations, whereas tissue levels in heart, liver, kidney, and particularly in the vasculature are comparably higher irrespective of the species investigated.\textsuperscript{35,45,46}

We and others have demonstrated recently that exogenous supplementation with pharmacological nitrite doses has implications for tissue protection under pathological conditions. Nitrite-related protection has been described for general disease states (eg, the cardiopulmonary resuscitation syndrome)\textsuperscript{47} and for chronic and acute intervention regimens.\textsuperscript{48,49} Organ-specific protection has also been described for ischemic conditions of the brain, kidney, liver, and heart.\textsuperscript{30,49,50}

To substantiate the role of endogenous nitrite as a key effector by its myoglobin-induced activation to NO\textsuperscript{−}, we investigated the involvement of the NO\textsuperscript{−}/sGC/cGMP signaling pathway. A timely vasodilatory response requires the activation of smooth muscle cell sGC.\textsuperscript{13} We showed significantly higher levels of cGMP when myoglobin was present, with this being a definitive indication of sGC activation on nitrite reduction. As expected, inhibition of sGC or scavenging of NO\textsuperscript{−} did not abolish the vasodilatory effects completely. Furthermore, a specific role for NOS was excluded with the use of eNOS\textsuperscript{−/−} and iNOS\textsuperscript{−/−} mice. In addition, our experiments with different exogenous nitrite doses further confirmed that nitrite is the principal source of NO\textsuperscript{−} via heme globin–driven signaling because exogenous nitrite at high and low pharmacological concentrations caused a significantly greater reduction in blood pressure in M\textsubscript{b}\textsuperscript{−/−} than in M\textsubscript{b}\textsuperscript{+/+} mice.

Taken together, these new observations demonstrate that endogenous nitrite and the heme globin myoglobin play an essential role in activating the hypoxic vasodilation response. Our principal finding was that nitrite-induced responses under hypoxia in terms of the effect on vasodilation and subsequently on blood pressure were substantially reduced or absent in mice lacking myoglobin. This relates specifically to NO\textsuperscript{−} generation from nitrite and the activation of the NO\textsuperscript{−}/sGC/cGMP signaling pathway. Critically, we also showed that the reduced blood pressure induced in vivo by hypoxia was largely impaired under the absence of myoglobin as nitrite-reductase, whereas knockout of eNOS or iNOS had no such effect. We also showed in vivo that vasodilation can be induced under sustained hypoxia by application of a physiological dose of exogenous nitrite. That hypoxic vasodilation occurred in aortic rings stripped of endothelium indicates that the vascular mechanism is located in the remaining smooth muscle cells, which we show is the cytolocalization of myoglobin transcripts and protein. However, it must be considered that these experimental models are an approximation of relevant vascular beds. In summary, these data provide evidence for a physiological role for endogenous nitrite and for a heme globin–related signaling mechanism in vivo.

**Sources of Funding**

This work was supported by the Deutsche Herzstiftung (scholarship to Dr Totzeck). Dr Luedeke was supported by a scholarship of the German Cardiac Society. Drs Rassaf and Kelm were supported by grants from the Deutsche Forschungsgemeinschaft (RA 969/4-2 and Kf405/5-1). Dr Rassaf is a Heisenberg professor of the Deutsche Forschungsgemeinschaft (RA969/7-1). Drs Cossins and Berenbrink received funding from the Biotechnology and Biological Sciences Research Council, UK. Drs Gladwin and Shiva received research support from National Institutes of Health grants R01HL098032, RO1HL096973, and PO1HL103455; Institute for Transfusion Medicine; and Hemophilia Center of Western Pennsylvania.

**Disclosures**

None.

**References**


CLINICAL PERSPECTIVE

Emerging evidence implicates that inorganic nitrate provides a source for bioactive nitric oxide (NO). Nitrite is reduced to NO under circumstances with decreased O2 or pH levels along physiological gradients or under pathological conditions. Hypoxic vasodilation is a physiological response to low O2 tension, and the present data elucidate a pivotal role in vivo role for endogenous nitrite in vascular responsiveness. Although exogenous nitrite supplementation has been associated previously with tissue protection in preclinical disease models (eg, myocardial infarction and several other local and general pathological conditions), the underlying mechanisms for the reduction of endogenous nitrite remained incompletely understood. We provide evidence for a crucial role for the endogenous nitrite/myoglobin pathway in this response. Ablation of vascular myoglobin in myoglobin-deficient mice impaired the reduction of exogenous and endogenous nitrite to NO, which subsequently caused an augmented ex vivo and in vivo vasodilatory response under hypoxia. Enhancing the endogenous nitrite levels via administration of nitrite or other NO sources may therefore provide beneficial effects in a wide variety of cardiovascular diseases but also points to a potential role of nitrite in other tissues where myoglobin has been located recently. We speculate that increased myoglobin concentrations, as occur during physical exercise training, may further enhance nitrite-dependent signaling. These findings both provide insight into physiological mechanisms and point to a tractable therapeutic strategy in which nitrite is used as a site-selective vasodilator to circumvent the potentially deleterious effects observed with unselective substances (eg, organic nitrates).
Nitrite Regulates Hypoxic Vasodilation via Myoglobin-Dependent Nitric Oxide Generation
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Circulation. 2012;126:325-334; originally published online June 9, 2012;
doi: 10.1161/CIRCULATIONAHA.111.087155

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/126/3/325

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2012/06/09/CIRCULATIONAHA.111.087155.DC1

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SUPPLEMENTAL MATERIAL

Nitrite Regulates Hypoxic Vasodilation via Myoglobin–Dependent Nitric Oxide Generation

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Supplemental Methods

Chemicals
All chemicals were bought from Sigma (Seelze, Germany) except for NOS inhibitor L-N (5)-(1-iminoethyl)-ornithine (L-NIO, Alexis, Lôrrach, Germany), sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, Alexis), phosphate-buffered saline (PBS, Serag-Wiessner, Naila, Germany), heparin (ratiopharm, Ulm, Germany), ketamine (Pfizer, Berlin, Germany), xylazine (aniMedica, Senden-Bösenfell, Germany), isoflurane (DeltaSelect, Pfullingen, Germany), diphenyleneiodonium chloride (DPI, Fluka, Seelze, Germany) and acetylcholine (Fluka).
**Animals**

Male NMRI (Naval Medical Research Institute, \(Mb^{+/+}\) and \(Mb^{-/-}\))^1 and C57BL/6 wild-type and \(eNOS^{-/-}\) mice^2 were obtained from the Duesseldorf animal house. \(iNOS^{-/-}\) mice (B6.129P2-Nos2^{tm1Lau/J}) were bought from the Jackson Laboratory, Sulzfeld, Germany. All groups did not differ significantly in age (12±3 weeks) or weight (32±6 g). Animals were kept on standard rodent chow, tap water *ad libitum* and on a 12/12 hours light/dark cycle. All experiments were approved by the responsible ethics committee.

**Determination of Mb gene regulation**

Single aortas (~10 mg wet mass) were snap frozen and total RNA extracted using RNasy Fibrous Tissue Kit (Qiagen, Ratingen, Germany) followed by synthesis of cDNA using Superscript III Reverse Transcription Kit (Invitrogen, Darmstadt, Germany) based on primers synthesized by Eurofins (MWG Operon, Ebersberg, Germany). Products were 5'-ATGGGGCTCAGTGATGGG-3' and 5'-GCCCTGGAAGCCTAGCTC-3' for Mb and 5'-AGCCGATTCTTGTACAC-3' and 5'-GTCATCATCTCCGCCCCTT-3' for GAPDH. Reverse transcription-PCR was carried out using Platinum GenoTYPE DNA Polymerase (Invitrogen, annealing temperature 57 °C) and products were run on a 1% agarose gel followed by elution and determination of concentration using a NanoDrop spectrophotometer (Fisher Scientific, Schwerte, Germany). The products were checked by sequencing.
**In situ analysis of Mb expression**

Aortic preparations from *Mb*<sup>+/−</sup> and *Mb*<sup>−/−</sup> mice were fixed in 4% paraformaldehyde as previously described<sup>1</sup> and routinely paraffin wax embedded. 3-5 µm sections were prepared for RNA-*in situ* hybridization (RNA-ISH) and immunohistology (IH). For RNA-ISH, riboprobes (sense and anti-sense) were prepared from a 225 bp cloned fragment of the Mb gene between the GLFKTHPET and KIPVKYLE amino acid regions (amino acids 32 to 106) to which PCR primers had been designed (forward TCTGTTTAAGACTCACCCCTGAGACC and reverse CTCCAGGTACTGACCGGGATCTTG). The product was ligated into pCRII cloning vector (Invitrogen) and transformed into TOPO-OneShot chemically competent cells (Invitrogen). The identity of selected clones was confirmed by sequencing. The PCR product from a single clone was gel-extracted to provide a template for dioxygenin riboprobe production (Roche, Mannheim, Germany). A dot blot assay was used to titer the riboprobes, using an anti-dioxygenin antibody and alkaline phosphatase detection. RNA-ISH was performed as previously described.<sup>3</sup> Slides incubated with the sense probe served as negative controls.

For IH, a custom made rabbit anti-mouse peptide antibody directed against a 14 amino acid long peptide at the C terminus (amino acids 136-149; C-LELFNIDAYKE-coNH2) of the protein and a rabbit anti-human myoglobin antibody (sc-2507, Santa Cruz Biotechnology) were used. Briefly, 3-5 µm sections were prepared and deparaffinised through graded alcohols. After antigen retrieval with citrate buffer pH 6.0 (30 min heating in microwave) and blocking of endogenous peroxidase activity (10 min at room temperature in DAKO REAL™ Peroxidase Blocking Solution; DAKO Cytomation, Glostrup, Denmark), slides were incubated at
4 °C for 15-18 h with the primary antibody (1:50 in TBS with 0.05% Tween), washed and incubated for 30 min at room temperature with Dako Envision+ System-HRP anti-rabbit (Dako Cytomation), followed by visualisation with diaminobenzidintetrahydrochloride (DAB). Negative controls were either incubated with the pre-immune serum of the custom made antibody or the primary antibody after it had been incubated with the peptide (27 mg/ml) to block specific binding.

Western analysis

Protein extracts were prepared by homogenizing 5 snap frozen mouse aortas to a fine powder, taken up in radioimmunoprecipitation assay buffer (RIPA Buffer containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) containing complete-mini protease inhibitor cocktail tablet (Roche) and boiled in 4x Laemmli buffer. For aortic tissue, a sample of 180 µg of supernatant protein was electrophoresed on polyacrylamide gels. Heart samples gave very strong signals, and 1 µg supernatant protein was sufficient to give signals comparable to those of the aortic tissue. Electrophoresis and blotting were performed using NuPAGE® MES Gels employing the XCell SureLockTM Mini-Cell apparatus (Invitrogen), following the manufacturer's instructions. Proteins were transferred to a nitrocellulose membrane (Life Technology, Darmstadt, Germany), saturated with 5% non-fat milk in 0.1% Tween-20 in Tris buffered saline (TBS) (blocking solution), and incubated overnight at 4 °C with α-myoglobin rabbit polyclonal antibody (Santa Cruz, (FL-154) sc-25607a) at 1:500 dilution. The membrane was washed for 4 x 10 min in 0.1% Tween-20 in TBS and then incubated with goat anti-rabbit IgG (HRP) (Abcam
ab2721-1) at 1:4000 dilution for 1 h at RT. The membrane was washed four times before application of ECL reagent (GE Healthcare) and exposure of the membrane to autoradiography film.

**Determination of vascular Mb nitrite-reductase activity**

Homogenates of four whole aortas from either $Mb^{+/+}$ or $Mb^{-/-}$ mice were prepared in PBS (pH 7.4) containing proteinase inhibitor (Roche) and centrifuged. The protein concentration of the supernatant was determined using the BioRad protein assay (BioRad, München, Germany). 280 µg of total protein was injected into a 40 ml sealed reaction chamber filled with PBS at pH 5.5 containing 100 µM nitrite that had been pre-equilibrated with helium (Linde, Pullach, Germany). NO$^*$ production under anaerobic conditions was determined by chemiluminescence over 20-30 min as described previously$^4$ and the rate of release was calculated as moles per g protein and per second. A small baseline NO$^*$ release was detectable prior to addition of protein, which may be caused by acidic disproportionation.$^5$ Inhibitors in dimethyl sulfoxide, ferricyanide, hemoglobin (Hb) and metHb were pre-incubated for 30 min prior to addition to the PBS/nitrite solution.

**Analysis of cGMP formation**

Five aortas were incubated in 10 ml Krebs-HEPES buffer and deoxygenated with nitrogen gas for 10 min with 1% final O$_2$ level. We injected nitrite (10 µM, final), and after 10 min the aortas were snap-frozen in liquid nitrogen. cGMP was determined using the BiotrakTM cGMP competitive enzyme immunoassay system (GE Healthcare, Munich, Germany) using the manufacturers protocol. The same assay
was used to measure cGMP levels in the plasma of the *in vivo* experiments without exogenous nitrite, which reflects an intracellular increase of cGMP concentration even at very low to moderate cGMP elevations.\(^6\)\(^7\) Tissue cGMP levels were expressed per mg wet tissue, plasma levels are presented per liter.

**Measurement of nitrite-derived NO\(^*\), nitrite and nitroso species (RSNO, RNNO)**

Electron paramagnetic (EPR) spectroscopy was used to quantify the formation of NO\(^*\) (Bruker Elexsys E580 EPR spectrometer [Karlsruhe, Germany] operating at X-band (\(~9.4\) GHz), equipped with a Super High Sensitivity Probehead (V2.0), recording of EPR spectra at 77 K). Spectra were averaged over 20 scans with a sweep time of 83 s, a time constant of 40.96 ms and a modulation frequency of 100 kHz. For spin trapping experiments a modulation amplitude of 0.5 mT G and 10 mW of microwave was used. Excised thoracic aortas were placed in deoxygenated Krebs-HEPES buffer containing nitrite (100 \(\mu\)M, final). After 3 min, 0.2 mM Fe-(DETC)\(_2\) was added and incubated for 10 min. Snap-frozen samples were placed into an EPR quartz capillary (3 mm inner diameter). Relative quantification of trapped NO\(^*\) was carried out by measuring the amplitude of the left (low field) line of the NO-Fe-(DETC)\(_2\) signal. Positive controls were carried out with NO\(^*\) donor S-nitroso-N-acetylpenicillamin (1 mM). Mb\([^{15}\text{N}]\)NO formed by incubation of deoxygenated tissue homogenates and \([^{15}\text{N}]\)nitrite at pH 5.5 was measured as previously described.\(^8\)

Nitrite and nitroso species in aortic tissue homogenates and in plasma were measured using HPLC (ENO20, Eicom, Dublin, Ireland) and chemiluminescence.\(^9\)-\(^13\)
Aortic ring bioassay and assessment of down-stream signaling pathway

*Ex vivo* vasodilation was assessed using an aortic ring bioassay. 2-3 mm rings of a single thoracic aorta were suspended in an organ bath containing 10 ml Krebs-Henseleit buffer and connected to force transducers (Hugo Sachs). The bath was purged with 5% CO$_2$/95% O$_2$ and equilibration was allowed for 60 min. Resting tension was set to 1 g. Following addition of KCl (40 mM, final), viability was checked with 10 µM phenylephrine (final) and by 10 µM acetylcholine (final). For hypoxia, aortic rings were equilibrated to 1% O$_2$ for 20 min.$^{14}$ After a second preconstriction with phenylephrine, nitrite (10 µM) was added and the relaxation was calculated as % of the maximum constriction. To remove the aortic endothelium, a small thread was introduced into the vessel lumen and rubbed gently. Removal of the endothelium was verified by lack of dilation when subjected to 10 µM acetylcholine. Inhibitors ODQ (10 µM, final), allopurinol (100 µM, final), DPI (10 µM, final), raloxifene (50 nM) and NO$^\cdot$ scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 1 mM, final) were added to the vessel containing organ bath 30 min prior to nitrite administration and relaxation was measured in comparison to untreated rings.
Supplemental Figure 2

A

B

C

D

D

E
Supplemental Figure 3
Supplemental Figure 4

Nitrite:

A

16.7 μmol kg⁻¹

Psyr

NO₂⁻

Pdias

NO₂⁻

B

1.67 μmol kg⁻¹

Psyr

NO₂⁻

Pdias

NO₂⁻
Supplemental Figure 5

A

B


P/sys (%) vs min

P/dias (%) vs min

\( \text{NO}_2^- \)

\( \text{Mb}^{++} \)

\( \text{Mb}^{-} \)
Supplemental Figure 6

Induction of hypoxia

EF (%)

-5 0 5 10 15 20 25 30

Induction of hypoxia

CO (μL/min⁻¹)

-5 0 5 10 15 20 25 30

min
Figure Legends

Supplemental Figure 1 of the online-only Data Supplement. Negative controls for *in situ* hybridization and immunohistology for Mb mRNA and protein in the aorta of wild type mice (*Mb*+/+). Using the sense riboprobe, no signal was seen (A, *Mb*+/+). Immunohistology controls showed marked reduction of the staining after peptide blocking (C) and no specific reaction after incubation with pre-immune serum (D).

Supplemental Figure 2. Nitrite, nitroso species and cGMP levels under normoxic and hypoxic conditions. A, Nitrite levels in aortic tissue of wild-type (*Mb*+/+) and myoglobin deficient (*Mb*−/−) mice under normoxia. No significant differences were detected (*P*=0.97, *n*=5). B, RSNO level under normoxia in aortic tissue of *Mb*+/+ and *Mb*−/− mice with no significant differences (*P*=0.74, *n*=3). C and D, Aortic tissue RNNO level in both mice under hypoxia (C) and normoxia (D) with no significant differences. E, cGMP plasma levels under normoxia in *Mb*+/+ and *Mb*−/− mice. No significant differences were detected between the two groups (*P*=0.37, *n*=4-5, mean±SD). F, cGMP levels (fmol/mg protein) in aortic tissue under normoxia in *Mb*+/+ and *Mb*−/− mice. No significant differences were detected between the groups (*P*=0.39, *n*=5, mean±SD).

Supplemental Figure 3. Absolute values for hemodynamic parameters presented as relative data in Fig. 3F. C57BL/6 wild-type (WT, *n*=5) and endothelial and inducible NO synthase deficient mice (*eNOS*−/−, *n*=6, and *iNOS*−/−, *n*=5) were anaesthetized, intubated and mechanically ventilated. Following a period with normoxic ventilation, the mice were challenge with hypoxia (10% oxygen, see corresponding schema in Fig. 2A). The effects on systolic (*P* _{sys} ) and diastolic (*P* _{dias} ) pressure were continuously monitored with a pressure volume catheter placed in the thoracic aorta. No significant differences were detected between the three different groups (*P*>0.05 for all time points, mean±SEM).
Supplemental Figure 4. Absolute effects of exogenous nitrite on in vivo hemodynamics under hypoxia (data presented as relative changes in Fig. 3B and 3C). Experiments were performed as indicated in Figure 3A. Mechanically ventilated mice were challenged with hypoxia for 30 min after inhibition of endogenous enzymatic NO• synthesis to establish steady state conditions. Nitrite was then injected intravenously and the effects on hemodynamics were recorded continuously by means of an indwelling pressure volume catheter. (A) Absolute effects of 16.7 µmol kg⁻¹ exogenous nitrite (NO₂⁻) on hemodynamics (systolic [P_{sys}] and diastolic [P_{dias}] pressure) in vivo under hypoxic ventilation (10% O₂) in wild-type (Mb⁺/+ ) and myoglobin-deficient mice (Mb⁻/- ). (B) Effects of 1.67 µmol kg⁻¹ nitrite. Values are mean±SEM (n=5).

Supplemental Figure 5. Effects of exogenous nitrite on in vivo hemodynamics under normoxia. A, Experimental protocol. L-NIO to inhibit endogenous NO• production was administered and 35 min later nitrite (1.67 µmol kg⁻¹) was injected intravenously. B, Relative changes in systolic (P_{sys}) and diastolic (P_{dias}) pressure in wild-type (Mb⁺/+ ) and myoglobin deficient (Mb⁻/- ) mice with no significant differences between the two sets of mice (n=5, mean±SEM).

Supplemental Figure 6. Heart functions upon induction of hypoxia. When challenged with hypoxia in vivo (10% oxygen) analogous to schema main Fig. 2A, analysis of heart functions through an indwelling pressure-volume catheter reveals an increase of ejection fraction (EF) and cardiac output (CO) (means±SEM of 5 specimens).


