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Review

Keeping the heart in balance: the functional interactions of myoglobin with nitrogen oxides

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Summary

Myoglobin (Mb) is an important intracellular oxygen-binding hemoprotein found in the cytoplasm of skeletal and cardiac muscle tissue playing a well-known role in O_2 storage and delivery. Within the last decade the knowledge about Mb's function has been considerably extended by the generation of myoglobin-deficient (myo$^{-/-}$) mice, which for the first time enabled the analysis of Mb's role in physiology without pharmacological intervention. Utilizing the myo$^{-/-}$ mice, it has been demonstrated that beyond its function in O_2 supply Mb substantially contributes to nitric oxide (NO) homeostasis in the heart. By a dynamic cycle, in which a decrease in tissue O_2 tension drives the conversion of Mb from being a NO scavenger under normoxia to a NO producer during hypoxia, mitochondrial respiration is reversibly adapted to the intracellular O_2 tension. Therefore, Mb may act as an important O_2 sensor through which NO can regulate muscle energetics and function. As Mb is widespread throughout the fauna, the diverse oxygen-dependent interactions between Mb and nitrogen oxides may not only be of relevance for mammals but also for other vertebrates as evidenced by comparable phenotypes of 'artificial' (myo$^{-/-}$ mice) and 'natural' Mb knockouts (icefish and amphibians). In conclusion, it seems likely that Mb's multifunctional properties create an environment characterized by a tightly adapted aerobic mitochondrial respiration and low levels of free radicals, and thus serve an essential and beneficial role within the myocardium, which appears to be functionally important over a wide range of species.

Key words: myoglobin, heart, oxygen consumption, nitric oxide, nitrite.

Outside the box

Interactions of myoglobin (Mb) and nitrogen oxides (NO_x) have been used for centuries as a means of meat preservation even though there was no concrete knowledge of the underlying biochemical processes. Although the origin of food curing is lost in history, it seems clear that preservation of meat and fish was practised as early as 3000 BC in Mesopotamia, and by the time of Homer (900 BC) the curing of meat with salt containing nitrates was already an old technique (Binkerd and Kolari, 1975). However, the discovery of Mb and nitrite as active components in the curing process of meat dates back to the late 19th century (Pegg and Shahidi, 2000). With modern spectroscopic methods, the principal pigment formed during nitrite curing has been characterized as a pentacoordinated nitrosylmyoglobin (Bonnett et al., 1980). Because the bond between nitric oxide (NO) and the ferrous iron is extremely strong (Cooper, 1999), the heme iron is protected from further oxidation. The formed nitrosylmyoglobin (MbNO) gives rise to a reddish-brown color of cured meat, which turns to the characteristic pink color when cooked. Of note, MbNO has also been identified in raw dry-cured ham specialities originating from the Mediterranean regions of Europe like Jamón Serrano (Møller et al., 2003).

From trophism to cardiovascular physiology

While at the food processing and also the subsequent hedonistic level the interactions between Mb and NO_x obviously proved to be valuable since the mists of ancient time, at the physiological level their functional roles within the cardiovascular system have been more or less neglected up until the beginning of the last decade. However, from *in vitro* studies it was well known that under normal O_2 tension an extremely rapid oxidation of NO by oxygenated Mb (MbO_2) occurs with the formation of nitrate and metmyoglobin (metMb) having the heme iron in the ferric state (Doyle and Hoekstra, 1981). These findings were already utilized in the early days of NO research, in that MbO_2 and also its molecular relative, oxygenated Hb (HbO_2), were used to inactivate NO released from cells, and thereby to provide evidence of NO-mediated processes (Ignarro et al., 1987). Nevertheless, it was not until 1994 when Lancaster suggested there was a possible physiological role for this reaction in the heart (Lancaster, 1994). In this context, he pointed out that this would also imply the activity of metMb reductase to regenerate the ferrous form of Mb. However, at the same time Reutov proposed that under hypoxic conditions, when deoxygenated Mb (deoxyMb) becomes the predominant form, the interaction of deoxyMb with nitrite might be of relevance for NO generation in tissues suffering from limited O_2 supply (Reutov et al., 1994).

With the spectroscopic detection of MbNO during ischemia in isolated rat hearts two years later (Konorev et al., 1996), it finally became clear that also in the heart, at least under pathophysiological conditions, the same species could be involved as during the curing process (i.e. MbNO). However, the functional role of the reactions between Mb and NO_x was experimentally resolved not until the advent of genetically modified mice. Only the use of Mb-deficient

(myo$^{-/-}$) mice, which were independently created by Garry and coworkers (Garry et al., 1998) and our group (Gödecke et al., 1999), enabled the identification of these pathways to be physiologically relevant. For the first time, these mutants enabled (i) studies of Mb's role in cardiac physiology without pharmacological intervention, and (ii) acute inhibition of Mb [e.g. by the highly toxic carbon monoxide (CO)] in normal wild-type (WT) hearts with myo$^{-/-}$ hearts as adequate control.

Mb and free NO in the murine heart under physiological oxygen tension

Using ^{1}H nuclear magnetic resonance (NMR) spectroscopy we could indeed show that the same reactions previously observed *in vitro* also took place in the beating heart in that we were able to directly measure the NO-induced conversion of MbO_2 to metMb in WT hearts (Flögel et al., 2001). After cessation of the NO challenge, MbO_2 was found to be rapidly regenerated, suggesting the presence of a robust metMb reductase activity (Hagler et al., 1979; Livingston et al., 1985; Chung et al., 1996). Thus, the metMb signal became detectable only when cardiac metMb production from MbO_2 and NO exceeded the capacity of metMb reductase to reconvert metMb into Mb. Of note, under unstressed conditions the basal NO formation in the heart by the diverse endogenous NO synthases (NOS, see also below) did not give rise to any traceable metMb signal in the ^{1}H NMR spectra.

From a mechanistic point of view it is important to keep in mind, that the formation of metMb may occur not only directly by the interaction of NO and MbO_2 as shown in Fig. 1A (left) but also by nitrosylation of deoxyMb, yielding MbNO as an intermediate and its subsequent reaction with O_2. The rate of NO binding to deoxyMb is of the same order of magnitude as that of NO reacting with MbO_2 to generate metMb and nitrate [k=1.7×10^7 *vs* 3.7×10^{7}mol^{-1} s^{-1} (Hoshino et al., 1993; Doyle and Hoekstra, 1981)] but the oxidation of MbNO is rather slow, because it is limited by the rate of dissociation into Mb and NO as shown by the corresponding reaction of nitrosylhemoglobin (HbNO) and O_2 (Herold and Rock, 2005). As already mentioned above, the NO–iron bond in MbNO is extremely strong, so that the dissociation into Mb + NO takes place at a rate almost 10^4-fold slower than the corresponding dissociation of MbO_2 [k=1×10^{-4} *vs* 10 s^{-1} (Cooper, 1999)]. Thus, it seems unlikely that under well-oxygenated conditions NO-induced metMb formation takes place *via* MbNO as an intermediate. Nevertheless, under conditions with enhanced deoxygenation of MbO_2, the formation of MbNO will become of greater relevance (see also next section). Furthermore, in the vicinity of the mitochondria, where the concentration of deoxyMb should be higher than in proximity to the capillaries, more MbNO is expected to be formed (Fig. 1A, right). Interestingly, for its molecular relative HbNO, a transfer of heme-bound NO to the thiol group of a cysteine in Hb's β-subunit has been shown to occur, and the formed S-nitrosated Hb has been suggested to play a crucial role in NO storage and long-distance transport (Stamler et al., 1997). However, a similar mechanism is less likely at least for mammalian Mbs, where the sulfur-containing amino acid cysteine, the prerequisite for nitrosothiol formation, is rare.

The interaction of MbO_2 with NO has important functional consequences in cardiac muscle. In hearts lacking Mb, changes in NO concentration have a much larger impact on the maintenance of vascular tone, cardiac function and energetic parameters compared with WT hearts (Flögel et al., 2001). Interestingly, Brunori proposed at the same time that Mb may protect cellular respiration by scavenging NO under well-oxygenated conditions

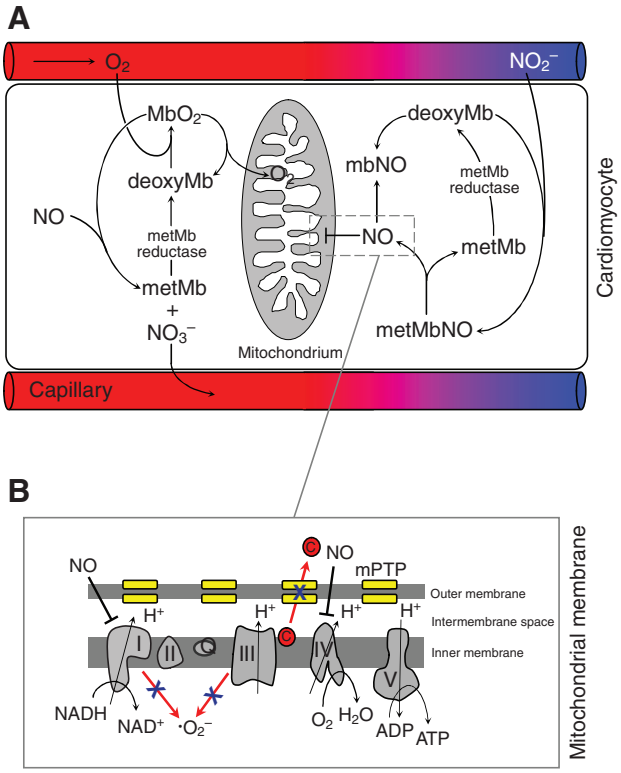

Fig. 1. Schematic drawing summarizing the distinct interactions of Mb and nitrogen oxides as a function of O_2 tension (A) and its functional consequences for mitochondrial respiration (B) in cardiomyocytes. Under fully oxygenated conditions MbO_2 acts mainly as a NO scavenger rapidly regenerated by the robust activity of metMb reductase, thereby acting as a molecular firewall, which protects mitochondrial respiration from NO inhibition (A left). With decreasing O_2 supply (A right), Mb gets increasingly deoxygenated uncovering its nitrite reductase activity, thereby releasing NO in proximity of the mitochondria which results in a reversible inhibition of cytochromes (B). As a consequence, myocardial O_2 consumption is reduced and cardiac contractility is dampened, representing an endogenous protecting mechanism for the heart under limited O_2 supply. For a detailed discussion please refer to the text. Abbreviations: I, II, III, IV, V represent complex I–V of the electron transport chain; ADP, adenosine diphosphate; ATP, adenosine triphosphate; C, cytochrome *c* oxidase; deoxyMb, deoxygenated myoglobin; NADH, nicotinamide adenine dinucleotide; MbNO, nitrosylmyoglobin; MbO_2, oxygenated myoglobin; metMb, metmyoglobin; metMbNO, nitrosylmetmyoglobin; mPTP, mitochondrial permeability transition pore; NO, nitric oxide; $\cdot O_2^-$, superoxide; Q, coenzyme Q.

(Brunori, 2001). Indeed, NO has been shown by several groups to be a potent but reversible inhibitor of cytochrome *c* oxidase, and nanomolar NO concentrations suffice to compete with O_2 for its binding site (Cleeter et al., 1994; Brown and Cooper, 1994; Bolaños et al., 1994). In support of Brunori's suggestions, we found that NO application over a substantial concentration range definitely resulted in a dose-dependent conversion of MbO_2 to metMb, while concomitantly acquired ^{31}P NMR spectra did not yet indicate any adverse effect on cardiac energy status (Flögel et al., 2001). Further substantiation of this hypothesis is derived from a transgenic (TG) mouse model with cardio-specific overexpression of inducible nitric oxide synthase (TGiNOS) where Mb's protective capacity on mitochondrial respiration was still sufficient even when cardiac NOS activities increased up to 300-fold over baseline. While TGiNOS mice showed no signs of heart failure or an obvious

pathological cardiac phenotype (Heger et al., 2002), overexpression of inducible NOS (iNOS) in a Mb-free background led to an impaired myocardial O_2 consumption and cardiac energy state, which was associated with cardiac hypertrophy, ventricular dilatation and the development of interstitial cardiac fibrosis (Gödecke et al., 2003; Flögel et al., 2007). Similarly, acute inactivation of Mb by CO (thereby also blocking the Mb-mediated detoxification of NO) in isolated hearts of TGiNOS mice resulted in an impaired heart function and a profound perturbation of cardiac energy state as shown by reduced phosphocreatine and ATP levels (Wunderlich et al., 2003). In line with these observations, Mammen et al. found that under hypoxic conditions myo$^{-/-}$ mice developed a systolic dysfunction, which could be inhibited by the NOS inhibitor nitro-L-arginine methyl ester (L-NAME) (Mammen et al., 2003).

As already pointed out by Lancaster, an appropriate reductive capacity to regenerate the ferrous form of Mb is required to constitute a protective cycle for the reaction of MbO_2 + NO to metMb + nitrate (Lancaster, 1994). In the well-oxygenated heart this high capacity of cardiac metMb reductase was demonstrated by us and others, because ^{1}H NMR spectroscopy gave no evidence for any metMb formation in normal mice or rat hearts under moderate NO challenges (Flögel et al., 2001; Kreutzer and Jue, 2004). Nevertheless, substantial functional differences were found between Mb-expressing and myo$^{-/-}$ hearts; action of both exogenously supplied NO and endogenously formed NO were significantly more effective in regulating coronary tone and myocardial contractility in myo$^{-/-}$ mice, indicating that Mb-free hearts are under a higher NO 'tone' than their WT counterparts. However, even when metMb became detectable under enhanced challenges, MbO_2 was quickly restored due to the strong reductase activity in the heart (Chung et al., 1996; Wunderlich et al., 2003; Flögel et al., 2001). Nonetheless, it should be noticed, that at NO doses sufficient to completely oxidize Mb to metMb the protective effect of Mb vanished. The same is true under *ex vivo* or *in vitro* conditions where only a limited reductive potential can be maintained (Gardner, 2005; Smagghe et al., 2008; Li et al., 2004).

Due to high concentrations of Mb within the mammalian heart [$\geq$200 μmol kg^{-1} wet mass (Wittenberg, 1970; Gödecke et al., 1999)], Mb can account for a substantial NO breakdown in cardiomyocytes. Thus, under conditions of proper O_2 supply Mb may be regarded as a molecular firewall (Fig. 1A, left), protecting cytochromes present at lower concentrations [<30 μmol kg^{-1} wet mass (Hickson, 1981; Balaban et al., 1996)] against transient increases in cytosolic NO brought about by stimulation of the diverse forms of NOS located in the endothelium, the sarcoplasmic reticulum and the mitochondria (Xu et al., 1999; Brahmajothi and Campbell, 1999; Bates et al., 1996; Giulivi et al., 1998). Interestingly, it has recently been shown that NO stimulates Mb gene and protein expression in vascular smooth muscle suggesting a feedback relationship between NO and Mb (Rayner et al., 2009) that regulates the concentration of the reactive signaling molecule NO [for a comprehensive review of the regulation of Mb expression refer to the paper by Kanatous et al. in this issue (Kanatous et al., 2010)]. The scavenging function of Mb may furthermore contribute to another important aspect of NO homeostasis; subcellular compartmentalization of the NOS isoforms enables distinct signaling pathways (Shaul, 2002) but due to the high diffusion capacity of NO, it remains widely unclear how a specific function can be exerted by different NOSs coexpressed within the same cell. In cardiac myocytes, endothelial NOS localized within caveolae of the sarcolemma has been shown to attenuate β-adrenergic stimulation (Barouch et al., 2002). However, neuronal NOS associated with the ryanodine receptor at the sarcoplasmic reticulum was reported to augment contractility (Barouch et al., 2002). Mb, with its high capacity to inactivate NO, most likely constitutes a cytoplasmic barrier that prevents spillover of NO from one compartment to others and therefore enables a specific localized action of NO released by the individual isoforms of NOS. Beneficial but also putative detrimental implications of Mb-mediated NO breakdown for a variety of human diseases are discussed in the paper by Hendgen-Cotta et al. in this issue (Hendgen-Cotta et al., 2010).

NO formation by Mb's nitrite reductase activity and its role in hypoxia and ischemia/reperfusion

In recent studies nitrite, the oxidation product of NO, has been shown to act as an endocrine storage pool of NO that can be activated along the physiological O_2 gradient to mediate a number of important responses, including hypoxic vasodilation (Cosby et al., 2003), cytoprotection during ischemia/reperfusion (Duranski et al., 2005), as well as NO-dependent and independent signaling (Bryan et al., 2005). In the circulation nitrite is converted to bioactive NO by deoxygenated hemoglobin (deoxyHb) in the red blood cells. The reduction of nitrite to NO is accompanied by the oxidation of deoxyHb resulting in the formation of metHb as given by nitrite + deoxyHb + $H^+ \rightarrow$ NO + metHb + OH^-. NO generated from this reaction may subsequently bind to a non-oxidized ferrous Hb to form HbNO (Gladwin et al., 2005). As shown for human Hb, these reactions are allosterically regulated by the structural conformation of Hb, in such a way that the maximal rate of nitrite reduction occurs at the transition of R-(oxy) to T-state (deoxy) around the P_{50} of Hb (Huang et al., 2005). The lower heme redox potential in the R-state oxygenated conformation of Hb is associated with an equilibrium distribution of heme electrons that thermodynamically favors the reduction of nitrite.

As Mb possesses a significant lower heme redox potential (i.e. a higher tendency to donate electrons) than R-state Hb (Spencer et al., 2000), we hypothesized that Mb in the heart – in analogy to Hb in the circulation – may also exert nitrite reductase activity when O_2 tension decreases and deoxyMb becomes the predominant form. The physiological relevance of this mechanism has to be considered in the context of hypoxic signaling within the tissue. O_2 is a requisite substrate for the production of NO by NOS with a Michaelis–Menton constant (K_m) of approximately 100 μmol l^{-1}. As O_2 supply is reduced and becomes limiting for NOS-dependent NO formation, the nitrite reservoir may be used for formation of NO by reaction with deoxyMb (Fig. 1A right). This would ensure a constant supply of NO almost independent of the tissue's O_2 level. Because Mb, similar to Hb, must be at least partially deoxygenated to act as a nitrite reductase, this reaction pathway becomes increasingly more favoured when the O_2 level falls below the physiological P_{50} of Mb [2–4 mmHg (1 mmHg≈133 Pa) (Antonini and Brunori, 1971)]. Since it has been shown that the reaction accelerates as tissue pH drops (Shiva et al., 2007a), Mb-dependent reduction of nitrite should further be enhanced under ischemia. As O_2 concentration within the subendocardium of the mammalian heart decreases to 4 mmHg during ventricular systole and to 2–5 mmHg in skeletal muscle during exercise or ischemic stress (Lösse et al., 1975), Mb-mediated formation of NO from nitrite might especially be of physiological relevance under these conditions.

Initial *in vitro* studies have revealed that deoxyMb reduces nitrite and generates NO by the analogue reaction as deoxyHb (see above and Fig. 1A right) but at a faster rate (Shiva et al., 2007a). As intermediate metMbNO is formed, which dissociates into metMb + NO at a rate comparable with the corresponding dissociation of MbO_2 [k=16 *vs* 10 s^{-1} (Wanat et al., 2002; Cooper, 1999)]. The bioactivity of the released NO should be sustained as long as it does not encounter another deoxyMb with formation of the very stable MbNO [$k=1\times10^{-4}$ s^{-1} (Cooper, 1999)]. Of note, the regeneration of the ferrous form of Mb by metMb reductase again is a prerequisite that these reactions may constitute an utile cycle. In rat heart homogenates containing both Mb and mitochondria, addition of nitrite in presence of succinate indeed resulted in NO generation and a modulation of mitochondrial respiration, strongly suggesting an NO release in proximity to mitochondria by Mb's reductase activity (Shiva et al., 2007a). To characterize the functional role of this reductase activity under *in vivo* conditions, we compared the effects of exogenously applied nitrite under both moderate hypoxia and severe ischemia in WT and myo$^{-/-}$ hearts.

By reducing Mb's oxygenation to approximately 50% we could show that a dynamic cycle exists in which a decrease in tissue O_2 tension drives the conversion of Mb from being a NO scavenger under normoxia to a NO producer during hypoxia. The NO generated by reaction of deoxyMb with nitrite interacted in a reversible manner with myocytic cytochromes and led to a downregulation of oxidative phosphorylation as detected by ^{31}P NMR spectroscopy – a similar decrease was not observed in mice lacking Mb. As a consequence, myocardial O_2 consumption was reduced and cardiac contractility dampened in WT mice (Rassaf et al., 2007). Interestingly, the impact of nitrite infusion under hypoxic conditions strikingly resembles a well-known adaptation process referred to as short-term hibernation (Heusch et al., 1996): When coronary blood supply is critically reduced, cardiac function and energy metabolism are actively downregulated – this 'perfusion–contraction matching' is a unique feature of the heart (Ross, 1991), which was up to now neither related to Mb nor to nitrite. Because contractile function of the ischemic region rapidly decreases resulting in a reduction in O_2 consumption, this attenuates the drop in high-energy phosphates and over time can even restore myocardial energy balance. Obviously, during nitrite infusion a scenario is observed which is strongly reminiscent of the specifics described for acute hibernation; a new steady state for O_2 consumption has been established and high-energy phosphate levels are maintained at lower steady-state levels. Therefore, the partial pressure of O_2 (P_{O_2})-dependent non-enzymatic formation of NO by reaction of Mb with nitrite may represent an important causal factor of short-term hibernation during periods of limited O_2 supply.

Under more severe conditions than hypoxia like myocardial infarction, blood flow to the myocardium is strongly impaired leading to a critical imbalance between O_2 demand and supply of the heart. Experimental studies have demonstrated that the required reperfusion, in particular the reoxygenation of ischemic myocardium generates reactive oxygen species (ROS), which trigger cellular injury (Yellon and Hausenloy, 2007). The detrimental effects of myocardial reperfusion occur within its initial phase, and enhanced ROS release has been established as a central mechanism of the early reperfusion injury. In cardiomyocytes especially the large amounts of mitochondria represent important sources of ROS production (Brookes and Darley-Usmar, 2002). The ability of NO to regulate both mitochondrial respiration and ROS generation has been intensively investigated (Brown and Cooper, 1994). It is well known that NO reversibly binds to complex IV of cytochrome *c* oxidase to inhibit the electron transport chain and furthermore induces S-nitrosation of complex I (Fig. 1B) resulting in a more prolonged but still reversible repression of mitochondrial respiration (Shiva et al., 2007b). A deficiency of NO has been shown to exacerbate reperfusion injury (Elrod et al., 2008), which becomes pathophysiologically even more relevant in consideration of the fact that particularly during the very early phase of ischemia/reperfusion the generation of NO by NOS is still compromised because of its requirement for O_2.

Using the above mentioned myo$^{-/-}$ mouse model in the setting of myocardial ischemia/reperfusion, studies from our groups revealed that also under these conditions a substantial Mb-dependent reduction of nitrite to NO occurred (Hendgen-Cotta et al., 2008), which was associated with important functional consequences. A cytoprotective role of exogenously applied nitrite has only been determined in WT mice accompanied with a significant increase in NO generation and NO–heme concentration within the reperfusion phase. This phenomenon has specifically been observed in the initial period of reperfusion. Infusion of nanomolar concentrations of nitrite dramatically decreased myocardial infarct size by more than 60% and improved the recovery of left ventricular developed pressure during the first minutes of reperfusion. This was critically dependent on the presence of Mb, because in myo$^{-/-}$ mice, application of nitrite had no effects. Parallel studies in isolated mitochondria revealed that nitrite modulates mitochondrial function during anoxia and that this regulation also depends on the presence of Mb (Hendgen-Cotta et al., 2008).

Together these data provide insight into the underlying biochemical mechanisms of nitrite protection and prove the important role of Mb in myocardial ischemia/reperfusion injury. Consistent with the attenuated ROS formation and the decreased oxidative inactivation of aconitase in the presence of Mb and applied nitrite (Hendgen-Cotta et al., 2008), it is likely that nitrite exerts its cytoprotective effect *in vivo* by inhibition of mitochondrial electron transfer, particularly through complex I (Fig. 1B), which is known to be a primary site of both injury and ROS production after ischemia/reperfusion (Lesnefsky et al., 2001). As already mentioned above NO can S-nitrosate critical thiols in complex I, leading to an inhibition of its activity (Brown and Borutaite, 2004). Furthermore, opening of the mitochondrial permeability transition pores (mPTP, Fig. 1B) has been linked to the release of cytochrome *c* oxidase and is important in initiating the mitochondrial apoptotic pathway (Kim et al., 1998). Since it has recently been demonstrated that the NO formed by reaction of Mb with nitrite successfully inhibits the opening of mPTP during reperfusion and cytochrome *c* release (Shiva et al., 2007b), this might represent an additional pathway by which nitrite contributes to the protection of the heart after ischemia.

The nitrite reductase activity of deoxyMb and thus the non-enzymatic formation of NO may not only be relevant for the heart but it also could contribute to hypoxic vasodilation in skeletal muscle and vasculature where Mb is expressed. At rest, the fraction of deoxyMb in skeletal muscle of healthy humans was found to be 9% (Richardson et al., 2006) but on medium exercise the deoxyMb signal increases to about 50% (Richardson et al., 2001) rising up to 70% under maximal voluntary torque (Vanderthommen et al., 2003), corresponding to an intracellular P_{O_2} lower than 4 mmHg. Given this significant deoxygenation of Mb in exercising muscle, it is most likely that this has profoundly increased the Mb-mediated formation of NO. Because of the low diffusion distances between

Mb and mitochondria, this NO may be critically involved in the observed inhibition of oxidative phosphorylation, which is known to be extremely NO sensitive (Xu et al., 2005). Therefore, this mechanism may also play an important role in limiting muscle O_2 consumption and thus the exercise capacity of skeletal muscle.

Beyond the mammalian system

The role of Mb in NO and nitrite metabolism in ectotherm species has received less attention than in mammals, with the exception of some recent studies on fish, amphibian and turtle species discussed below. In contrast to mammals, ectotherms display a wide range of physiological and biochemical adaptations to face even extreme changes in environmental conditions. As notable examples of such extreme adaptations, the red-eared turtle (*Trachemys scripta*) and goldfish (*Carussus auratus*) are able to survive prolonged anoxia by a drastic downregulation of oxidative phosphorylation (Bickler and Buck, 2007) whereas Antarctic fish have adapted to live at constant subzero temperatures in the coldest marine environment on Earth (Sidell and O'Brien, 2006). For such reasons ectotherm species are suitable models for investigations on how interactions of Mb with NO or nitrite may regulate oxidative metabolism, particularly during conditions of low O_2 tension not attainable for mammalian species.

Earlier studies performed on ventricular heart strip preparations from sculpin have shown that Mb plays an important role in maintaining heart function during hypoxia (Canty and Driedzic, 1987). Furthermore, natural knockout species that do not express Mb, including frogs [*Xenopus* (Fuchs et al., 2006)] and few Antarctic icefish [all of which do not express Hb (Sidell and O'Brien, 2006)], represent an unique opportunity to pinpoint the role of Mb in the regulation of physiological NO levels. An interesting hypothesis proposes that an increased basal level of NO due to lack of the major NO scavengers Hb and Mb in blood and muscle may have prompted the development of the changes in the icefish's cardiovascular system (Sidell and O'Brien, 2006; Garofalo et al., 2009b), which include increased blood volume, heart enlargement, increase in number and diameters of capillaries, and increased number of mitochondria that to some extent are reminiscent of those found in myo$^{-/-}$ mice (Gödecke et al., 1999; Meeson et al., 2001). Of course, such adaptations maximize the diffusion gradient for O_2 in the absence of a blood O_2 carrier and have been proved successful because of the increase in O_2 solubilization and low metabolic rate at the low temperature of the Antarctic Ocean.

Among ectotherms, fish have been extensively studied and may contain several Mb isoforms, as found in carp (Fraser et al., 2006) and goldfish (Roesner et al., 2008) – two related hypoxia-tolerant species expressing Mb-1 at high levels in the heart and a tissue specific Mb-2 at very low levels in the brain. Interestingly, the Mb-2 isoform expressed in the brain is distinct from neuroglobin (Ngb), which has also been identified in the goldfish brain (Roesner et al., 2008). In both carp and goldfish, Mb-1 is upregulated in the heart during hypoxia whereas the levels of Mb-2 in the brain are not affected by exposure to hypoxia (Fraser et al., 2006; Roesner et al., 2008). Although the functional characterization of the two carp isoforms of Mb is still in progress, an enzymatic role of Mb-2 in NO metabolism rather than in O_2 transport appears plausible due to its low tissue concentration. Notably, fish appear unique among vertebrates as they may express multiple Hb and Mb isoforms.

Numerous studies suggest that NO is generated in non-mammalian cardiomyocytes. Histochemical methods using NADPH diaphorase and 4,5-diamino-fluorescein diacetate (DAF-2 DA) fluorescent probe have revealed NO generation in fish and turtle cardiac tissue, respectively (Garofalo et al., 2009a; Misfeldt et al., 2009). Although there exists some controversy regarding the origin of NO (Donald and Broughton, 2005), immunostaining data indicate that the fish heart may express endothelial NOS (eNOS) and iNOS isoforms (Tota et al., 2005; Amelio et al., 2008; Garofalo et al., 2009b). Moreover, as discussed below, effects of L-arginine (substrate for NOS), NOS inhibitors and NO scavengers are all consistent with their expected effects on NO metabolism (cf. Fig. 2).

The addition of L-arginine decreases O_2 consumption in turtle myocardial preparations, particularly during hypoxia (Misfeldt et al., 2009), indicating that NOS-derived NO decreases the rate of respiration, most likely by interaction with cytochrome *c* oxidase as known for mammals (Fig. 1B). As force generation remains unaffected by L-arginine, this produces an enhanced work

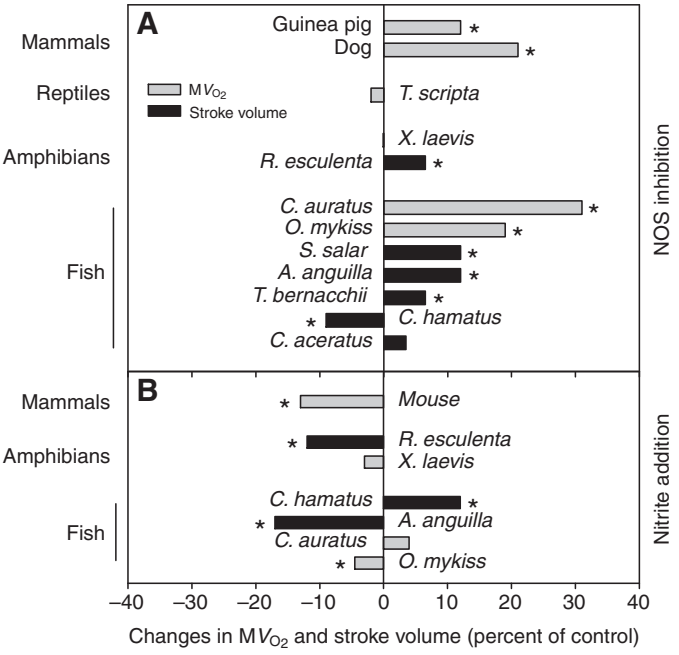

Fig. 2. Effects of nitric oxide synthase (NOS) inhibition (A) and added nitrite (B) on myocardial O_2 consumption ($M\dot{V}_{O_2}$, gray bars) and stroke volume (black bars) measured in vertebrates. (A) Values given are relative changes *vs* control (in %) after treatment with NOS inhibitors for guinea pig [50 mmol l^{-1} L-NNA (Shen et al., 2001)]; dog [150 g min^{-1} L-NAME (Setty et al., 2001)]; freshwater turtle *Trachemys scripta* [1 mmol l^{-1} ADMA (Misfeldt et al., 2009)]; clawed frog *Xenopus laevis* [150 mmol l^{-1} ADMA (C. L. Pedersen, H. Gesser and A.F., unpublished)]; common European frog *Rana esculenta* [100 mmol l^{-1} L-NMMA (Gattuso et al., 1999)]; goldfish *Carassius auratus* and trout *Oncorhynchus mykiss* [150 mmol l^{-1} ADMA (Pedersen et al., 2010)]; salmon *Salmo salar* [10 mmol l^{-1} L-NMMA (Gattuso et al., 2002)]; eel *Anguilla anguilla* [10 mmol l^{-1} L-NIO (Imbrogno et al., 2001)]; Antarctic *Trematomus bernacchii* and icefish *Chionodracus hamatus* and *Chaenocephalus aceratus* [10 mmol l^{-1} L-NIO (Garofalo et al., 2009a)]. (B) Values shown are relative changes *vs* control (in %) after the addition of nitrite for mouse [100 mmol l^{-1} nitrite (Rassaf et al., 2007)]; *R. esculenta, C. hamatus* and *A. anguilla* [10 mmol l^{-1} nitrite (Cerra et al., 2009)]; *X. laevis* [100 mmol l^{-1} nitrite, 150 mmol l^{-1} ADMA (C. L. Pedersen, H. Gesser and A.F., unpublished)]; *C. auratus* (100 mmol l^{-1} nitrite, 150 mmol l^{-1} ADMA) and *O. mykiss* [13 mmol l^{-1} nitrite, 150 mmol l^{-1} ADMA (Pedersen et al., 2010)]. Significant difference from control is indicated (*P<0.05). L-NNA, nitro-L-arginine; L-NAME, nitro-L-arginine methyl ester; ADMA, asymmetric dimethylarginine; L-NMMA, monomethyl-L-arginine methyl ester; L-NIO, nitro-iminoethyl-L-ornithine.

efficiency (force to O_2 consumption ratio) (Misfeldt et al., 2009), which may be important to maintain cardiac function in spite of limited O_2 availability and contribute to the overall excellent hypoxia tolerance of this species. Conversely, blocking NOS activity with asymmetric dimethylarginine (ADMA) increases O_2 consumption in hypoxic trout and goldfish heart preparations and decreases myocardial efficiency, again showing a beneficial effect of physiological NO on heart function (Pedersen et al., 2010).

Interestingly, when Mb is not present, as in the heart of the icefish *C. aceratus* and of the frog *Rana esculenta*, NOS appears to be expressed at low levels and basal NO production is low (Garofalo et al., 2009a; Cerra et al., 2009). NOS stimulation by externally added L-arginine has a larger effect on stroke volume in the Mb-null icefish *C. aceratus* than in the Mb-expressing *C. hamatus* whereas inhibition by nitro-iminoethyl-L-ornithine (L-NIO) in *C. aceratus* has virtually no effect, indicating a very low level of basal NO (Garofalo et al., 2009b). Similarly, small effects of L-arginine on stroke volume have been observed in the frog *R. esculenta* (Cerra et al., 2009). In another amphibian, *Xenopus*, O_2 consumption and force development of ventricular heart preparations are not affected when NOS is inhibited (ADMA), indicating low basal levels of NO also in this species (C. L. Pedersen, H. Gesser and A.F., unpublished results). Although a direct role of MbO_2 in NO scavenging *in vivo* has only been demonstrated in mammals, taken together the direct relationship between basal NO levels and Mb expression strongly suggests that a similar function must be present as well in the heart of ectotherm vertebrates.

The effect of nitrite in O_2 consumption and contractility of hearts from ectotherms has just begun to be investigated (cf. Fig. 2). In *Xenopus*, no effect of nitrite has been detected on either O_2 consumption or force development of hypoxic heart preparations (C. L. Pedersen, H. Gesser and A.F., unpublished), consistent with the absence of Mb that could function as a nitrite reductase in this species. Other studies on perfused eel, icefish and frog hearts (Cerra et al., 2009) showed dose-dependent changes in stroke volumes to nitrite levels similar to those seen when NOS activity is increased by L-arginine and thus consistent with generation of NO from nitrite. As so far no Mb isoform has been identified in amphibians and thus Mb is believed to be absent in these species, these results indicate that other pathways are responsible for the conversion of nitrite to NO in their heart. In the frog, such effects of nitrite are independent of NOS activity and disappear when the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) is added whereas in eel and icefish they depend on NOS activity but not on the addition of PTIO (Cerra et al., 2009). Whether Mb is involved in NO generation in eel and icefish hearts remains to be established, although it appears that NOS might represent a source of NO from added nitrite, as some studies have suggested (Vanin et al., 2007).

Under hypoxia, trout but not goldfish heart preparations show a small but significant decrease in O_2 consumption when nitrite is added, again consistent with NO generation (Pedersen et al., 2010). The diverse O_2 affinity of trout and goldfish Mbs is likely to account for the difference between the two species, as the lower O_2 affinity of Mb in trout, a hypoxia-intolerant species, would allow reduction by the deoxygenated heme at a higher O_2 tension than in goldfish, a species showing an exceptional tolerance to hypoxia. Thus, nitrite conversion to NO appears to occur at O_2 gradients that are physiologically relevant for each species and that are reflected by the oxygenation state of Mb. *In vitro* experiments under fully anaerobic conditions show a nitrite reductase activity of trout Mb comparable with that of mammalian Mbs whereas goldfish Mb reacts faster with nitrite (Pedersen et al., 2010). It is possible that such high rates may be of importance in effectively reducing mitochondrial respiration under conditions of severe hypoxia, as those experienced and extremely well tolerated by goldfish.

An overall comparison of the NO-mediated effects on myocardial O_2 consumption and stroke volume shows a remarkably good agreement in the responses observed throughout the species investigated (Fig. 2), suggesting that the interaction of Mb and NO_x may be part of a basal regulatory mechanism common for vertebrates. Interestingly, the relationship between NO, mitochondrial respiration and globins is not restricted to Mb and the heart of vertebrates. The existence of diverse globins found in bacteria and plants supports the view that NO interaction is an ancestral functions of all globins. In *Escherichia coli*, flavoHb provides an efficient mechanism to protect these bacteria from NO-induced toxicity (Gardner et al., 1998; Gardner, 2005). In baker's yeast the expression of a flavoHb is induced under conditions where the respiratory chain is blocked by, e.g. antimycin (Zhu and Riggs, 1992; Zhao et al., 1996). More recent results demonstrate that yeast Hb is also upregulated in response to NO and plays an important role in the defense against NO action (Sarver and DeRisi, 2005), indicating that preservation of mitochondrial function is one important evolutionary conserved task of globins. This may also apply to the recently discovered members of the globin family cytoglobin (Cygb) and Ngb, for which mechanistically unresolved protections in tumor expansion (Shivapurkar et al., 2008) and stroke (Sun et al., 2003), respectively, have been reported. As both Cygb and Ngb are expressed only at low levels [$<1\,mmol\,l^{-1}$ (Burmester et al., 2007)] in contrast to Mb ($\geq 200\,mmol\,l^{-1}$), it seems likely that their physiological significance is rather related to a regional control of NO fluxes than to an enhancement of O_2 transport into mitochondria (Brunori et al., 2005; Fago et al., 2004).

Conclusions

Beyond the initial finding of Mb's O_2-binding properties – set into a physiological context especially by the seminal work of Beatrice and Jonathan Wittenberg [see also the paper by Gros et al., in this issue (Gros et al., 2010)] – within the last decade, the field of Mb biology was particularly invigorated by the generation of $myo^{-/-}$ mutants. Based on recent work, the role of Mb in muscle physiology has been reassessed and its scope of function has been considerably extended beyond O_2 storage and delivery to include a crucial role in modulating the cytosolic levels of the major signaling molecule NO. Depending on its oxygenation state Mb may not only act as an NO scavenger but also *via* its nitrite reductase activity as an NO producer, thereby reversibly adapting mitochondrial respiration to the tissue's O_2 tension. Because Mb is not only expressed in mammals, the diverse interactions between Mb and NO_x are also likely to be of relevance for other vertebrates as evidenced from comparable phenotypes of 'artificial' ($myo^{-/-}$ mice) and 'natural' Mb knockouts (icefish and amphibians). Analysis of these $myo^{-/-}$ species revealed that the dynamic relationship between Mb and NO_x plays an important role both under physiological and pathological conditions, like hypoxia and ischemia/reperfusion. Future studies will provide insight to what extent compartmentalization of these distinct interactions is required (or exists) to avoid competing reactions, with respect to the fact that reaction kinetics of NO generation from nitrite and deoxyMb is rather slow compared with NO scavenging by MbO_2.

List of abbreviations

ADMA	asymmetric dimethylarginine
ADP	adenosine diphosphate
ATP	adenosine triphosphate
CO	carbon monoxide
Cygb	cytoglobin
DAF-2 DA	4,5-diamino-fluorescein diacetate
eNOS	endothelial NOS
Hb	hemoglobin
HbNO	nitrosylhemoglobin
HbO_2	oxygenated hemoglobin
iNOS	inducible NOS
Mb	myoglobin
MbNO	nitrosylmyoglobin
MbO_2	oxygenated myoglobin
metHb	methemoglobin
metMb	metmyoglobin
metMbNO	nitrosylmetmyoglobin
mPTP	mitochondrial permeability transition pore
$myo^{-/-}$	myoglobin deficient
NADPH	nicotinamide adenine dinucleotide phosphate
Ngb	neuroglobin
L-NAME	nitro-L-arginine methyl ester
L-NIO	nitro-iminoethyl-L-ornithine
L-NMMA	monomethyl-L-arginine methyl ester
L-NNA	nitro-L-arginine
$M\dot{V}_{O_2}$	myocardial oxygen consumption
NO	nitric oxide
NOS	nitric oxide synthase
NO_x	nitrogen oxides
PTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
ROS	reactive oxygen species
TG	transgene
WT	wild-type

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