Myoglobin: A scavenger of bioactive NO

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The present study explored the role of myoglobin (Mb) in cardiac NO homeostasis and its functional relevance by employing isolated hearts of wild-type (WT) and myoglobin knockout mice. ^{1}H NMR spectroscopy was used to measure directly the conversion of oxygenated Mb (MbO_2) to metmyoglobin (metMb) by reaction with NO. NO was applied intracoronarily (5 nM to 25 μM), or its endogenous production was stimulated with bradykinin (Bk; 10 nM to 2 μM). We found that infusion of authentic NO solutions dose-dependently ($\geq$ 2.5 μM NO) increased metMb formation in WT hearts that was rapidly reversible on cessation of NO infusion. Likewise, Bk-induced release of NO was associated with significant metMb formation in the WT ($\geq$1 μM Bk). Hearts lacking Mb reacted more sensitively to infused NO in that vasodilatation and the cardiodepressant actions of NO were more pronounced. Similar results were obtained with Bk. The lower sensitivity of WT hearts to changes in NO concentration fits well with the hypothesis that in the presence of Mb, a continuous degradation of NO takes place by reaction of MbO_2 + NO to metMb + NO_3^-, thereby effectively reducing cytosolic NO concentration. This breakdown protects myocytic cytochromes against transient rises in cytosolic NO. Regeneration of metMb by metMb reductase to Mb and subsequent association with O_2 leads to reformation of MbO_2 available for another NO degradation cycle. Our data indicate that this cycle is crucial in the breakdown of NO and substantially determines the dose–response curve of the NO effects on coronary blood flow and cardiac contractility.

Myoglobin (Mb) is an important intracellular O_2-binding hemoprotein found in the cytoplasm of vertebrate type I and IIa skeletal and cardiac muscle tissue (1). As a major breakthrough in understanding globular protein structure, its tertiary structure was derived from x-ray diffraction studies by John Kendrew and his colleagues as early as the 1950s (2). Mb is a relatively small (M_r 16,700) and densely packed protein consisting of a single polypeptide chain of 153 amino acid residues. It contains an iron-porphyrin heme group identical to that of hemoglobin (Hb), and like Hb is capable of reversible oxygenation and deoxygenation. In mammals, half O_2 saturation of Mb is achieved at an intracellular O_2 partial pressure as low as 2.4 mmHg (1 mmHg = 133 Pa; ref. 3), suggesting a predominance of oxygenated Mb (MbO_2) under basal conditions.

Mb's function as an oxygen store is well accepted. Mb serves as a short-term O_2 reservoir in exercising skeletal muscle and in the beating heart, tiding the muscle over from one contraction to the next (4). In diving mammals, the concentrations of Mb exceed those of terrestrial mammals up to 10-fold, and Mb most likely serves for the extension of diving time when pulmonary ventilation ceases (5). Similarly, in mammals and humans adapted to high altitudes, Mb is expressed in high concentrations in skeletal muscle (6).

It has been proposed that Mb facilitates intracellular delivery of O_2, in that Mb adjacent to the cell membrane picks up oxygen, traverses the cytosol by translational diffusion to unload O_2 in the vicinity of mitochondria, and finally diffuses back to the cell membrane in the deoxygenated state (7). This circuit, termed "facilitated O_2 diffusion," may be a critical link between capillary O_2 supply and O_2-consuming cytochromes within mitochondria in the steady state. Facilitated O_2 diffusion has been unambiguously demonstrated in concentrated Mb solutions (8), but experiments carried out in isolated cells, papillary muscle, and at the whole organ level have yielded conflicting results (9–11). Likewise, model calculations have both refuted and supported the contribution of Mb-bound O_2 to total O_2 flux (11, 12).

The recent generation of transgenic mice lacking Mb has shed new light on the role of Mb in the intracellular delivery of O_2 (13, 14). Loss of Mb led to a surprisingly benign phenotype, with exercise and reproductive capacity, as well as cardiac and skeletal function, largely unaltered (13). Maintenance of function was accomplished by the activation of numerous compensatory mechanisms (14). However, direct evidence for an important role of Mb in facilitating O_2 diffusion was only recently produced by experiments employing CO to acutely inactivate Mb in the isolated wild-type (WT) heart by using hearts of Mb knockout ($myo^{-/-}$) mice as appropriate controls (15). Additionally, supportive evidence is derived from observations on single isolated cardiomyocytes (15, 16).

Mb is a molecular relative of Hb and together these hemoproteins play vital roles in one of the most important aspects of animal metabolism: the acquisition and utilization of O_2. With the advent of NO research there is now also abundant literature about the interaction of Hb with NO. It is generally accepted that Hb is crucial for oxidative inactivation of NO by reaction to nitrate and methemoglobin (17). However, the role of Hb, through *S*-nitrosothiol formation, in providing a protected route for delivery of bioactive NO is a matter of intense debate (18, 19). A comparison of the kinetic and thermodynamic properties of NO interactions with Mb and Hb (derived from *in vitro* investigations) reveals similar data for NO association and dissociation as well as for chemical reactions for both proteins (for review, see ref. 20), suggesting similar biological functions in NO homeostasis. However, there are at present no studies on the role of Mb in the *in vivo* metabolism of NO. Likewise, it is not known whether the presence of Mb alters the biological response to NO, whether exogenously supplied or endogenously formed.

The aim of the present study was to explore the role of Mb in cardiac NO homeostasis by using isolated hearts of WT and $myo^{-/-}$ mice, recently generated in our laboratory (14), as models. We were able to directly measure the NO-induced conversion of MbO_2 to metmyoglobin (metMb) in the WT heart with ^{1}H NMR spectroscopy. Furthermore, we found that Mb is important in the inactivation of NO and substantially determines the dose–response curve of the NO effects on coronary blood flow and cardiac contractility.

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Abbreviations: Mb, myoglobin; Bk, bradykinin; ETU, *S*-ethylisothiourea; $myo^{-/-}$, myoglobin knockout mice; L-NMMA, N^G-monomethyl-L-arginine methyl ester; LVDP, left ventricular developed pressure; MbO_2, oxygenated myoglobin; metMb, metmyoglobin; PCr, phosphocreatine; WT, wild type.

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Materials and Methods

Animals. Myo$^{-/-}$ mice were generated by deletion of the essential exon-2 via homologous recombination in embryonic stem cells, as described (14). Body weight ranged from 27 to 38 g and heart weight ranged from 170 to 250 mg, with no significant differences between the two groups.

Langendorff Experimental Setup for NMR Measurements. The preparation of murine hearts and retrograde perfusion at constant pressure of 100 mmHg with modified Krebs–Henseleit buffer—gassed at 95% O_2/5% CO_2 (carbogen), resulting in a pH of 7.4—were performed essentially as described (21). In brief, mice were injected with 250 units of heparin i.p. and anesthetized with urethane (1.5 g/kg) i.p. Hearts were rapidly excised and the aortas were cannulated. Nonrecirculating perfusion at 100 mmHg was initiated. Each heart was placed inside a 10-mm NMR tube and transferred into a heated (37°C) 10-mm probe inside a Bruker AMX 400-MHz WB NMR spectrometer. Perfusion pressure, coronary flow, and left ventricular developed pressure (LVDP) were measured continuously, the latter via a home-made fluid-filled balloon inserted into the left ventricle. Signals were recorded by using a PC with dedicated software (EMKA Technologies, Paris). After hearts stabilized inside the magnet, cardiac pacing (500 beats per min) was initiated and continued throughout. Left ventricular end-diastolic pressure was set to 5 mmHg. After the onset of cardiac pacing (30 min), the coronary perfusion rate was fixed to the steady flow already established and was maintained constant. After the switch to constant flow, baseline spectra were recorded. Subsequently, solutions with increasing concentrations of NO (5 nM to 25 μM), Bk (10 nM to 2 μM), adenosine (5–500 nM), N^G-monomethyl-L-arginine methyl ester (L-NMMA; 100 μM), or *S*-ethylisothiourea (ETU; 50 μM) were infused stepwise, and hemodynamic data as well as NMR spectra were acquired. After application of the highest respective concentration, infusion was stopped, hearts were allowed to recover for 10 min, and data/spectra were collected once more.

Infusion of NO Solutions. Aqueous solutions of NO were prepared essentially as described (22). Briefly, argon (quality = 5.0, >99.99%; Linde, Unterschleissheim, Germany) was passed through a closed all-glass system, composed of two scrubbing bottles, one containing an alkaline pyrogallol solution (5%, wt/vol) to remove traces of oxygen, and the other one containing potassium hydroxide (10%, wt/vol) to scavenge higher oxides of nitrogen. The bottles were connected in series with a three-necked beaker containing saline (0.9% NaCl) to dissolve NO. After flushing with argon for 30 min, the gas flow was switched to NO (quality = 3.0, >99.9%; AGA Gas, Hamburg, Germany) and maintained for an additional 45 min. Aliquots were transferred to air-tight syringes through a septum. The system was kept under positive pressure with NO to avoid changes in NO concentration because of re-equilibration between the aqueous and gas phases. Concentrations ranged between 1.6 and 1.9 mM NO, depending on the ambient pressure and temperature.

Dilutions were made in deoxygenated and argon-flushed saline, and concentrations were determined immediately before application by injecting 200 μl of the samples into a NO chemiluminescence analyzer (Sievers Instruments, Boulder, CO). Infusion of the NO solutions was performed by using air-tight fused-silica capillaries (inside diameter 0.2 mm; Supelco, Deisenhofen, Germany), the ends of which were positioned right in front of the aorta to minimize the contact time of NO solution and oxygenated buffer.

NMR Spectroscopy. Spectra were recorded by a Bruker AMX 400 WB NMR spectrometer, operating at frequencies of 400 MHz for ^{1}H and 161.97 MHz for ^{31}P. Shimming was done on the free-induction decay of the water signal. A line width at half height of 15 Hz could be routinely obtained.

^{1}H NMR. For selective excitation of the MbO_2 and metMb resonances at −2 to −4 ppm (23, 24), the standard $1\bar{3}3\bar{1}$ pulse sequence of the Bruker library was used. The delay for binomial H_2O suppression was set to 166 μs, resulting in maximal excitation of the region of interest. A 45° pulse (12.5 μs, estimated from the H_2O signal) was used; 16K transients were averaged for a typical ^{1}H NMR spectrum requiring 15 min of signal accumulation (acquisition time, 42 ms; sweep width, 12,195 Hz; data size, 1K; zero filling to 2K; exponential weighting resulting in a 40-Hz line broadening; chemical shifts were referenced to the H_2O resonance at 4.8 ppm). For 8K experiments 4K transients (≈4 min accumulation time) were averaged. Excitation with the $1\bar{3}3\bar{1}$ sequence led to large phase dispersion resulting in positive lipid signals and negative signals for the MbO_2 and metMb resonances (compare to ref. 15; for a theoretical explanation, see ref. 25). For a better presentation, these signals were inverted in Fig. 1 and Fig. 5. Relative peak areas were obtained by integration after baseline correction.

^{31}P NMR. Transients ($n = 240$) were accumulated with a 75° flip angle, a repetition time of 1 s, a spectral width of 5,882 Hz, a data size of 4K, zero filling to 8K, and exponential weighting resulting in a 10-Hz line broadening (≈4 min of signal accumulation). Chemical shifts were referenced to the phosphocreatine (PCr) resonance at −2.52 ppm. Relative peak areas were obtained by integration. These areas were scaled according to saturation factors of the respective phosphorus compounds determined from fully relaxed ^{31}P NMR spectra in control experiments for each strain.

After equilibration of the hearts within the magnet, NMR spectra were continuously recorded. For absolute quantification, baseline spectra of each experiment were related to the respective HPLC and SDS/PAGE data for ATP and Mb (14), respectively (assuming 100% NMR visibility of the particular compounds). Peak areas were converted to concentrations as described before (21). PCr and metMb concentrations were determined from the PCr/ATP and MbO_2/metMb ratios, respectively, measured by NMR spectroscopy. Cytosolic free ADP concentration was determined from the creatine kinase equilibrium as described before (21). Because ^{1}H NMR spectra during NO infusion were acquired in 15 min, a maximum of five different NO concentrations were applied within one experiment to avoid exceeding 2 h of total protocol time.

Materials. All compounds used in this study were either analytical grade or of the highest purity available. Bk, ETU, L-NMMA, and Mb were obtained from Sigma. All other reagents were obtained from Merck.

Statistical Analysis. All results are expressed as means ± SD. For multiple comparisons, ANOVA was applied, followed by the Bonferroni correction. A *P* value of <0.05 was considered significant.

Results

Functional and Metabolic Effects of Authentic NO Solutions. Fig. 1 shows a representative experiment on the effects of intracoronary infusion of NO on the Mb and high-energy phosphate levels as measured with ^{1}H and ^{31}P NMR spectroscopy. In WT hearts, the MbO_2 signal at −2.7 ppm (26) remained unchanged up to [NO] = 1 μM. At [NO] ≥ 2.5 μM, spectra showed a decrease of the MbO_2 signal and concomitantly a new signal at −3.8 ppm became detectable. This signal could be unequivocally assigned to metMb by both control measurements of commercially available Mb and comparison with literature data (24, 27). At [NO] = 5 μM, approximately 50% of the MbO_2 was converted to

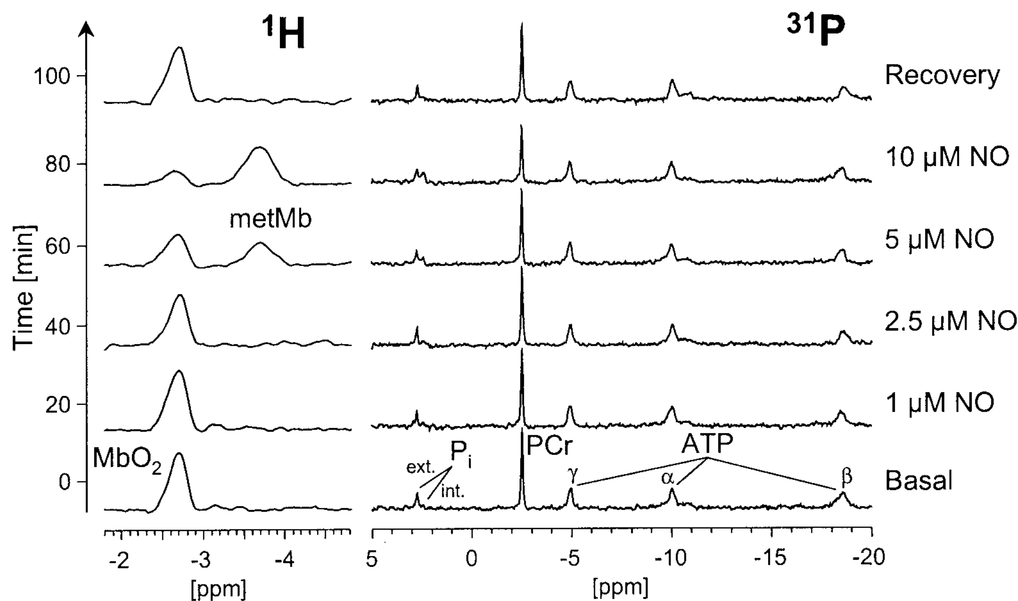

Fig. 1. ^{1}H (*Left*) and ^{31}P NMR (*Right*) spectra of WT hearts showing the effect of increasing NO concentration on cardiac Mb and energy status. Assignments: ATP, adenosine triphosphate (γ-, α-, and β-phosphorus); MbO_2, oxygenated myoglobin; metMb, metmyoglobin; PCr, phosphocreatine; P_i (ext.) and (int.), extracellular and intracellular inorganic phosphate, respectively.

metMb, reaching nearly 100% conversion at 25 μM NO (Fig. 2 *Top*). This reaction was reversible after cessation of the NO infusion (Fig. 1), reflecting the rapid regeneration of MbO_2 by metMb reductase activity in the WT (28, 29). It is noteworthy that the conversion of MbO_2 to metMb is already detectable at NO concentrations at which ^{31}P NMR spectra did not yet show any adverse effect on cardiac energy status (Fig. 1, third trace from top).

The dose-dependent effects of NO on Mb, cardiac contractile force, and energetic parameters in WT and Mb-deficient hearts are summarized in Fig. 2. As can be seen, cardiac contractility progressively decreased at [NO] $>$ 100 nM, resulting in a 50% reduction of LVDP at the maximal [NO] applied. In the NO dose range of 1–5 μM, a significantly greater decrease in LVDP was observed in myo$^{-/-}$ as compared with WT hearts, with most pronounced differences at 5 μM NO (62.4 $\pm$ 6.4 vs. 74.0 $\pm$ 3.9% of control, n = 8 each, $P < 0.01$; Fig. 2 *Middle*). Cardiodepression was accompanied by a severe deterioration of energy status, which was indicated by a reduction of the myocardial PCr content by more than 50% of the respective control level at [NO] = 25 μM (Fig. 1, bottom). At [NO] = 5 μM, impairment of energy homeostasis again was greater in the myo$^{-/-}$ group, as reflected by significantly increased myocardial ADP in myo$^{-/-}$ compared with WT hearts (42.3 $\pm$ 9.5 vs. 30.5 $\pm$ 8.4 μM, n = 8 each, $P < 0.05$; Fig. 1, bottom; calculated from ^{31}P NMR data). It should be noted that the differences in contractility and energetics between WT and myo$^{-/-}$ hearts became significant only in the NO concentration range that caused the formation of metMb (shaded area in Fig. 2). When [NO] $>$ 10 μM was applied, the differences with regard to LVDP and energetic parameters were blurred.

At [NO] lower than 1 μM we observed a significant enhancement of NO-induced vasodilatation corresponding to a left shift of the NO dose–response curve in myo$^{-/-}$ compared with WT hearts (data not shown). In this concentration range cardiac function and energy status were not substantially affected in both groups. Control experiments with the NO-independent vasodilator adenosine (5–500 nM) revealed no differences between WT and myo$^{-/-}$ hearts (n = 8 each, data not shown) for all parameters measured; even at maximal vasodilatation no changes in energy status or MbO_2 concentration were observed.

Role of Endogenous NO Synthesis. To assess whether endogenously synthesized NO contributes differently to the setting of basal vascular tone in WT and myo$^{-/-}$ hearts, experiments with the NO synthase inhibitors L-NMMA and ETU were undertaken. As shown in Fig. 3, there was no difference in coronary perfusion pressure under basal conditions; however, both inhibitors induced a significantly stronger vasoconstriction in the myo$^{-/-}$ as compared with the WT group, resulting in an increase of perfusion pressure from 100 mmHg to approximately 150 and 125 mmHg (n = 6 each, $P < 0.01$), respectively. Cardiac function and energy status remained unchanged in both groups and no effect on MbO_2 concentration could be detected in WT hearts (data not shown).

To increase endogenous NO release, hearts were stimulated by application of Bk. We found that the Mb-deficient group displayed a more sensitive response (Fig. 4), that was similar to previous results. The Bk-induced vasodilatation was more pronounced in myo$^{-/-}$ than in WT hearts, resulting in a drop of coronary perfusion pressure from 100 mmHg to 46.7 $\pm$ 5.1 and 64.1 $\pm$ 9.6 mmHg, respectively (n = 8 each, $P < 0.01$, at maximal [Bk]). Furthermore, at [Bk] $>$ 1 μM there was a perceptible LVDP decrease in myo$^{-/-}$ hearts only (78.3 $\pm$ 7.6% vs. 90.1 $\pm$ 4.9% of control, n = 8, $P < 0.01$; Fig. 4 *Lower*). ^{31}P NMR spectra did not reveal any changes in energy status (data not shown). However, we found significant differences in ^{1}H NMR spectra of WT hearts acquired within 4 min before, during, and after 1 μM Bk stimulation (Fig. 5). There was a small but significant decrease in the MbO_2 signal with the concomitant appearance of the metMb signal. This effect was rapidly reversed after the cessation of Bk infusion.

Discussion

This study reports a previously undescribed function of Mb: the reaction of MbO_2 with NO to metMb and nitrate as a major mechanism of attenuating intracellular NO bioactivity in cardiac muscle. This mechanism is physiologically relevant, because not only did NO infusion result in enhanced formation of metMb,

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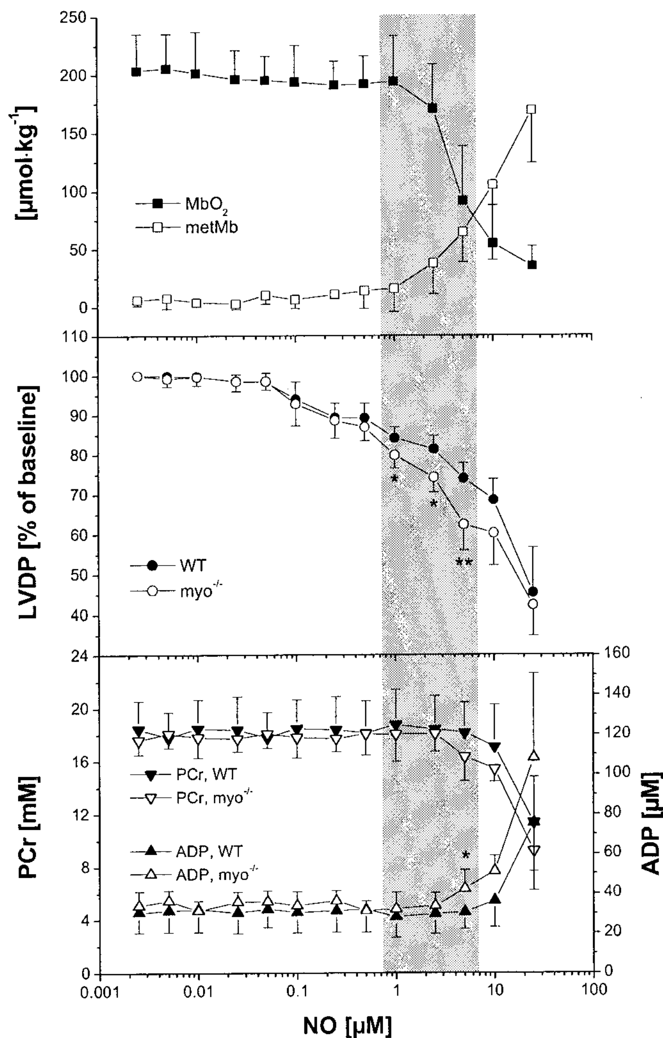

Fig. 2. Analysis of cardiac Mb (*Top*), function (*Middle*), and energetics (*Bottom*) of isolated perfused hearts with increasing NO concentration. Symbols show means ± SD for n = 8 hearts; * = $P < 0.05$; ** = $P < 0.01$; For abbreviations see Fig. 1. The shaded area emphasizes the association of metMb formation in WT hearts and the more pronounced impairment of cardiac function and energy status in Mb-deficient as compared with WT hearts.

but stimulation of endogenous NO release by Bk did also. Hearts from Mb-deficient (myo$^{-/-}$) mice were consistently more sensitive to endogenously formed and exogenously applied NO; the vasodilatory response and the depression of contractile as well as energetic parameters were more pronounced when compared with WT hearts.

^{1}H NMR spectroscopy enabled us to directly monitor the NO-induced formation of metMb from MbO_2 in the beating heart. Although this very fast reaction (K = 3.7×10^7 $M^{-1} \cdot s^{-1}$) is well known to occur *in vitro* (30), no conclusive experimental evidence existed until now that this reaction has functional relevance in cardiac NO homeostasis *in vivo*. After cessation of NO infusion or Bk stimulation, we found MbO_2 is rapidly regenerated, reflecting the presence of cardiac metMb reductase (28, 29). Obviously, the metMb signal becomes detectable only when metMb production from MbO_2 and NO exceeds the capacity of metMb reductase to reconvert metMb into Mb.

As shown in Fig. 6, the formation of metMb can occur via two different reactions: (*i*) directly by the interaction of NO and MbO_2 or (*ii*) by nitrosylation of deoxygenated Mb, yielding MbNO as intermediate and its subsequent reaction with O_2. On the basis of our data, it cannot be decided by which of these pathways metMb is primarily formed. The rate of NO binding to deoxygenated Mb (K = 1.7×10^7 $M^{-1} \cdot s^{-1}$; ref. 31) is in the same order of magnitude as the conversion of MbO_2 to metMb by reaction with NO (K = 3.7×10^7 $M^{-1} \cdot s^{-1}$; ref. 30). However, because MbO_2 is the dominant form under fully oxygenated conditions (3), the probability of the encounter of NO and MbO_2 by far exceeds that of NO and deoxygenated Mb. Under *in situ* conditions with partial deoxygenation of MbO_2, the formation of MbNO might be of greater relevance. Especially in the vicinity of the mitochondria, where the concentration of deoxygenated

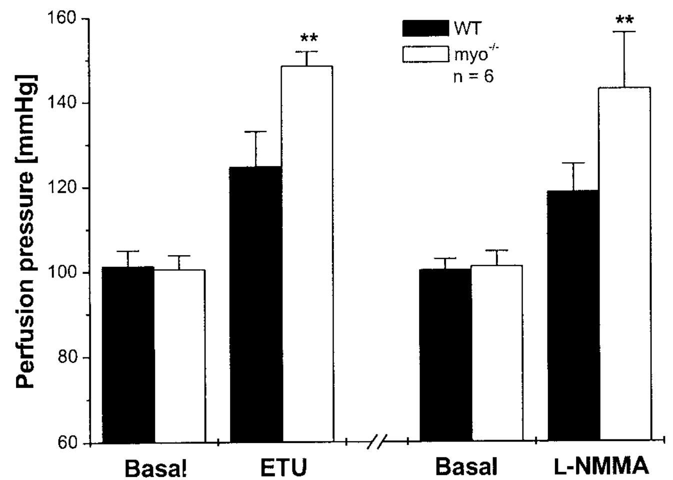

Fig. 3. Effect of the NO synthase inhibitors ETU (50 µM) and L-NMMA (100 µM) on coronary perfusion pressure of constant-flow-perfused hearts of WT and myo$^{-/-}$ mice. Symbols show means ± SD for n = 6 hearts; ** = $P < 0.01$.

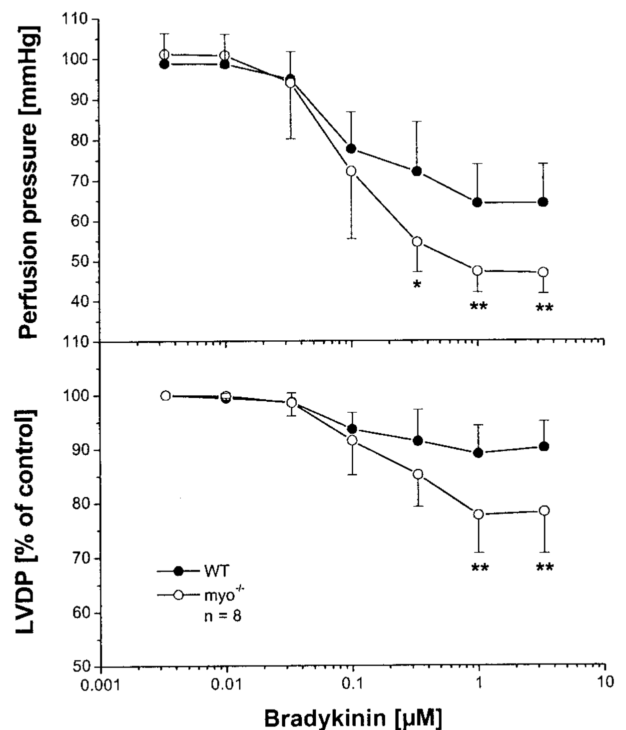

Fig. 4. Analysis of coronary perfusion pressure (*Upper*) and cardiac function (*Lower*) of constant flow perfused hearts under Bk stimulation. Symbols show means ± SD for n = 8 hearts; * = $P < 0.05$; ** = $P < 0.01$.

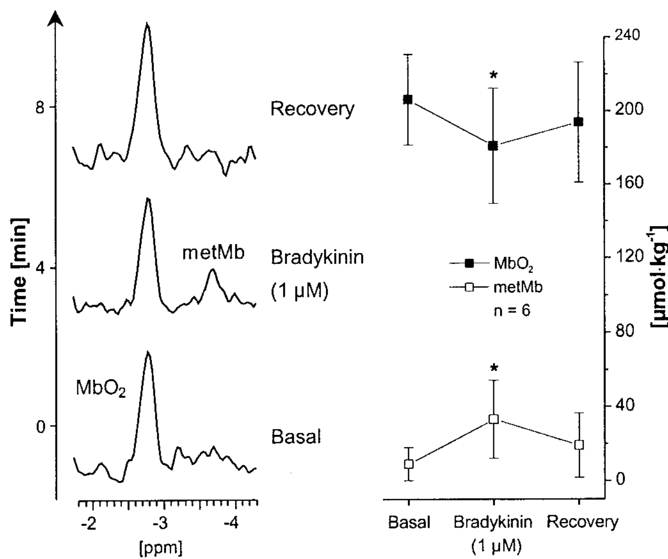

Fig. 5. ^{1}H NMR spectra (*Left*, 4 min accumulation time) of WT hearts showing the effect of Bk stimulation on cardiac Mb. Symbols on the Right show means ± SD for $n = 6$ hearts, * = $P < 0.05$.

Mb should be higher than in proximity to the capillaries, more MbNO is expected to be formed (Fig. 6). In the presence of O_2, MbNO is preferentially converted to metMb ($K = 1 \times 10^{-3} \cdot s^{-1}$; ref. 20) being kinetically favored over the dissociation into Mb + NO ($K = 1 \times 10^{-4} \cdot s^{-1}$; ref. 20), the latter taking place at a rate almost 10^4-fold slower than the corresponding dissociation of MbO_2 ($K = 10\ s^{-1}$; ref. 20). Furthermore, it is noteworthy that the reaction of NO with the O_2 bound to the heme group was estimated to be at least 20-fold faster compared with both the rate of NO reaction with free O_2 ($K = 1.6 \times 10^6\ M^{-2} \cdot s^{-1}$; ref. 32) and to O_2 displacement by NO (30).

Considering the large amount of Mb in the heart (approx. 200 μmol/kg wet weight; refs. 7 and 14), both pathways—the direct reaction of MbO_2 with NO and the intermediate formation of MbNO—can account for a substantial NO breakdown in cardiomyocytes. Thus, Mb may be regarded as a molecular scavenger protecting the lower concentrated cytochromes ($\approx$30 μmol/kg wet weight; refs. 33 and 34) against transient increases in cytosolic NO brought about by stimulation of NO synthase located in the endothelium, the sarcoplasmatic reticulum, and the mitochondria (35–38). Support for this hypothesis can be derived from comparison of the ^{1}H and ^{31}P NMR spectroscopic data presented in this study, because the conversion of MbO_2 to metMb is already detectable at NO concentrations at which ^{31}P NMR spectra do not indicate any adverse effect on cardiac energy status.

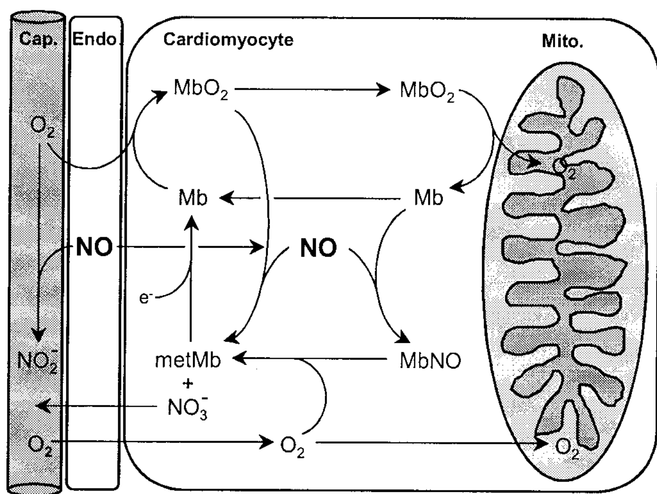

Fig. 6. Schematic drawing summarizing possible interactions of the different Mb compounds with O_2 and NO. For a detailed discussion refer to the text. Cap., capillary; Endo., endothelium; Mito., mitochondrion.

NO scavenging by Mb in cardiac muscle has important functional consequences: in hearts lacking Mb, changes in NO concentration have a much larger impact on the maintenance of vascular tone and cardiac function, as well as on energetic parameters compared with WT hearts. The lower sensitivity of Mb-containing WT hearts to alterations in NO concentration fits well with the assumption that in the presence of Mb, a continuous breakdown of NO takes place by reaction of MbO_2 with NO to metMb + NO_3^-, thereby reducing the effective cytosolic NO concentration. The recovery of MbO_2 and its availability for another NO degradation cycle is ensured by regeneration of metMb by metMb reductase to Mb and subsequent association with O_2 (cf. Fig. 6). Thus, the inhibition of NO synthase caused a more pronounced vasoconstriction in Mb-deficient hearts, which was most likely caused by the increased contribution of NO to the setting of basal vascular tone, in turn caused by the lower rate of NO bioinactivation. Along the same line, maximally stimulated endogenous NO release by Bk resulted in a more pronounced vasodilatation and, additionally, resulted in a more substantial depression of cardiac function in myo$^{-/-}$ compared with WT hearts. Similar effects were achieved by infusion of NO solutions > 1 μM. It should be noted that cardiac function was significantly better maintained in WT than myo$^{-/-}$ hearts in the NO dose range in which metMb becomes detectable (1–5 μM NO). These findings very likely reflect a reduced NO interference with heme enzymes of the mitochondrial electron-transport chain as well as creatine kinase and guanylate cyclase, all of which have been described as contributing to NO-mediated cardiodepression (39–43). However, when concentrations of infused NO exceeded 10 μM, functional and energetic parameters in WT and Mb-deficient hearts converged to similar levels. At such high NO levels, the protective capacity of Mb in WT hearts is exhausted, as indicated by the almost complete conversion of MbO_2 to metMb, resulting in a depression of myocardial energy generation and induction of contractile dysfunction, as observed in the present and other studies (42, 44).

It is difficult to assess the effective NO concentration that cardiomyocytes are exposed to when hearts are perfused with authentic NO solutions. It is well known that a considerable amount of NO will be inactivated by reaction with buffer O_2 or scavenged along its route to the interstitial space (45). We have reported before (46) that during a single passage through the heart, more than 85% of the infused NO is converted to nitrite ions and the NO half-life is as short as 100 ms. In addition, because there is lateral diffusion of NO, there is most likely a steep concentration gradient from the sarcolemma to the mitochondria. Therefore, the given concentrations of the infused NO solutions can only be regarded as an upper estimate of the amount of NO acting on the cardiomyocytes.

Our data indicate that Mb is crucial to the inactivation of NO and substantially determines the dose–response curve of the NO effects on coronary blood flow and cardiac contractility. These results uncover an additional physiological function of Mb besides its known role in oxygen storage and delivery. In this context, Mb's function seems to be limited to NO degradation, whereas its molecular relative Hb interacts with NO homeostasis not only by oxidative inactivation of NO (17), but also through (*i*) binding of NO to deoxygenated heme moieties (47) and (*ii*) formation of *S*-nitrosothiols (18). However, the latter reactions

are rather unlikely for Mb because (*i*) the oxygen binding curve for Mb in comparison to Hb is both shifted to the left and much steeper, resulting in a substantially lower amount of deoxygenated Mb than Hb at the same oxygen partial pressure, and (*ii*) the sulfur-containing amino acid cysteine, the prerequisite for nitrosothiol formation (18), is nonexistent in almost all Mb species characterized to this day.

Considering the large amounts of Mb in red muscle [0.5 mmol/kg (7, 33)], Mb may be important for NO turnover of the entire organism. On this larger scale, the reaction of NO with Hb to metHb and nitrate is generally considered to be the main route of intravascular NO breakdown. However, the recent observation that at physiological NO concentrations the binding of NO to the minor population of Hb's deoxygenated hemes outweighs the inactivation of NO by reaction with oxygenated Hb to metHb (47) challenged this hypothesis, thus raising the question about which other mechanisms might additionally contribute to NO breakdown (48). Estimation of the total body Mb content reveals that the quantities of Mb and Hb are within the same range. Assuming skeletal muscle in humans makes up 40–45% of total body weight (70 kg), and skeletal muscle consists of approximately 2/3 red muscle, the total body content of Mb can be approximated to 200 g (10 mmol), compared to 750 g of Hb (12 mmol). Furthermore, rate constants for NO-mediated conversion of the oxygenated hemoproteins to their respective met-compounds are almost equal for Hb and Mb ($K = 3.4 \times 10^7$ vs. $3.7 \times 10^7\ M^{-1} \cdot s^{-1}$; refs. 30 and 49), suggesting that Mb may indeed contribute to whole body NO homeostasis. We therefore propose that Mb not only is a key element determining the magnitude of the NO response in muscle but also plays an important role in overall NO inactivation *in vivo*.

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