Nitrite Regulates Hypoxic Vasodilation via Myoglobin–Dependent Nitric Oxide Generation

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Matthias Totzeck, MD¹*; Ulrike B. Hendgen-Cotta, PhD¹*; Peter Luedike, MD¹; Michael Berenbrink, PhD²; Johann P. Klare, PhD³; Heinz-Juergen Steinhoff, PhD³; Dominik Semmler, BSc¹; Sruti Shiva, PhD⁴; Daryl Williams, PhD²; Anja Kipar, Dr.med.vet.habil.⁵; Mark T. Gladwin, MD⁶; Juergen Schrader, MD⁷; Malte Kelm, MD¹; Andrew R. Cossins, PhD²**; Tienush Rassaf, MD¹**

¹Dept of Med, Division of Cardiology, Pulmonology & Vascular Med, Med Faculty, University Hospital Duesseldorf, Duesseldorf, Germany; ²Institute of Integrative Biology, University of Liverpool, Liverpool, United Kingdom; ³Dept of Physics, University Osnabruceck, Osnabruceck, Germany; ⁴Dept of Pharmacology & Chemical Biology, University of Pittsburgh, Pittsburgh, PA; ⁵Veterinary Pathology, University of Liverpool, Liverpool, United Kingdom; ⁶Pulmonary, Allergy, and Critical Care Med, University of Pittsburgh, Pittsburgh, PA; ⁷Dept of Cardiovascular Physiology, Heinrich-Heine-University Duesseldorf, Duesseldorf, Germany

* contributed equally / ** share senior-authorship

Correspondence:
Prof. Dr. Tienush Rassaf
Department of Medicine, Division of Cardiology
Pulmonology and Vascular Medicine
University Hospital Duesseldorf
Moorenstrasse 5
D-40225 Duesseldorf, Germany
Tel: +49-211-811-8800
Fax: +49-211-811-8812
E-mail: Tienush.Rassaf@med.uni-duesseldorf.de

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Abstract:

Background - Hypoxic vasodilation is a physiological response to low oxygen (O₂) tension that increases blood supply to match metabolic demands. While this response has been characterized for more than 100 years, the underlying hypoxic sensing and effector signaling mechanisms remain uncertain. We have shown that deoxygenated myoglobin (deoxyMb) in the heart can reduce nitrite to nitric oxide (NO⁺) and thereby contribute to cardiomyocyte NO⁺ signaling during ischemia. Based on recent observations that Mb is expressed in the vasculature of hypoxia-tolerant fish, we hypothesized that endogenous nitrite may contribute to physiological hypoxic vasodilation via reactions with vascular Mb to form NO⁺.

Methods and Results - We here show that Mb 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 deoxyMb activates canonical soluble guanylate cyclase (sGC)/cyclic guanosine monophosphate (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 (Mb⁻/⁻). Hypoxic vasodilation studies in Mb, endothelial and inducible NO synthase knockout models (eNOS⁻/⁻, iNOS⁻/⁻) suggest that only Mb 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 Mb enhances blood flow and matches O₂ supply to increased metabolic demands under hypoxic conditions.

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. Hypoxic vasodilation occurs in conduit and resistance-size arteries. While 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 concentration to local mediators such as adenosine, and ATP-sensitive potassium channels, as well as prostacyclin. NO is known to contribute to the mechanism of hypoxic vasodilation via the down-stream activation of an extended signaling pathway, which culminates in the decrease of intracellular [Ca²⁺] in smooth muscle cells and relaxation of vascular tone. The origin of NO under hypoxia, however, has not yet been formally identified, though the source of NO contributing to normoxic vasodilation is widely believed to be the endothelial NO-synthase (eNOS). However, NO formation by eNOS requires O₂, with a Kₘ estimated at 25-100 μM 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.

High concentrations of the inorganic anion, nitrite, has 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 more recently been shown that nitrite functions as a more potent vasodilator
under mild hypoxic or acidic conditions and in the human circulation.\textsuperscript{4,5,19} Moreover, hypoxia effectively enhances the effects of exogenously administered nitrite.\textsuperscript{4,5,19,20} The pioneering study from Cosby and coworkers have 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 (Hb) O\textsubscript{2} saturation and directly correlated with the formation of NO-modified Hb (iron-nitrosylated Hb and, to a lesser extent, S-nitrosated Hb).\textsuperscript{4} These results were recapitulated in aortic ring preparations, in which the addition of nitrite to deoxyHb and deoxygenated erythrocytes resulted in vessel relaxation.\textsuperscript{4} Furthermore, reactions between nitrite and Hb in isolated aortic rings along physiological Hb fractional O\textsubscript{2} saturations exhibited a distinct interaction: nitrite-dependent vasodilation is inhibited at high Hb O\textsubscript{2} fractional saturation, whereas vasodilation is promoted when Hb unloads to 50\% saturation.\textsuperscript{21} Taken together, these findings and the ability of Hb to reduce nitrite to NO\textsuperscript{*} \textit{in vitro}, shown by Brooks and Doyle\textsuperscript{22,23} suggested a role of Hb, and of the heme globin family more generally, in exogenous nitrite-mediated hypoxic vasodilation.\textsuperscript{24}

Following these investigations on Hb, our groups have shown that Mb and neuroglobin also react with and reduce nitrite to NO\textsuperscript{*}, suggesting a generalizable biological role for heme globins in hypoxic NO\textsuperscript{*} signaling.\textsuperscript{4,25-28} Importantly, Mb reduces nitrite to NO\textsuperscript{*} 60 times faster than deoxyHb due to its low heme redox potential.\textsuperscript{27} While the biochemical reaction mechanisms between nitrite and these heme globins have been thoroughly characterized, the NO\textsuperscript{*} produced \textit{via} these reactions will bind to excess deoxyHb or deoxyMb to form an iron-nitrosyl-complex, effectively limiting NO\textsuperscript{*} signaling. For this reason, the notion that heme globins can signal \textit{via} nitrite reduction has remained controversial. While other enzymes, such as eNOS and xanthine oxidoreductase (XOR), have been shown to reduce nitrite to NO\textsuperscript{*} at low O\textsubscript{2} tensions and pH
values, the inhibition of both eNOS and XOR does not block nitrite-dependent vasodilation in the human circulation.\textsuperscript{4,29}

The availability of a viable \(Mb^{-/-}\) mouse model allows for more definitive testing of these still controversial candidate pathways. Using this model we have shown that Mb is required for hypoxic and ischemic nitrite signaling in the heart. The reduction of exogenous nitrite by cardiac Mb mediates cardioprotection during ischemia and reperfusion.\textsuperscript{26,30} Mb-dependent nitrite reduction to NO\(^*\) regulates \(O_2\) consumption under moderate hypoxia\textsuperscript{25,26} and this limits the generation of reactive oxygen species under ischemia.\textsuperscript{30} Despite the widely held belief that Mb is expressed only in oxidative striated and cardiac myocytes, it is now known to be localized in a wide variety of tissues\textsuperscript{31} including smooth muscles\textsuperscript{32} with yet unidentified physiological functions. It is therefore tempting to speculate, that nitrite-deoxyMb 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.\textsuperscript{4,5,19,20} In addition, dietary approaches using nitrate to elevate circulating nitrite levels are emerging as a potential treatment regimen for high blood pressure.\textsuperscript{20} While 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, we here demonstrate that endogenous nitrite is activated by Mb in vascular tissue and that this crosstalk is relevant under physiological conditions.
Methods

Assessment of in vivo hemodynamics

Mice were anesthetized by intraperitoneal (i.p.) injection of ketamine (45 mg kg⁻¹) and xylazin (Rompun, 10 mg kg⁻¹). A tracheal tube was inserted and mechanical ventilation initiated according to the body weight (volume controlled ventilation, Inspira, Harvard Apparatus, March-Hugstetten, Germany). Ventilation was controlled by end-tidal capnography (Hugo-Sachs, March-Hugstetten, Germany). 1.2 vol% isoflurane was added as anesthetic to O₂ and nitrogen. A pressure volume catheter (Millar Instruments) was inserted via the right carotid artery into the thoracic aorta and heparin (70 IU kg⁻¹) was injected i.p.. Heart rate, systolic (Psys) and diastolic blood pressure (Pdias) 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 (1.4 F, Millar Instruments, Seeheim-Ober Beerbach, Germany).

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⁻¹) i.p. to inhibit endogenous NO⁺ synthesis via NOS and unmask the genuine effects of exogenous nitrite.⁴ After 30 min, we injected nitrite (1.67 μmol kg⁻¹ or 16.7 μmol kg⁻¹) intravenously (i.v.) and measured hemodynamics for the following 20 min. 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 min 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 mmHg (standard deviation [SD]) and with normal CO₂ and pH were included.

**Supplemental Methodology**

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

**Statistical analysis**

Descriptive statistics such as mean±SD or mean±SEM (standard error of the mean) were used to summarize continuous variables. The data were analyzed by Student’s t-test (two groups) and ANOVA (multiple groups) with post hoc Bonferroni adjustment using statistical software Prism 5.0 (GraphPad) and Sigma Plot 11 (SigmaPlot). Two-way ANOVA (for [i] mouse species and [ii] time) with pairwise comparisons (Holm-Sidak procedure) was used for comparisons on in vivo basal hypoxic vasodilation. P-value < 0.05 was considered to be statistically significant. However, given the smaller sample size of some approaches (n=3), P-values should not be over-interpreted.

**Results**

**Mb is expressed in vascular smooth muscle and reduces nitrite to NO***

The expression of Mb transcript in mouse aorta tissue was confirmed by two methods. First, we used reverse transcription-PCR, checked by sequencing, to demonstrate the presence of Mb RNA
transcripts in mouse vascular tissue (data not shown). Second, we used RNA-in situ hybridization of an anti-sense riboprobe directed against the murine Mb transcript to identify the smooth muscle cells of the media as the source of Mb mRNA (Figure 1A). The corresponding sense riboprobe was inactive (Figure 1 of the online-only Data Supplement) and no signal was obtained in Mb−/− mice (Figure 1B). The presence of Mb protein was confirmed using both immunohistology, using a custom-made polyclonal antibody directed against the C-terminus of mouse Mb (Figure 1C and D; negative controls in Figure 1 of the online-only Data Supplement) and Western blotting (Figure 1E). Figures 1C-E shows that aortas from Mb+/+ mice contain Mb, namely in all smooth muscle cells; vessel 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 deoxyMb. Homogenates of mouse aortas were added to a PBS solution (pH 5.5) containing nitrite (100 μM, 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 the XOR, by simultaneous addition of its inhibitors allopurinol (100 μM) and diphenyliodonium (DPI, 10 μM) and the inhibition of the electron transfer from ubiquinol to bc1-complex in the mitochondrial respiratory chain by incubation with 300 μM myxothiazol had no effect in Mb+/+ and Mb−/− tissue, consistent with a negligible contribution in previous observations.26,33,34 Preincubation with 50 mM 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 μM Hb 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\(^{\bullet}\) 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 Hb which is consistent with our previously reported observations under the same experimental conditions.\(^{27}\)

**Regulation of physiological in vivo vasodilation under hypoxia depends on reduction of endogenous nitrite via Mb**

In light of relatively high levels of nitrite in the vessel walls compared to other tissues and blood\(^{35}\) and the demonstrated nitrite-reductase activity of vascular Mb, we investigated acute *in vivo* responses to hypoxia using \(Mb^{+/+}\) and \(Mb^{-/-}\) mice, which have comparable levels of nitrite (Figure 2A of the online-only Data Supplement). Anaesthetized mice were intubated and mechanically ventilated (scheme in Figure 2A). Hypoxia was then induced and the immediate effects on hemodynamics were recorded. In \(Mb^{+/+}\) mice, we observed a reduction in systolic \(P_{sys}\) and diastolic blood pressure \(P_{dia}\) within 2-4 min of hypoxia to a lower plateau after 5-10 min. In \(Mb^{-/-}\) mice, the acute blood pressure response was reduced by up to 54\% (Figure 2B and C) and instead we saw a continuing but gradual decline in pressure.

**Nitrite-induced hypoxic vasodilation depends on NO\(^{\bullet}/sGC/cGMP signaling pathway**

The *in vivo* differences between \(Mb^{+/+}\) and \(Mb^{-/-}\) mice were consistent with significantly higher levels of nitroso species in aortic tissue relating to higher rates of NO\(^{\bullet}\) production in \(Mb^{+/+}\) mice (Figure 3A; comparable normoxic values and RNNO levels in Figure 2B-D of the online-only Data Supplement). Given the smaller sample size of these approaches, \(P\)-values should not be over-interpreted. However, this was further evidenced by higher plasma cGMP levels and in aortic tissue of \(Mb^{-/-}\) mice (Figure 3B; relevant normoxic baseline values Figure 2E and F of the online-only Data Supplement). These cGMP levels were quantified by competitive enzyme
immonoassay in excised thoracic aortas incubated under hypoxia with 10 μM nitrite for 10 min. This suggests that nitrite-dependent formation of NO\(^*\) via deoxyMb 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 sGC inhibitor ODQ upon vasorelaxation under 10 μM nitrite. In \(Mb^{+/+}\) vessels both inhibitors significantly but not totally\(^{33}\) inhibited the nitrite-dependent vasodilatory response (Figure 3C). To quantify the production of NO\(^*\) we used EPR spectroscopy on single excised aortas that were pre-incubated with 100 μM nitrite under hypoxia (1% O\(_2\), pH 7.4). A spin trap was generated anoxically from ferrous sulfate and diethylthiocarbamate (0.2 mM Fe-(DETC)\(_2\)) and added to trap any NO\(^*\). The resulting NO-Fe-(DETC)\(_2\) EPR signal was used to quantify the amount of NO\(^*\) generated over a 10 min period. Figure 3D shows that the signal obtained from \(Mb^{+/+}\) mice was distinctive from that of \(Mb^{-/-}\) mice. Quantification of the NO-Fe-(DETC)\(_2\) EPR signal (Figure 3E) revealed a ~50% decrease of NO\(^*\) formation in \(Mb^{-/-}\) aortas relative to vessels from \(Mb^{+/+}\) mice. Taken together these data suggest that the nitrite-induced, Mb-dependent, *in vivo* vasodilation directly depends upon the canonical NO\(^*\)/sGC/cGMP pathway located in the vessel wall.

**Hypoxic vasodilation is independent of enzymatic NO\(^*\) production with normal responses in \(eNOS^{-/-}\) and \(iNOS^{-/-}\) mice**

NO\(^*\) could be generated by the O\(_2\)-dependent eNOS or iNOS rather than by Mb. 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, which in relative terms
were indistinguishable from their respective wild-type controls (Figure 3F and Figure 3 of the online-only Data Supplement). This is incompatible with a role for eNOS and iNOS for hypoxic vasodilation.

**Effects of escalating nitrite doses on hypoxic vasodilation in vivo depend on the presence of Mb**

To further demonstrate the role of nitrite as the principal source of NO*, we assessed the *in vivo* Mb-dependent effects on blood pressure under steady state hypoxia using different exogenous nitrite doses. Pharmacological concentrations of exogenous nitrite have been demonstrated to relax pre-constricted isolated arteries.5,17,18 In our experimental protocol endogenous NO* synthesis was inhibited pharmacologically using L-NIO4, and mice were exposed for 30 min to hypoxia (ventilation with 10% O2 to reach a final paO2 of 30 mmHg). Exogenous nitrite was then administered i.v. and hemodynamics were monitored (schema in Figure 4A). Nitrite at 16.7 μmol kg⁻¹ 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⁻¹ nitrite, which is a physiological dose (Figure 4C and absolute values and the effects of exogenous nitrite under normoxia as controls in Figure 4 and 5 of the online-only Data Supplement).

If NO* was generated from applied nitrite *via* vascular Mb then we anticipated the formation of iron-nitrosylated Mb products (MbNO)30,37 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 EPR spectroscopy in Mb+/+ but not Mb-/- aortas (Figure 5A). The EPR spectrum was identical to that obtained for an authentic Mb[¹⁵N]NO solution which directly links the nitrite-dependent production of NO* to the Mb protein itself and not to other heme proteins in smooth muscle, including cytoglobin38, which should occur in Mb-/-
mice. Other potential nitrite reduction mechanisms\textsuperscript{33,39,40} could also be negated by using \textit{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 μM) vasodilation or the impact of Mb.

**Nitrite-driven hypoxic vasodilation \textit{via} vascular Mb 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\textsuperscript{*} to vascular smooth muscles. The former is unlikely since 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 6 of the online-only Data Supplement), perhaps due to a decrease in afterload. Also Figure 6B shows that the circulating NO\textsuperscript{*} pool represented by nitrite and nitroso compounds in the plasma was identical in both species under hypoxia and unaffected by absence of Mb, which argues against any influence on vasodilation of NO\textsuperscript{*} generated by the heart.

**Discussion**

Although NO\textsuperscript{*} is known to be involved as trigger in hypoxic vasodilation, the source of NO\textsuperscript{*} remains unclear. Plasma and vascular nitrite may provide an alternative source of NO\textsuperscript{*}, but questions remain relating to the activator of nitrite, the exact role of heme globins and the relevance for endogenous nitrite in regulation of physiological functions. Our present study aims to address these current controversies.

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

**Role of heme globins**

A nitrite reductase activity has been identified in a variety of different proteins ranging from XOR, cytochrome c oxidase, cytochrome c to eNOS. A comparable activity was also described for heme globins, e.g. Hb and Mb, and neuroglobin has recently emerged as novel redox-regulated nitrite-reductase. Since heme globins can also react with NO to form stable nitrosyl-heme complexes which may limit NO bioavailability, a role for heme globin driven NO signaling has been questioned. Despite a general belief that Mb expression and function is limited to cardiomyocytes and striated muscle cells, recent experimental studies confirmed the existence of Mb in the vasculature and in a wide range of non-muscle tissues of the hypoxia-tolerant carp. Here, we have confirmed the presence of Mb transcript in smooth muscle layers of vessels and using EPR spectroscopy we have confirmed Mb protein expression according to its distinct spectrum. No transcript signal was evident in endothelial cells as recently described for capillaries located in the central nervous system consistent with its presence in smooth muscle cells. We also showed that ablation of Mb in vascular tissue led to a marked decrease in reduction of nitrite to NO, an observation that was independently verified by chemiluminescence and by EPR spectroscopy. By contrast, we failed to detect a role for XOR-mediated nitrite reduction, and the contribution of acidic disproportionation was negligible. The local presence of Mb in the vessel wall and a relevant Mb-dependent NO generation implicates 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 Mb-knockout mouse. We were thus able to demonstrate the relevance of cardiac Mb in nitrite reduction along the physiological O₂ gradient thus restoring myocardial energy balance and yielding a much reduced infarct size²⁶,³⁰. Here we showed that genetic ablation of Mb impaired the hypoxia-induced vasodilation response by up to 54% which points directly at Mb as a key component in the signal transduction mechanism necessary for hypoxic vasodilation. While these observations specifically assess a role for myoglobin which accounts for more than half of the vasodilatory response, it is likely that multiple overlapping enzymatic and non-enzymatic pathways for nitrite reduction are present in both 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 Hb 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 endogenous generation by NOS, while a smaller percentage is derived from nutritional sources.⁴⁴ Nitrite is present in the blood in nanomolar concentrations while tissue levels in heart, liver, kidney and particularly in the vasculature are comparably higher irrespective of the species investigated.³⁵,⁴⁵,⁴⁶ We and others have recently demonstrated 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, e.g. the cardiopulmonary resuscitation syndrome⁴⁷ and for chronic and acute intervention regimens.⁴⁸,⁴⁹
Organ-specific protection has also been described for ischemic conditions of the brain, the kidney, the liver, and heart.\textsuperscript{30,49,50}

To substantiate the role of endogenous nitrite as a key effector by its Mb-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 Mb is present, this being a definitive indication of sGC activation upon nitrite reduction. As expected, an inhibition of sGC or scavenging of NO\textsuperscript{*} did not abolish the vasodilatory effects completely. Furthermore, a specific role for NOS was excluded using 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 as exogenous nitrite at high and low pharmacological concentrations caused a significantly greater reduction in blood pressure in Mb\textsuperscript{+/+} than in Mb\textsuperscript{−/−} mice.

Taken together, these new observations demonstrate that endogenous nitrite and the heme globin Mb play an essential role in activating the hypoxic vasodilation response. Our principal finding was that a range of nitrite-induced responses under hypoxia on vasodilation and subsequently on blood pressure was substantially reduced or absent in mice lacking Mb. 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 \textit{in vivo} by hypoxia, was largely impaired under the absence of Mb as nitrite-reductase, while knockout of eNOS or iNOS had no such effect. We also showed \textit{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 cyto-location
of Mb transcripts and protein. However, it has to 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 \textit{in vivo}.

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**Conflict of Interest Disclosures:** None.

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**Figure Legends:**

**Figure 1.** Cyto-localization and myoglobin (Mb)-dependent nitrite reduction. **A** and **B**, Cyto-localization of Mb transcripts in the aorta of wild-type (**A**, *Mb*+/+) and Mb deficient mice (**B**, *Mb*−/−) by RNA-*in situ* hybridization. Using the anti-sense Mb riboprobe, smooth muscle cells exhibited a focal cytoplasmic signal (arrows) whilst the endothelium was negative. For both **A** and **B** Papanicolaou’s haematoxylin counterstain was used. **C** and **D**, Immunohistology, using an antibody directed against the mouse Mb protein. While the *Mb*+/+ aorta contains Mb in all smooth muscle cells (**C**), there is no evidence of Mb expression in *Mb*−/− mice (**D**). **E**, Presence of...
Mb protein was confirmed using Western blotting. Mb protein and Mb+/+ hearts served as positive controls. F through H, Nitrite-reductase activity of aortas from Mb+/+ and Mb−/− mice. F, Representative traces showing a decreased nitric oxide (NO\(^{•}\)) formation in Mb−/− compared to 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+/+ and Mb−/− mice, while pre-incubation with ferricyanide to oxidize all cellular heme proteins significantly decreased NO\(^{•}\) generation in Mb+/+ mice (mean±SD, #P<0.05 compared to untreated control). H, Addition of 20 \(\mu\)M hemoglobin (Hb) did not significantly change the rate of NO\(^{•}\) formation; control experiments using 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 Hb.

**Figure 2.** Basal hypoxic vasodilation *in vivo* depends on nitrite reduction *via* myoglobin (Mb). A, Experimental design. Mice were mechanically ventilated and a Millar catheter was inserted into the right carotid artery to record systolic (P\(_{\text{sys}}\)) and diastolic (P\(_{\text{dia}}\)) pressure continuously. Following a normoxic equilibration period, the ventilation gas mixture was changed to 10% O\(_2\)/90% N\(_2\) (induction of hypoxia). B and C, absolute and relative changes of hemodynamics, respectively, following the induction of hypoxia showing a greater decrease of pressures in wild-type (Mb+/+) vs. Mb deficient (Mb−/−) mice (asterisks and bars indicate time points and intervals with P<0.05 with n=7 and 6 respectively; values are means±SEM).

**Figure 3.** Nitrite-induced myoglobin (Mb)-dependent hypoxia vasodilation relies on
NO*/sGC/cGMP signaling pathway and is independent of NO synthases. A compares the concentrations of RSNO levels in aortic tissue of wild-type (Mb+/+) with Mb deficient (Mb−/) mice (mean±SD, n=3, *P<0.05), while B shows the same comparison for plasma and aortic tissue cGMP levels (mean±SD, n=3, *P<0.05). C shows the dependence of hypoxic vasodilation upon NO*/sGC/cGMP signaling in isolated aortic rings. Selective scavenging of NO* with cPTIO or blocking of sGC via ODQ nearly abolished the vasodilatory response in vessel equilibrated under hypoxia and challenged with 10 μM nitrite. (mean±SD, *P<0.05 compared to controls, n=5). D, Formation of NO* from nitrite was significantly higher in aortas of Mb+/+ mice as detected by EPR spectroscopy. The displayed signals are representative of three independent experiments. Controls showed that spin trap (Fe-(DETC)2) incubated with 1 mM [15N]nitrite and spin trap alone displayed no EPR signal. E, Quantitative analysis of these data revealed significantly higher amplitudes in Mb+/+ aortas compared to Mb−/− vessels (mean±SD, n=3, *P=0.018). F, In vivo vasodilation is sustained in mice lacking endothelial or inducible NO synthase (eNOS/iNOS). eNOS and iNOS are major alternative sources for NO* under normoxia but ablation of either gene failed to affect the vasodilatory responses in vivo. Thus, hypoxically-induced vasodilation following the schema in Figure 2A was not reduced in eNOS−/− (n=6) and iNOS−/− (n=5) compared to the corresponding wild-type (C57BL/6) mice (n=5). Under normoxia the baseline pressures remained stable throughout (Figure 2 of the online-only Data Supplement), (values are means±SEM).

**Figure 4.** Nitrite-evoked vasodilation under hypoxia is dose dependent and reduced under myoglobin (Mb) deficiency. A, Experimental design and (B and C) relative effects of 16.7 and 1.67 μmol kg⁻¹ exogenous nitrite, respectively, upon hemodynamics under hypoxic ventilation.
The relative decrease in $P_{\text{sys}}$ and $P_{\text{dias}}$ in wild-type ($Mb^{+/+}$) was significantly higher than in Mb deficient ($Mb^{-/-}$) mice (values are means±SEM, $n=5$, *$P<0.05$).

**Figure 5.** Formation of nitrosyl-myoglobin (MbNO) as an indirect marker for the formation of NO$^\cdot$ and independence of other nitrite-reductases. **A**, Exogenously applied nitrite was converted to NO$^\cdot$ and this nitrosylated Mb. Incubation of [$^{15}\text{N}$]-labeled nitrite led to the formation of Mb[$^{15}\text{N}$]NO as detected by EPR spectroscopy (deoxygenated Mb solution as authentic control). **B**, Nitrite reduction to vasodilatory NO$^\cdot$ was independent of xanthine oxidoreductase (inhibited by 100 μM allopurinol, 10 μM diphenyliodonium [DPI]), aldehyde oxidase (50 nM raloxifen) or mechanisms located in the endothelial layer (Endothel.) Compared to untreated controls, no significant decrease in vasorelaxation was detected whilst a significant difference between wild-type ($Mb^{+/+}$) and Mb deficient ($Mb^{-/-}$) aortic rings remained detectable (means±SD, $n=3$, *$P<0.05$, **$P<0.01$).

**Figure 6.** Cardiac function does not contribute to the decrease in blood pressure under hypoxia. **A**, Cardiac functions upon the induction of hypoxia as measured by an indwelling left ventricular pressure volume catheter. Either parameter shows a small increase which is incompatible with a contribution of cardiac function on hypoxic vasodilation (means±SEM). **B**, Wild-type ($Mb^{+/+}$) and myoglobin deficient ($Mb^{-/-}$) mice were anaesthetized and tracheally intubated. After stabilization, mice were challenged with hypoxia (10% O$_2$/90% N$_2$ – analogous to our *in vivo* protocol in Figure 2A). Chemiluminescence and HPLC 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$^\cdot$ (RXNO, e.g., N-nitroso compounds). No significant differences were measured between the two strains for either compound (means±SEM).
A

Physiological effects of endogenous nitrite in vivo

induction of hypoxia

blood gas analysis

absolute and relative effects on Psys and Pdias

B

Psys

Pdias

Induction of hypoxia

C

Relative changes Psys (%)

Relative changes Pdias (%)

Induction of hypoxia

Mbh

Mbb
In vivo effects of exogenous nitrite

A

induction of hypoxia

NO₂⁻ i.v.

blood gas analysis

absolute and relative effects on Psys and Pdias

Inhibition of endogenous NO* synthesis

-60 -40 -20 0 20 40

Psys (%)

-60 -40 -20 0 20 40

Pdias (%)

B

Nitrite:

16.7 μmol kg⁻¹

C

1.67 μmol kg⁻¹
Nitrite Regulates Hypoxic Vasodilation via Myoglobin-Dependent Nitric Oxide Generation
Matthias Totzeck, Ulrike B. Hendgen-Cotta, Peter Luedike, Michael Berenbrink, Johann P. Klare, Heinz-Juergen Steinhoff, Dominik Semmler, Sruti Shiva, Daryl Williams, Anja Kipar, Mark T. Gladwin, Juergen Schrader, Malte Kelm, Andrew R. Cossins and Tienush Rassaf

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SUPPLEMENTAL MATERIAL

Nitrite Regulates Hypoxic Vasodilation via Myoglobin–Dependent Nitric Oxide Generation

Matthias Totzeck, MD¹*, Ulrike B. Hendgen-Cotta, PhD¹*, Peter Luedike, MD¹,
Michael Berenbrink, PhD², Johann P. Klare, PhDB³, Heinz-Juergen Steinhoff, PhD³,
Dominik Semmler, BSc¹, Sruti Shiva, PhD⁴, Daryl Williams, PhD², Anja Kipar,
Dr.med.vet.habil.⁵, Mark T. Gladwin, MD⁶, Juergen Schrader, MD⁷, Malte Kelm, MD¹,
Andrew R. Cossins, PhD²** & Tienush Rassaf, MD¹**

Supplemental Methods

Chemicals

All chemicals were bought from Sigma (Seelze, Germany) except for NOS inhibitor L-N (5)-(1-iminoethyl)-ornithine (L-NIO, Alexis, Löffach, 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-Bösensell, 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 \textit{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 RNeasy Fibrous Tissue Kit (Quiagen, 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'-AGCCGATTTCATTGTCACTAC-3' and 5'-GTGATCATCTCCGCCCCTT-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 $M_b^{+/+}$ and $M_b^{-/-}$ mice were fixed in 4% paraformaldehyde as previously described\(^1\) 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 TCTGTTTAAGACTCACCCTGAGACC and reverse CTCCAGGTACTTGACCGGGATCTTG). 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.\(^3\) 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-LELFRNDIAAKYKE-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 previously4 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 O2 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. Tissue cGMP levels were expressed per mg wet tissue, plasma levels are presented per liter.

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

Electron paramagnetic (EPR) spectroscopy was used to quantify the formation of NO\(^\bullet\) (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 µM, 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\(^\bullet\) 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\(^\bullet\) 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.

Nitrite and nitroso species in aortic tissue homogenates and in plasma were measured using HPLC (ENO20, Eicom, Dublin, Ireland) and chemiluminescence.
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$^\bullet$ 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 1
Supplemental Figure 3

Psyst

Induction of hypoxia

Pdias

Induction of hypoxia

-5 0 5 10 15 20 25 30

min

Psyst (mmHg)

40 60 80 100 120 140 160

-5 0 5 10 15 20 25 30

min

Pdias (mmHg)

40 60 80 100 120

C57BL/6 WT
eNOS-

INOS+
Supplemental Figure 4

**Nitrite:**

**A**

16.7 $\mu$mol kg$^{-1}$

**B**

1.67 $\mu$mol kg$^{-1}$
Supplemental Figure 5

A

B
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
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 (Psys) 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 (NO\(_2^-\)) on hemodynamics (systolic [P\(_{\text{sys}}\)] and diastolic [P\(_{\text{dias}}\)] pressure) in vivo under hypoxic ventilation (10% O\(_2\)) 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\(_{\text{sys}}\)) and diastolic (P\(_{\text{dias}}\)) 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).


