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Nitrite Regulates Hypoxic Vasodilation *via* Myoglobin–Dependent Nitric Oxide Generation

Matthias Totzeck, MD[1,*], **Ulrike B. Hendgen-Cotta, PhD**[1,*], **Peter Luedike, MD**[1], **Michael Berenbrink, PhD**[2], **Johann P. Klare, PhD**[3], **Heinz-Juergen Steinhoff, PhD**[3], **Dominik Semmler, BSc**[1], **Sruti Shiva, PhD**[4], **Daryl Williams, PhD**[2], **Anja Kipar, Dr.med.vet.habil.**[5], **Mark T. Gladwin, MD**[6], **Juergen Schrader, MD**[7], **Malte Kelm, MD**[1], **Andrew R. Cossins, PhD**[2,**], and **Tienush Rassaf, MD**[1,**]

[1]Dept of Med, Division of Cardiology, Pulmonology & Vascular Med, Med Faculty, University Hospital Duesseldorf, Duesseldorf, Germany

[2]Institute of Integrative Biology, University of Liverpool, Liverpool, United Kingdom

[3]Dept of Physics, University Osnabrueck, Osnabrueck, Germany

[4]Dept of Pharmacology & Chemical Biology, University of Pittsburgh, Pittsburgh, PA

[5]Veterinary Pathology, University of Liverpool, Liverpool, United Kingdom

[6]Pulmonary, Allergy, and Critical Care Med, University of Pittsburgh, Pittsburgh, PA

[7]Dept of Cardiovascular Physiology, Heinrich-Heine-University Duesseldorf, Duesseldorf, Germany

Abstract

Background—Hypoxic vasodilation is a physiological response to low oxygen (O_2) 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 ($NO^{\cdot}$) and thereby contribute to cardiomyocyte $NO^{\cdot}$ 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 $NO^{\cdot}$.

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 $NO^{\cdot}$ 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 $NO^{\cdot}$ and the subsequent signal transduction mechanisms were all significantly impaired in mice without myoglobin ($Mb^{-/-}$). Hypoxic

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 Tienush.Rassaf@med.uni-duesseldorf.de.
*contributed equally
**share senior-authorship

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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 $NO^{\cdot}$ *via* the heme globin Mb enhances blood flow and matches O_2 supply to increased metabolic demands under hypoxic conditions.

Keywords

hypoxic vasodilation; myoglobin; nitrite

Hypoxic vasodilation occurs as an adaptive response to a developing imbalance between demand and supply for O_2. This mechanism ensures an adequate increase of local blood flow to fulfill the need for delivery of O_2 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 conduit[2–6] and resistance-size arteries.[6–9] 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[10] to local mediators such as adenosine, and ATP-sensitive potassium channels, as well as prostacyclin.[11,12] $NO^{\cdot}$ 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 [Ca^{2+}] in smooth muscle cells and relaxation of vascular tone.[13–15] The origin of $NO^{\cdot}$ under hypoxia, however, has not yet been formally identified, though the source of $NO^{\cdot}$ contributing to normoxic vasodilation is widely believed to be the endothelial NO-synthase (eNOS).[16] However, $NO^{\cdot}$ formation by eNOS requires O_2, with a K_m estimated at 25–100 μM O_2, suggesting that under hypoxic conditions $NO^{\cdot}$ formation by eNOS would decrease, rather than increase. Moreover, eNOS does not possess an intrinsic mechanism for increased $NO^{\cdot}$ production in response to hypoxia, suggesting that NOS-independent $NO^{\cdot}$ 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.[17] Indeed, millimolar to high micromolar pharmacological concentrations of exogenous nitrite have been demonstrated to relax preconstricted isolated vessels.[5,17,18] 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.[4,5,19] Moreover, hypoxia effectively enhances the effects of exogenously administered nitrite.[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_2 saturation and directly correlated with the formation of NO-modified Hb (iron-nitrosylated Hb and, to a lesser extent, S-nitrosated Hb).[4] These results were recapitulated in aortic ring preparations, in which the addition of nitrite to deoxyHb and deoxygenated erythrocytes resulted in vessel relaxation.[4] Furthermore, reactions between nitrite and Hb in isolated aortic rings along physiological Hb fractional O_2 saturations exhibited a distinct interaction: nitrite-dependent vasodilation is inhibited at high Hb O_2 fractional saturation, whereas vasodilation is promoted when Hb unloads to 50% saturation.[21] Taken together, these findings and the ability of Hb to reduce nitrite to $NO^{\cdot}$ *in vitro*, shown by Brooks and Doyle[22,23] suggested a role of Hb, and of the heme globin family more generally, in exogenous nitrite-mediated hypoxic vasodilation.[24]

Following these investigations on Hb, our groups have shown that Mb and neuroglobin also react with and reduce nitrite to $NO^{\cdot}$, suggesting a generalizable biological role for heme

globins in hypoxic $NO^{\cdot}$ signaling.[4,25–28] Importantly, Mb reduces nitrite to $NO^{\cdot}$ 60 times faster than deoxyHb due to its low heme redox potential.[27] While the biochemical reaction mechanisms between nitrite and these heme globins have been thoroughly characterized, the $NO^{\cdot}$ produced *via* these reactions will bind to excess deoxyHb or deoxyMb to form an iron-nitrosyl-complex, effectively limiting $NO^{\cdot}$ signaling. For this reason, the notion that heme globins can signal *via* nitrite reduction has remained controversial. While other enzymes, such as eNOS and xanthine oxidoreductase (XOR), have been shown to reduce nitrite to $NO^{\cdot}$ at low O_2 tensions and pH values, the inhibition of both eNOS and XOR does not block nitrite-dependent vasodilation in the human circulation.[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.[26,30] Mb-dependent nitrite reduction to $NO^{\cdot}$ regulates O_2 consumption under moderate hypoxia[25,26] and this limits the generation of reactive oxygen species under ischemia.[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[31] including smooth muscles[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.[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.[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 O_2 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 (P_{sys}) and diastolic blood pressure (P_{dias}) were recorded beat-to-beat.

To assess basal hypoxic vasodilation, hypoxia was induced by ventilation with 10% O_2/90% N_2 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 $NO^{\cdot}$ 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% O_2/90% N_2 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 pO_2 of 36±6 mmHg (standard deviation [SD]) and with normal CO_2 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 $NO^{\cdot}$ 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 $NO^{\cdot}$

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 $NO^{\cdot}$ 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 $NO^{\cdot}$ was monitored by headspace gas-phase chemiluminescence. Figure 1F shows that the rate of $NO^{\cdot}$ 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 $NO^{\cdot}$ generation in $Mb^{+/+}$. By contrast, no significant $NO^{\cdot}$ 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 $NO^{\cdot}$ generation in both $Mb^{+/+}$ and $Mb^{-/-}$ tissue. However, we observed a tendency to greater rates of $NO^{\cdot}$ 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_{dias}) 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 $NO^{\cdot}$/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 $NO^{\cdot}$ 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 immunoassay in excised thoracic aortas incubated under hypoxia with 10 μM nitrite for 10 min.[36] This suggests that nitrite-dependent formation of $NO^{\cdot}$ *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 $NO^{\cdot}$ 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 $NO^{\cdot}$ 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 diethyldithiocarbamate (0.2 mM Fe-$(DETC)_2$) and added to trap any $NO^{\cdot}$. The resulting NO-Fe-$(DETC)_2$ EPR signal was used to quantify the amount of $NO^{\cdot}$ 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 $NO^{\cdot}$ 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 $NO^{\cdot}$/sGC/cGMP pathway located in the vessel wall.

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

$NO^{\cdot}$ 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 $NO^{\cdot}$, 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 $NO^{\cdot}$ synthesis was inhibited pharmacologically using L-NIO[4], and mice were exposed for 30 min to hypoxia (ventilation with 10% O_2 to reach a final paO_2 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 $NO^{\cdot}$ 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 [^{15}N]-labeled nitrite we detected the Mb[^{15}N]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[^{15}N]NO solution which directly links the nitrite-dependent production of $NO^{\cdot}$ to the Mb protein itself and not to other heme proteins in smooth muscle, including cytoglobin[38], which should occur in *Mb*$^{-/-}$ mice. Other potential nitrite reduction mechanisms[33,39,40] 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 μM) vasodilation or the impact of Mb.

Nitrite-driven hypoxic vasodilation *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 $NO^{\cdot}$ 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 $NO^{\cdot}$ 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 $NO^{\cdot}$ generated by the heart.

Discussion

Although $NO^{\cdot}$ is known to be involved as trigger in hypoxic vasodilation, the source of $NO^{\cdot}$ remains unclear. Plasma and vascular nitrite may provide an alternative source of $NO^{\cdot}$, 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 $NO^{\cdot}$, is a regulator of the physiological vasodilatory response to hypoxia; 2.) heme globin-related nitrite signaling *via* vascular Mb activates the well-established $NO^{\cdot}$/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[36], cytochrome *c* oxidase[41], cytochrome c[42], to eNOS[40]. A comparable activity was also described for heme globins, e.g. Hb and Mb.[4,19,26] and, neuroglobin has recently emerged as novel redox-regulated nitrite-reductase.[28] Since heme globins can also react with $NO^{\cdot}$ to form stable nitrosyl-heme complexes which may limit $NO^{\cdot}$ bioavailability, a role for heme globin driven $NO^{\cdot}$ 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.[31,43] 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.[37] No transcript signal was evident in endothelial cells as recently described for capillaries located in the central nervous system[31] 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 $NO^{\cdot}$, 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[36], and the contribution of acidic disproportionation[37] was negligible. The local presence of Mb in the vessel wall and a relevant Mb-dependent $NO^{\cdot}$ 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 O_2 gradient thus restoring myocardial energy balance and yielding a much reduced infarct size[26,30]. 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 $NO^{\cdot}$ 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 $NO^{\cdot}$ but also a key reservoir for $NO^{\cdot}$ 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 syndrome[47] 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.[30,49,50]

To substantiate the role of endogenous nitrite as a key effector by its Mb-induced activation to $NO^{\cdot}$ we investigated the involvement of the $NO^{\cdot}$/sGC/cGMP signaling pathway. A timely vasodilatory response requires the activation of smooth muscle cell sGC.[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 $NO^{\cdot}$ did not abolish the vasodilatory effects completely. Furthermore, a specific role for NOS was excluded using $eNOS^{-/-}$ and $iNOS^{-/-}$ mice. In addition, our experiments with different exogenous nitrite doses further confirmed that nitrite is the principal source of $NO^{\cdot}$ *via* heme globin driven signaling as exogenous nitrite at high and low pharmacological concentrations caused a significantly greater reduction in blood pressure in $Mb^{+/+}$ than in $Mb^{-/-}$ 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 $NO^{\cdot}$ generation from nitrite and the activation of the $NO^{\cdot}$/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 Mb as nitrite-reductase, while 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 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 *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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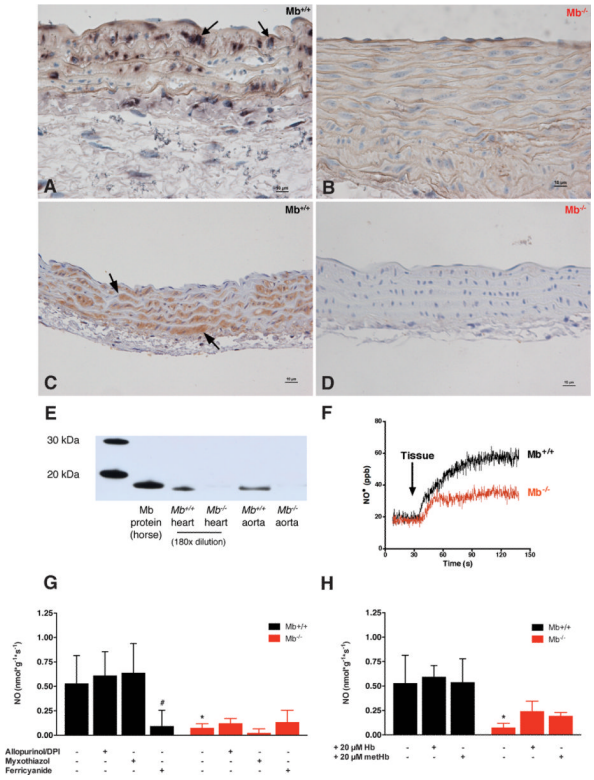

Figure 1.

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 ($NO^{\cdot}$) 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 $NO^{\cdot}$ release in either $Mb^{+/+}$ and $Mb^{-/-}$ mice, while pre-incubation with ferricyanide to oxidize all cellular heme proteins significantly decreased $NO^{\cdot}$ generation in $Mb^{+/+}$ mice (mean±SD, $^{\#}P<0.05$ compared to untreated control). **H**, Addition of 20 μM hemoglobin (Hb) did not significantly change the rate of $NO^{\cdot}$ 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.

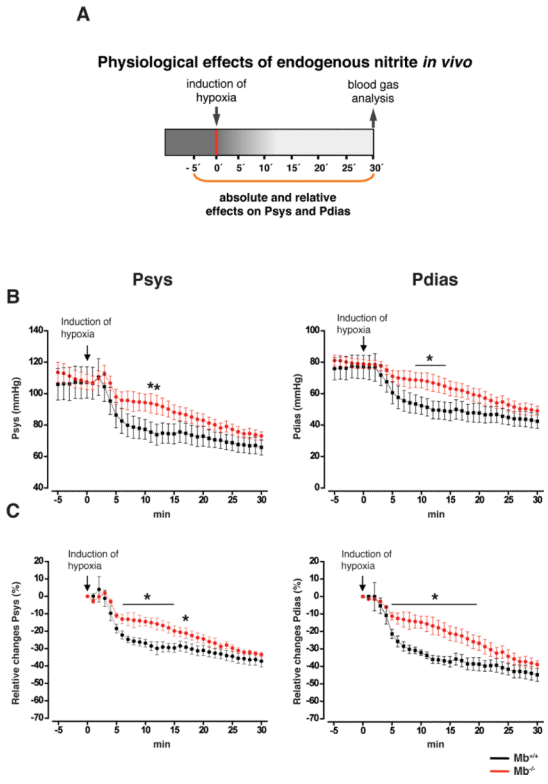

Figure 2.

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_{sys}) and diastolic (P_{dias}) 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).

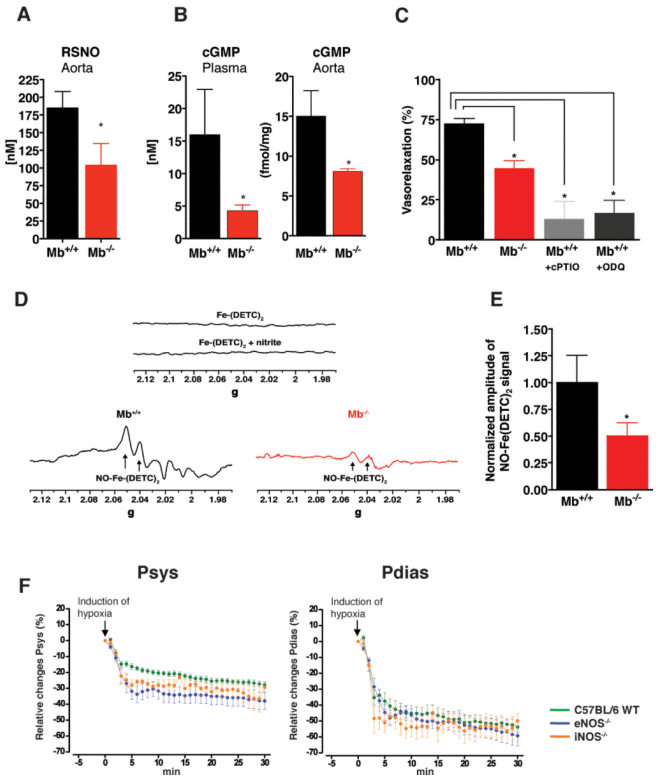

Figure 3.

Nitrite-induced myoglobin (Mb)-dependent hypoxia vasodilation relies on $NO^{\cdot}$/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 $NO^{\cdot}$/sGC/cGMP signaling in isolated aortic rings. Selective scavenging of $NO^{\cdot}$ 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 $NO^{\cdot}$ 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 [^{15}N]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 $NO^{\cdot}$ 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).

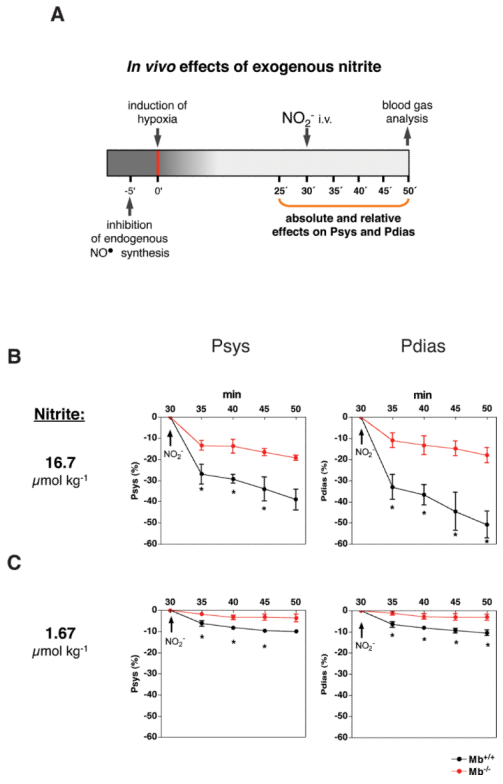

Figure 4.

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_{sys} and P_{dias} in wild-type ($Mb^{+/+}$) was significantly higher than in Mb deficient ($Mb^{-/-}$) mice (values are means±SEM, n=5, *P<0.05).

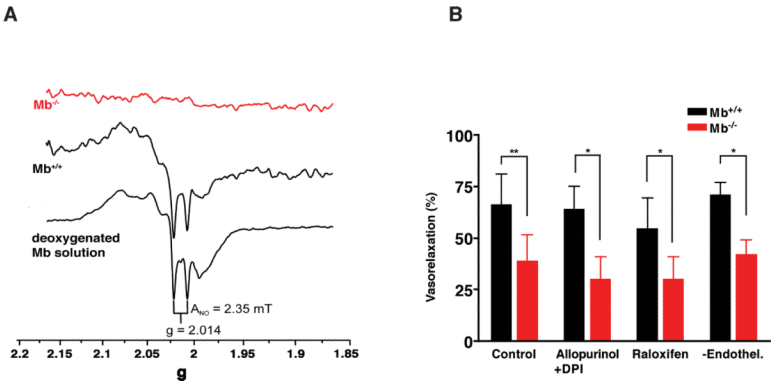

Figure 5.

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}N]-labeled nitrite led to the formation of Mb[^{15}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).

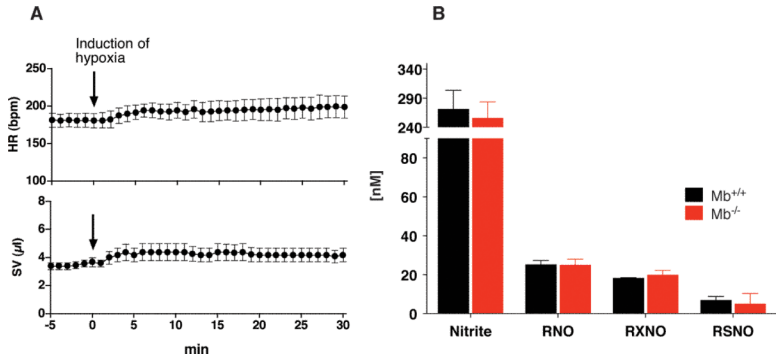

Figure 6.

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).