Aus der Klinik für Kardiologie, Pneumologie und Angiologie
der Heinrich-Heine-Universität Düsseldorf
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Assessment of the Functional Properties of Human Myoglobin

Dissertation

Zur Erlangung des Grades eines Doktors der Medizin
der Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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2016

Als Inauguraldissertation gedruckt mit der Genehmigung der

Medizinischen Fakultät der Heinrich-Heine Universität Düsseldorf

gez.:

Dekan: Univ.-Prof. Dr. med. Joachim Windolf

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For my parents who always believed in me

Teile dieser Arbeit wurden veröffentlicht:

Peer-reviewed Originalarbeiten:

- Totzeck M, Hendgen-Cotta UB, Rammos C, **Petrescu AM**, Klare JP, Meyer C, Balzer J, Kelm M, Rassaf T. Assessment of the Functional Diversity of Human Myoglobin. *Nitric Oxide.* 2012 May; 26(4): 211-216.

Zusammenfassung

Myoglobin (Mb) gehört zu den meist-untersuchten Proteinen. Dabei sind die funktionellen Eigenschaften von Mb als myokardialer Sauerstoffspeicher weitestgehend bekannt. Neuere Arbeiten zeigen, dass Mb darüber hinaus eine bedeutende Rolle im Herz-Kreislaufsystem und im Besonderen in der Regulation des Stickstoffmonoxidsignalweges einnimmt. Unter normoxischen Bedingungen fängt Mb Stickstoffmonoxid (NO) ab. Eine Abnahme des Sauerstoffpartialdrucks erhöht die Konzentration von Desoxymoglobin (DesoxyMb), welches dann über seine Reduktasefunktion Nitrit zu bioaktivem NO reduzieren kann. Diese Eigenschaft von Mb wurde bisher für murines und equines Mb beschrieben. Es ist jedoch nicht bekannt, ob humanes Mb vergleichbare Eigenschaften besitzt.

In der vorliegenden Studie wurde mit Hilfe von Gasphasen-Chemilumineszens und UV-Vis-Spektrophotometrie demonstriert, dass zum einen humanes Myoglobin unter normoxischen Bedingungen als NO-Fänger agieren, als auch zum anderen unter hypoxischen Bedingungen als Nitrit-Reduktase fungieren kann. Weiterhin zeigen die vorliegenden Ergebnisse, dass die Reaktionsgeschwindigkeiten von humanem Mb als NO-Fänger und Nitrit-Reduktase höher im Vergleich zu denen des equinen Mb's sind.

Hämproteine können mit Nitrit als Oxidationsprodukt und Speicherpool von NO paramagnetische Komplexe bilden. Dies wurde zuvor bereits für Hämoglobin (Hb) in Erythrozyten nachgewiesen. Zur weiteren Charakterisierung der Reaktion von Nitrit mit Mb wurde Metmyoglobin (MetMb), in Analogie zu Methämoglobin (MetHb), in einer Reaktion mit Nitrit untersucht. Mittels Elektronenspinresonanz-Spektroskopie-Messung (EPR) konnte in der vorliegenden Arbeit erstmals der *in vitro* Nachweis in der Interaktion zwischen Nitrit und MetMb erbracht werden.

Zusammengefasst liefern die vorliegenden Ergebnisse Hinweise, dass die NO Bioverfügbarkeit im humanen Myokard durch Mb-abhängige Mechanismen beeinflusst werden kann. Auf Grundlage dieser Mechanismen können zukünftige Ansätze durchgeführt werden, die die Rolle von humanem Mb in der Modulation der NO-Nitrit-Homöostase näher untersuchen.

Abstract

Myoglobin (Mb) is one of the most studied proteins in natural sciences. Its functional role in the metabolism of oxygen (O_2) is widely known. More recently, it has been shown that Mb also plays an essential role in nitric oxide (NO) signaling in the cardiovascular system. Under normoxic conditions, Mb can act as a scavenger of bioactive NO. A decrease in O_2 tension, in turn, augments the pool of deoxygenated myoglobin (deoxyMb). DeoxyMb acts as a generator of NO *via* reduction of inorganic nitrite, a circulating and tissue storage form of NO. This property of Mb in reducing nitrite, with formation of NO and metmyoglobin (metMb), has previously been implemented for mouse and horse Mb. However, it has not been known whether human Mb also exhibits comparable functions.

Using gas-phase chemiluminescence and ultraviolet-visible spectrophotometry (UV-Vis spectrophotometry), this study demonstrates that human Mb acts as a potent scavenger of NO under normoxia and as a nitrite reductase under hypoxia. Moreover, the results suggest that both NO scavenging and nitrite reduction activity of Mb are more potent in human compared to horse Mb.

Nitrite can furthermore form paramagnetic complexes with heme proteins. This has previously been demonstrated for hemoglobin (Hb) in red blood cells. To further characterize the reaction of nitrite with Mb, it was investigated whether metMb analogous to methemoglobin (metHb) further interacts with nitrite, resulting in detectable nitrite-metMb formation. Using electron paramagnetic resonance (EPR) spectroscopy measurements, this study provides *in vitro* evidence of the interaction between nitrite and metMb.

These findings provide evidence of mechanisms that maintain the NO bioavailability in the human cardiac muscle. Such mechanisms may be an important experimental basis for future translational studies to investigate the role of Mb in modulating NO-nitrite homeostasis in humans.

Abbreviations list

°C	Degree Celsius
μM	Micromolar
Abs	Absorbance
a.u.	Absorbance units
AUC	Area under the curve
BSA	Bovine serum albumin
C	Concentration
cGMP	Guanosine 3'5'-cyclic monophosphate
CLD	Chemiluminescence detector
DeoxyHb	Deoxygenated hemoglobin
DeoxyMb	Deoxygenated myoglobin
DPI	Diphenyliodonium
DTPA	Diethylene triamine pentaacetic acid
e.g.	*Exempli gratia, lat.* (for example)
EPR spectroscopy	Electron paramagnetic resonance spectroscopy
Eqs.	Equations
Fe^{2+}/Fe^{3+}	Ferrous ion/ferric ion
Fig.	Figure
g	Gram
GTP	Guanosine triphosphate
H^+	Proton
H_2O	Water
HbCO	Carboxyhemoglobin
HbNO	Nitrosylhemoglobin
HbSNO	S-nitroso-hemoglobin
HCl	Hydrochloric acid
He	Helium
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

Hg	Mercury
$HgCl_2$	Mercuric chloride
I_2	Iod
I_3^-	Acidic tri-iodide
i.e.	*Id est, lat.* (that is)
K	Potassium
KI	Potassium iodide
l	Liter
L-NMMA	NG-monomethyl L- arginine
Mb	Myoglobin
MbCO	Carboxymyoglobin
MbNO	Nitrosylmyoglobin
MbSNO	S-nitroso-myoglobin
MetMb	Metmyoglobin
mg	Milligram
ml	Milliliter
mM	Millimolar
N_2O_3	Dinitrogen trioxide
nM	Nanomolar
NaOH	Sodium hydroxide
$Na^{15}NO_2$	Isotope-labeled sodium nitrite
NEM	N-Ethylmaleimide
NMR	Nuclear magnetic resonance spectroscopy
NO^-	Nitrosonium ion
NO•	Nitric oxide
NO_2^-/NO_3^-	Nitrite/Nitrate
NO_x	Nitrogen oxides (Here: nitrate and nitrite)
NOA	Nitric oxide analyzer
NOS	Nitric oxide synthase
nM	Nanomolar

O_2	Oxygen
OxyHb	Oxygenated hemoglobin
OxyMb	Oxygenated myoglobin
PBS	Phosphate buffered saline
ppb	Parts per billion
ppm	Parts per million
RNNO	N-nitroso-compounds
RNO	Sum of nitroso-compounds
ROS	Reactive oxygen species
RSNO	S-nitroso-compunds
RSSR	Di-sulphide compounds
RXNO	Nitroso compounds
s	Second
SD	Standard deviation
UV	Ultraviolet
V	Volume
Vis	Visible
WT	Wild-type
XOR	Xanthine oxidoreductase

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1. Introduction

Myoglobin (Mb) is a monomeric heme protein which can be found in the cytoplasm of cardiac and skeletal muscle cells. Its functional role in the metabolism of oxygen (O_2) is widely acknowledged.[1] More recently, however, it has been shown that Mb plays an essential role in regulating nitric oxide (NO) homeostasis in the cardiovascular system.[2]

NO is a ubiquitous signaling molecule of great interest due to its numerous biological activities both under physiologic and pathologic conditions.[3] In the cardiovascular system, NO plays several important roles ranging from modulation of vascular tone[4], platelet activity[5, 6], inflammatory[7] and proliferative processes[8] to the regulation of the excitation-contraction coupling.[9, 10] The primary endogenous source of NO are NO synthases (NOS).[11] NO is also synthesized by alternative NOS-independent pathways during hypoxia, when the production by NOS is impaired.[12] Under these circumstances, nitrite, an endogenous storage form of NO, is reduced to NO by various proteins (e.g. deoxyhemoglobin (deoxyHb), deoxymyoglobin (deoxyMb)).[12-16]

The following introduction will discuss the structure of Mb and its role in different biological processes. Subsequently, an overview of NO characteristics, including metabolism and functions, shall be presented. Finally, the interaction between NO species and Mb will be described both in physiological and in hypoxic conditions.

1.1 Structure and function of myoglobin

Structure of myoglobin

The structure of Mb has been known since 1957, when John Kendrew determined the composition of the protein by high-resolution X-ray crystallography.[17] Nuclear magnetic resonance spectroscopy (NMR spectroscopy) could further provide information about the protein's structure by using the properties of certain atomic

nuclei that depend on the local molecular environment, thus generating a three-dimensional molecular model.[18-20]

The molecular weight of Mb is approximately 17,600 daltons.[21] Its concentration in the heart is about 200-300 μmol·kg^{-1}, and may reach 400-500 μmol·kg^{-1} in skeletal striated muscles.[2, 21] Mb consists of a single polypeptide chain of about 153 amino-acids residues and has an iron-porphyrin complex (heme prosthetic group), which is situated in the center of the molecule.[2, 22, 23] The tertiary structure of Mb consists of eight α-helices, whose folding pattern forms a pocket for the heme group.[22] The heme group consists of a tetrapyrrole macrocycle that surrounds the central iron atom, which is attached to the protein moiety through a proximal histidine residue.[22] On the opposite face of the heme, another histidine residue exists. This plays an essential role in redox reactions and ligand binding (e.g. O_2, NO).[24, 25] The heme iron can exist either in a ferrous (Fe^{2+}) or a ferric (Fe^{3+}) state. In a ferrous state, the heme iron can bind O_2 yielding oxyMb. Therefore, Mb can occur either in its O_2-free form, deoxyMb, or in its oxygenated form called oxyMb.[21] *In vivo* experiments have suggested that under baseline conditions only 10% of the total cardiac Mb exists in a deoxygenated form.[26, 27]

Under certain conditions (e.g. reaction with NO), Mb - in its oxygenated form - is further oxidized to metmyoglobin (metMb). Having the heme iron in the ferric state, metMb is no longer able of binding O_2.[22, 28] The concentration of metMb in the muscle is low because of the existence of the metMb reductase, the enzyme that reduces Fe^{3+} back to Fe^{2+}.[29]

Function of myoglobin

Mb has various roles within the cytosol of vertebrate muscle tissues.[21, 30] Firstly, Mb is an O_2 storage protein.[21] This function is evident in aquatic mammals which are apneic for extended periods of time, as a result of prolonged dives beneath the surface. They are characterized by skeletal Mb concentrations as great as ten times those of terrestrial mammals.[2, 31] Through similar physiological mechanisms, mammals living at high altitudes demonstrate increased concentrations of Mb, as the protein develops within red muscle in response to mitochondrial demand for

O_2.[2, 30, 32] In the absence of O_2, oxyMb releases previously stored O_2 to sustain the aerobic metabolism in active muscles, thereby compensating for the atmospheric conditions.[2]

Secondly, Mb can facilitate the diffusion of O_2, thereby contributing to intracellular O_2 transport.[33] The total flux of O_2 is the sum of two complementary processes: a simple in-flow of dissolved O_2, and an Mb-facilitated O_2 flux[30]. The simple diffusion of O_2 depends upon the properties of a tissue cylinder, described by Krogh as a fragment of tissue supplied by a single capillary.[29] The O_2 diffusion mediated by Mb is due to the intracellular O_2 gradient.[1] This gradient is generated between the extracellular space and Mb, as well as from Mb to the inner mitochondrial membrane.[1] Therefore, Mb in close proximity to the cell membrane binds to the available O_2, and crosses the cytosol to discharge the gas molecule close to the mitochondria. In this subsequent deoxygenated state, the Mb then diffuses back towards the cell membrane.[2, 30]

In addition, Mb has been shown to act as a buffer of intracellular pO_2 in mammals.[33, 34] Mb is half saturated at a pO_2 of 2.3 mmHg; as the capillary pO_2 is around 20-25 mmHg, Mb occurs - under basal conditions - in a largely oxygenated state.[34] Mb therefore becomes a source of O_2 when muscle activity increases, allowing the intracellular concentration of O_2 to remain relatively constant despite activity-induced oscillations in O_2.[30] It also acts as a buffer for short tissue fluctuations of pO_2 during cardiac systole; the coronary arteries are fully patent during the diastolic phase of the cardiac cycle, thereby allowing restoration of the O_2 reserve.[30, 34]

Beyond the role played in the metabolism and transport of O_2, it has recently been demonstrated that Mb has additional properties as a modulator of NO bioavailability.[2, 14, 15, 25, 35] Mb performs a role in NO scavenging under normoxic conditions, protecting the heart from the detrimental effects of excessive NO, including apoptosis and inhibition of the mitochondrial respiratory chain.[2, 36, 37, 38] Under hypoxic conditions, Mb is able to reduce nitrite to NO, which functions as a downregulator of cardiac energy status. [14, 15, 35] This feature will be discussed further below.

1.2 Metabolism and biological functions of nitric oxide

Interaction of nitric oxide with biological targets

NO exists in a variety of forms: as a free radical (NO•), which can be reduced to nitroxyl anion (NO^-), or oxidized either to nitrosonium (NO^+) or more complex compounds ($NO_2^-/NO_3^-/N_2O_3$).[11] NO has a half-life of a few milliseconds[39] as a consequence of the rapid inactivation process in blood and tissues - either through oxidation[40], or interaction with different biological species such as heme moieties[2, 25, 41, 42], thiols[11, 39] and amines[43].

The major oxidation product of NO is nitrite, which can preserve the NO bioactivity for an extended period of time.[40] In the presence of O_2, nitrite will consecutively oxidize to nitrate, a more stable compound with a plasma half-life of approximately 6 hours.[44] However, the nitrite concentration in blood is a more accurate reflection of the plasma NO level synthesized from NOS[45] and it can be helpful in estimating the formation of NO, considering its short half-life.[39]

NO reacts with plasma proteins containing thiol groups (such as cysteine, glutathione, homocysteine) resulting in the formation of RSNOs (e.g. S-nitrosocysteine, S-nitrosoglutathione, S-nitrosoalbumin), which are stable compounds that act as a reservoir for NO. This process enables NO to be protected from oxidative inactivation while the overall bioavailability increases.[11, 39] Furthermore, RSNOs can act as donors of NO in a deoxygenated state.[39]

NO also reacts with amines, yielding endogenous nitrosamines (RNNOs). These compounds are not only related to inflammatory processes, they are also physiological constituents of human plasma with a function that has yet to be defined.[43]

In addition, NO can be inactivated *via* a reaction with oxygenated heme-proteins including oxyhemoglobin (oxyHb) and oxyMb.[2, 25, 41, 42] The reaction with oxyHb produces nitrate and metHb at a constant rate of 3-4 x 10^7 $M^{-1}*s^{-1}$.[46] In the deoxygenated state, Hb can also react with NO to form iron-nitrosyl-hemoglobin (HbNO), a reaction which can occur in any of the four heme-groups of the Hb

molecule. Subsequently, after reoxygenation of Hb and consecutive conformational changes, NO is transferred from the heme to the cysteine 93 of the β-chain to form S-nitroso-hemoglobin (HbSNO).[13, 39, 47] Experimental studies have demonstrated that - during exercise - after an infusion with nitrite, these compounds form across the vascular bed in less than 10 seconds.[13] The magnitude of these reactions is inversely correlated with the O_2 saturation of Hb.[13] It has been reported that Mb also plays an essential role in the inactivation of NO within the cardiovascular system, not only by directly oxidating NO, but also by binding NO yielding iron-nitrosyl-myoglobin (MbNO) or S-nitroso-myoglobin (MbSNO). [2, 48- 51]

As soon as NO is generated from L-arginine and O_2, a part of it acts in a paracrine manner *via* guanylyl cyclase, within the close vicinity of its production. This enzyme catalyzes the dephosphorylation of GTP to cGMP, which is a second messenger molecule responsible for various intracellular functions, ranging from vasodilation[52], prevention of blood clots formation[53], slowdown of atherosclerotic plaque accumulation[54], to neurotransmission[55] and immune response[56, 57]. However, the major part reacts with other molecules, resulting in more stable compounds (nitrite, nitrate, RSNOs, RNNOs).[11] Through this mechanism the bioavailability of NO within the bloodstream is conserved.[11]

Synthesis of nitric oxide

NO can be synthesized both enzymatically and non-enzymatically. The enzymatic pathway occurs in a variety of cell types and tissues within the vascular system, including vascular endothelium[11], red blood cells[58, 59] and neurons[60]. Three isoforms of NOS have been identified: the neuronal isoform (type I or nNOS), the inducible isoform (type II or iNOS) and the endothelial isoform (type III or eNOS).[57] All three produce NO and L-citrulline from L-arginine, with formation of N-hydroxy-L-arginine as an intermediate in NO synthesis. This has been defined as the “L-arginine - NO pathway”.[57] L-arginine is predominantly a urea cycle metabolite, but is also obtained from dietary intake.[61] NO is synthesized from L-arginine only in the presence of O_2, which acts as a co-factor.[11, 62]

It has been demonstrated that the highest expression of NOS can be found in neuronal cells, white blood cells, vascular endothelium, and cardiomyocytes.[63] It should be noted, however, that the distribution of each of the three isoforms differs from species to species.[63] Both eNOS and nNOS are constitutively expressed within cardiomyocytes, but higher levels of eNOS have been identified in rabbits than in rats; conversely, nNOS is more important in rats.[63] iNOS is the inducible isoform of NOS which can also be expressed in the myocardium, primarily, it is believed, in response to inflammation.[64] However, the role of iNOS in the heart remains a source of controversy.[36, 64-66] Among the important functions performed in transporting metabolic gases, red blood cells also express eNOS, thus contributing to the circulating NO pool, and performing a role in cardioprotection.[58, 59]

Under normoxic conditions, the production of NO in the myocardium is within the nanomolar range.[63, 67] It has been demonstrated that during hypoxia the concentration of NO and its oxidation products is increased.[63] As O_2 is required for enzymatic NO synthesis, this generation pathway should theoretically be reduced under hypoxic conditions.[60] While experimental studies have indicated that the increase in NO concentration during hypoxia may also be due to an activation of iNOS and eNOS[68], the majority of NO is derived from NOS-independent pathways.[12, 63, 69]

Therefore, despite NO being predominantly generated from NOS under normoxic conditions[11], the NOS-independent pathways work as a backup mechanism, providing sufficient quantities of NO under hypoxic conditions when conventional NO production is impaired.[12, 69, 70]

In addition, cardiac nitrite acts as an endogenous store of NO.[12, 35, 71] This is particularly important during ischemia, when pH falls to approximately 5.5.[12, 71] Several pathways for nitrite reduction to NO have been described, including heme-based nitrite-reductases (e.g. Hb, Mb).[13-15, 47, 69, 72] In a deoxygenated state, Hb can interact with nitrite to form NO and methemoglobin (metHb).[39] Nevertheless, it has been postulated that the escape of NO formed inside the red cells is limited due to the rapid scavenging process of the remaining oxyHb.[46, 73-75] This represents a

major challenge to the hypothesis that Hb, in the deoxygenated state, plays an essential role in the process of generating NO from nitrite.[47] However, it is believed that the nitrite-Hb reaction ends by generating dinitrogen trioxide (N_2O_3) - a stable, small, uncharged molecule - which can diffuse through the red cell membrane and than undergoes homolysis to NO_2^- and NO.[69, 73, 74, 76] The exact mechanism of this reaction is unclear, but it has been hypothesized that metHb has a high binding affinity for nitrite, which leads to the formation of metHb-nitrite. This subsequently reacts with NO to N_2O_3 (eqs. (1), (2), and (3)).[73]

$$(1)\ NO_2^- + \text{metHb} \rightarrow \text{metHb-}NO_2^-$$

$$(2)\ \text{metHb-}NO_2^- + NO \rightarrow N_2O_3 + Hb(Fe^{2+})$$

$$(3)\ N_2O_3 \rightarrow NO + NO_2^-$$

Then, NO can either provide direct effects or form S-nitrosothiols (RSNOs), which enables its transportation.[74, 77] Electron paramagnetic resonance (EPR) spectroscopy is a useful technique to show the affinity of metHb for nitrite.[73] Using EPR, it has been revealed that the affinity of metHb for nitrite increases when the ratio of nitrite to metHb decreases, and in acidic conditions. These studies showed that under certain conditions the affinity of metHb for nitrite is high, and may constitute a major pathway for NO-dependent signaling in hypoxia. Similarly, the bioactivity of NO after its production from nitrite through deoxyMb could be demonstrated in both smooth cells and cardiomyocytes.[14, 15, 78]

Neuroglobin and cytoglobin are additional heme proteins capable of mediating the production of NO from nitrite.[69, 79, 80] NO can also be generated from nitrite by components of the mitochondrial respiratory chain, as they can serve as electron carriers in a pH-dependent manner. In 1998, Reutov and Sorokina demonstrated that cytochrome c oxidase can reduce nitrite.[81] Its reductase function increases under hypoxic conditions and acidosis.[82]

It has been postulated that xanthine oxidoreductase (XOR), a member of the molybdenum oxotransferase enzyme family, may also reduce nitrite to NO.[12] However, whether XOR is responsible for the reduction of nitrite to NO in the ischemic heart is still unclear. [14, 83]

Nitrite can be also reduced to NO by reaction with protons (H^+); it is acidified to nitrous acid, which decomposes into NO and other nitrogen species (see (4)-(6)).[12, 39]

$$(4)\ NO_2^- + H^+ \rightarrow HNO_2$$

$$(5)\ 2HNO_2 \rightarrow N_2O_3 + H_2O$$

$$(6)\ N_2O_3 \rightarrow NO + NO_2$$

However, no more than 15-20% of the total available NO is generated from nitrite *via* this reaction.[84, 85] Therefore, there are several pathways capable of producing NO, depending upon the tissue O_2 tension. These include an enzymatic NOS-dependent pathway - which will dominate under normoxia - and a NOS-independent pathway, including heme-based nitrite reductases, which are responsible for the nitrite-dependent NO synthesis in hypoxic conditions.[40, 69]

The structural similarity between Hb and Mb has provided the scientific basis for exploring the functions of Mb.[86] It has been shown that the mechanism by which Mb leads to NO formation *via* nitrite bioactivation is similar to that of Hb, and plays a central role in both cardiomyocytes and smooth muscle cells under hypoxic conditions.[14, 78] While horse and mouse Mb have been already described to reduce nitrite under these conditions[14], it is as yet unclear whether human Mb also exerts a nitrite reductase activity.

1.3 Function of nitric oxide in the heart

It is already established that NO, both endogenous and exogenous, plays an important role in cardioprotection.[12, 15] NO exerts some of its most important effects within the myocardium, as a mediator of ischemia/reperfusion (I/R) injury[15], and regulation of cardiac contraction.[9, 64, 87, 88] These will be discussed at length in the following section.

Coronary heart disease is one of the most common causes of death and chronic disability worldwide. [89] Timely reperfusion in acute myocardial infarction has been demonstrated to significantly reduce morbidity and mortality.[89] This process has, however, been noted to cause additional cellular damage by inducing the death of cardiomyocytes (so-called "myocardial ischemia/reperfusion injury").[89] The restoration of blood flow is vital to ensure maximal tissue survival, but the consequent production of reactive oxygen species (ROS) results in inflammation and oxidative damage. Finally, this process leads to necrosis and programmed cell death (apoptosis).[90, 91] NO plays a key role in ischemia/reperfusion (I/R) injury. [49, 92, 93] It can inhibit the O_2-binding center of the complex IV (cytochrome oxidase) in a reversible manner, as well as the O_2-binding center of the complex I, thereby limiting mitochondrial respiration. This not only prevents the generation of ROS after I/R injury, but also calcium uptake and preserves mitochondrial function.[15, 93] Nitrite represents an "endocrine bioavailable storage pool of NO".[94] During myocardial I/R, nitrite is able to reduce cellular damage in a pH- and NO-dependent manner, regulating a number of cellular functions, including cellular respiration and viability.[95] It has been demonstrated that the intraventricular administration of exogenous nitrite 5 minutes before reperfusion reduces infarct size by 61% and improves left ventricular function.[15, 35] In addition, recent studies have shown that NO diminishes the rate of cardiac fibrosis and hypertrophy, and stimulates angiogenesis, key determinants of the remodeling process after myocardial infarction.[96-99]

With regards to the role of NO in myocardial function, there are contradictory studies, proposing both negative and positive inotropic effects.[100-102] Nevertheless,

it has been shown that low concentrations of NO, derived from constitutively expressed eNOS, maintain left ventricular function in contrast to high NO concentrations, which are iNOS-induced and are associated with cytotoxicity and heart failure.[9, 64, 68, 87, 88]

As mentioned above, under hypoxic conditions the conventional NO production through NOS is reduced, with NO being principally released from nitrite in NOS-independent ways.[40, 72] Experimental studies have already explored the contribution of Mb to NO supply in the myocardium under hypoxic conditions, and its influence on the biological response to NO.[14, 15, 35] Depending upon O_2 tension, Mb interacts with nitrogen oxides (NO_x), maintaining the cardiac NO homeostasis.[25, 35]

1.4 Interaction between myoglobin and nitric oxide species / nitrite and its biological impact

The ability of NO to interact with heme groups is one of its important chemical characteristics.[49] Several studies have revealed the reaction between Mb and NO and its role in maintaining NO homeostasis. It has been shown that under normoxic conditions Mb can act as a scavenger of bioactive NO, while under hypoxic conditions deoxyMb becomes the predominant form, acting as an NO producer by reducing nitrite.[2, 14, 15, 25] The nitrite reductase function of horse and mouse Mb has been already described[14, 15, 25], however, it is not known whether human Mb also exerts a comparable characteristic.

In 2001, Brunori et al. proposed that Mb may act as an intracellular scavenger of NO, in order to prevent the NO-mediated inhibition of mitochondrial respiration in the skeletal muscle and the heart.[27] Shortly after this publication, experimental evidence proved that in wild-type ($Mb^{+/+}$) mouse hearts, application of either exogenous NO or stimulation of endogenous NO production can lead to the formation of metMb and nitrate, as a major mechanism for decreasing the intracellular bioactivity of NO. This reaction could not be observed in myoglobin

deficient ($Mb^{-/-}$) mouse hearts.[2] The end-product - metMb - is no longer capable of binding O_2, but it is reduced back to deoxyMb very rapidly by metMb reductase, thereby avoiding accumulation.[2]

The formation of metMb can occur either through direct interaction between NO and MbO_2 or through an intermediate reaction (nitrosylation of deoxyMb), resulting in MbNO and its consecutive reaction with O_2.[2] By employing isolated perfused mouse hearts, Flögel et al. demonstrated that metMb forms predominantly in the vicinity of capillaries, which is most likely to be due to the higher concentration of oxyMb in this region (Eq. (7)).[2] In the proximity of mitochondria, the concentration of deoxyMb was higher than oxyMb, causing an intermediate reaction to take place, that resulted in the formation of MbNO (Eqs. (8), (9)).[2] It has been shown that the reaction of MbO_2 with NO is about twenty times faster when compared to both of the intermediate reactions which result in the formation of metMb and nitrate.[2]

$$(7)\ \text{oxyMb} + NO \rightarrow \text{metMb} + NO_3^-$$

$$(8)\ \text{deoxyMb} + NO \rightarrow \text{MbNO}$$

$$(9)\ \text{MbNO} + O_2 \rightarrow \text{metMb} + NO_3^-$$

The physiological role of this reaction was proven using transgenic mice with cardiac specific overexpression of iNOS, and Mb-deficiency ($tg\text{-}iNOS^+/Mb^{-/-}$). They developed signs of cardiac failure with ventricular hypertrophy, dilatation and interstitial fibrosis. The results in the $tg\text{-}iNOS^+/Mb^{-/-}$ mice showed higher levels of NO than the $Mb^{+/+}$mice. Such high levels of NO are known to induce a negative inotropic effect, apoptosis and inhibition of the respiratory chain, which may explain the phenotypic changes described above.[36, 64]

However, dependent upon O_2 tension, Mb is able to “shift its role from being an NO scavenger to an NO producer”.[14, 35, 49, 103] The mechanism of nitrite reduction by deoxyMb is similar to that of deoxyHb, yet the reaction is faster, being governed by

a molecular rate constant of 12 x 10^7 $M^{-1}s^{-1}$ at pH 7.4 and 37º C.[69, 83] In ischemic conditions, this rate increases to an estimated ten times faster.[69, 104]

The reaction between myoglobin and NO is modulated in an allosteric, pO_2-dependent manner.[14] Nitrite represents a "storage pool" of NO, which can be bioactivated through a reductive reaction at a low O_2 gradient, resulting in NO formation.[13, 71] Subsequently, NO may react with deoxyMb to form iron-nitrosylated Mb (MbNO), an indirect marker of NO production (Eqs. (10), (11)).[15, 105] Whether MbNO represents only an intermediate compound, or also has an impact on cardiovascular function is still currently a matter for debate.

$$(10)\ NO_2^- + \text{deoxyMb} + H^+ \rightarrow NO + \text{metMb} + OH^-$$

$$(11)\ NO + \text{deoxyMb} \rightarrow \text{MbNO}$$

The NO generated from nitrite by deoxyMb protects the heart against I/R injury by regulating many physiological and pathological processes, resulting in limitation of ROS generation, reduction of infarct size, amelioration of cardiac function, and vasodilation.[14, 15, 40, 49, 78] The fact that Mb has a central role in modulating NO hypoxic generation in myocardium, independent of Hb, was demonstrated by using $Mb^{-/-}$ mice. In this case, nitrite exerted no protective effects in contrast to $Mb^{+/+}$ mice, where nitrite was proven to reduce the myocardial infarct size.[15]

In recent years, it has been revealed that the products of nitrite-deoxyHb reaction generate nitrite-metHb, which is involved in the heme-protein dependent NO signaling through formation of N_2O_3.[73, 74] In analogy to the process described for metHb, it has been shown that metMb also reacts with nitrite, resulting in nitrite-metMb formation. This compound has been detected by EPR spectroscopy using metMb from horse skeletal muscle.[77] However, it remains unknown whether metMb from horse and human heart also exhibits a binding affinity for nitrite.

Interaction between human myoglobin and nitric oxide

In contrast to the Mb structure in other mammals, human Mb contains a sulfhydryl group at position 110 of the amino acid sequence (Cys^{110}).[106] The reaction of human oxyMb with NO therefore results in oxidation of NO to nitrate with metMb formation, and also S-nitrosation at Cys^{110}.[107] As RSNOs are stable compounds which can preserve NO bioactivity and regulate its bioavailability, S-nitrosated human myoglobin (MbSNO) may also represent an important pool of biological NO.[43, 107] Furthermore, in normoxic conditions, human Mb can inactivate NO not only *via* scavenging, but also by binding NO to its sulfur-containing cysteine, as Hb does.[2,107]

Starting from the premise that the structure of human Mb is similar to other mammalian Mb - with the exception of aforementioned unique cysteine residue (Cys^{110}) - it is tempting to speculate that human Mb is capable of eliciting analogous chemical reactions. Therefore, it has been hypothesized in this study that human deoxyMb may also function as a nitrite-reductase, generating NO under hypoxic conditions. Furthermore, it is yet to be established whether human oxyMb reacts with NO at the same rate as oxyMb originating from other mammals.

1.5 Open questions

Given the current knowledge about the interaction between NO species and Mb in horses and mice, the aim of this study is to elucidate the following:

1) In analogy to horse and mice Mb, does human Mb exhibit a nitrite reductase activity?
2) Is the rate of both NO scavenging and nitrite reduction comparable in human and horse Mb?
3) Can horse and human heart metMb also exert a binding affinity for nitrite as described for metHb?

2. Material and methods

If not otherwise specified, the materials used in the following experiments were obtained from Sigma-Aldrich Chemie GmbH, Munich, Germany.

2.1 Preparation of myoglobin solution from horse and human heart

The *in vitro* experiments, designed to analyze the chemical properties of human and horse Mb, were carried out using purified Mb from horse heart and commercially available purified human Mb. In order to produce horse Mb solutions with a final concentration of 200 *µ*mol/l - an amount equivalent to the *in vivo* concentration of Mb within the myocardium[14] - 0.351 g Mb (17,600 daltons[21]) were suspended in 10 ml phosphate buffered saline (PBS, Serag Wiessner, Germany), and rotated in the dark for 10 minutes at 4°C. This produced a metMb solution with an oxidized iron (ferric form, Fe^{3+}). In order to reduce the central iron atom (Fe^{3+} to Fe^{2+}), the resulting horse metMb was subsequently incubated with 0.016 g sodium dithionite. Deoxygenation was indicated by a colour change from brown to dark red.

An identical protocol was applied to human Mb. Sodium dithionite was added to 10 *µ*l human Mb (the commercially available assay volume). In control experiments, 10 *µ*l N-ethylamide (NEM) solution 10 mM was added in order to block the additional thiol group which human Mb is known to exhibit, thereby preventing S-nitrosation.[43]

The resulting deoxyMb solutions were then subjected to 100% O_2 (Linde, Unterschleissheim, Germany) for 15 minutes, yielding a completely oxygenated Mb preparation. To remove the excess sodium dithionite that had previously been added, gel chromatography was performed. After ten times equilibration of the Sephadex G 25 M column (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) with 2 ml PBS solution, fractions of 2 ml Mb solution were added and eluted with PBS. The middle section - with the aforementioned dark red coloring - was collected. For each elusion, 10 x 2 ml PBS were added to the column.

The resulting solution was again gassed with 100% O_2 (Linde, Germany) at atmospheric pressure for 15 minutes. This ultimately yielded oxyMb.

2.2 UV-Vis Spectroscopy for the assessment of myoglobin

To prove the presence of pure oxyMb, photometric wavelength scans were performed with a UV-Vis Spectrophotometer (Beckman Coulter DU 800). The increase of absorbance at 418 nm, 542 nm and 580 nm, which corresponds to oxyMb, was then recorded.

Principle of spectrophotometry

A spectrophotometer determines the quantity of light a sample can absorb when a beam of light passes through it. The measure is called 'absorbance', and according to Lambert Beer's law[108], is directly proportional to the concentration of absorbing compounds in that sample:

$$C = Abs \times 300\ (Dilution) / \varepsilon,$$

C = concentration, measured in moles per liter (l); Abs = absorbance; ε = molar extinction coefficient.

Identification of myoglobin using UV-Vis Spectroscopy

Spectrophotometry was applied in order to determine the presence and the concentration of oxyMb in the stock solution. This method was applicable as a result of Mb's property to absorb the radiation emitted by an external source, due to the heme particularities and the double bonds from that level.[109] The spectral collection range is 260-800 nm, the absorption peak being around 418 nm, 542 nm and 580 nm, respectively. The registered values in this study are comparable to those from published data.[110]

After the preparations of the horse and human oxyMb solutions were complete, aliquots of 10 μl of each solution were introduced with a Glass Pipette (Typ

1710/RN, Hamilton, Bonaduz, Schweiz) into one of the cuvettes of the Beckman Spectrophotometer, which contained 2990 μl PBS (1:300 dilution). Subsequently, a repeated wavelength scan (λ 300-650 nm) was performed. The absorbance at 418 nm, 542 nm and 580 nm - which corresponds to oxyMb - was recorded (Fig. 1), and the concentration of oxyMb was calculated using Lambert Beer's formula:

[Mb] = Absorbance x 300 (Dilution) x molar extinction coefficient (ε) (see Table 1).

Given the C1 and C2 concentrations, as well as the V1 and V2 containing volumes in cuvettes and Eppendorf tubes respectively, the volume of oxyMb required to prepare the 2 μM oxyMb solution was determined according to the Law of Equivalence:

$$C1 \times V1 = C2 \times V2.$$

Table 1: Molar extinction coefficients of Mb (34) (115)

MetMb	409 nm ε = 15.3		
OxyMb	418 nm ε = 12.8	542 nm ε = 13.9	580 nm ε = 14.4

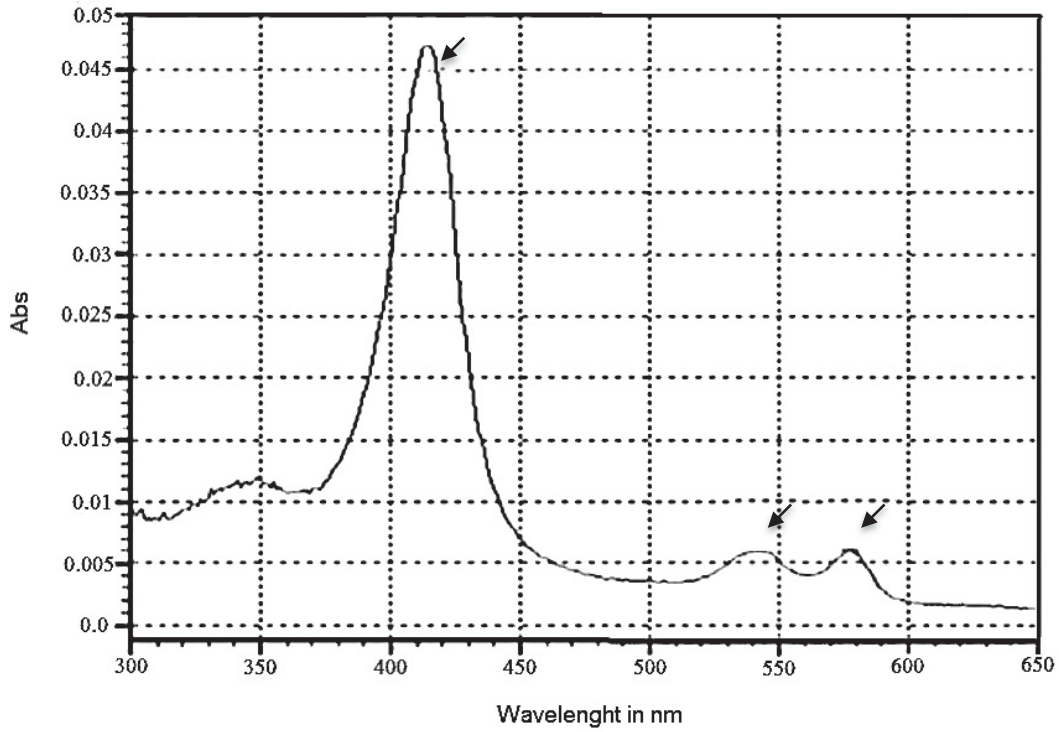

Fig. 1: **Spectra of oxyMb:** Original recording of oxyMb showing the absorbance peaks at 418 nm, 542 nm and 580 nm (arrows).

2.3 Assessment of nitric oxide scavenging by UV-Vis Spectroscopy

Inorganic sodium nitrite ($NaNO_2$) was used to prepare a 100 μM nitrite solution at pH = 5.0 as previously described.[14, 111] This acidic condition was chosen in order to create *in vitro* the acidic environment which is seen under circumstances of organ I/R (e.g. myocardial infarction). For this to be achieved, 690 mg NO_2^- was added to 10 ml PBS, followed by the addition of 1 ml of this stock solution to 9 ml PBS (100 mM). Finally, 500 μl of the 100 mM nitrite solution were added to 499.5 ml PBS, and titrated to pH 5.0 with HCl.

In order to assess the production of metMb from NO and oxyMb, the human and horse oxyMb solutions were prepared as described (see 2.1). At the beginning of the experiment, photometric wavelength scans were performed with UV-Vis spectrophotometer in order to prove the presence of pure oxyMb. An aliquot of 10 μl of each oxyMb solution was then added into one of the cuvettes of the Beckman

Spectrophotometer, which contained 990 μl nitrite solution (100 μM) at pH 5.0. Under acidic conditions, nitrite generates NO through acidic disproportionation[112], which may further react with oxyMb to form metMb[50].

Using the spectrophotometer, a continuous wavelength scan in the range of 300 to 650 nm was carried out at 60 second intervals, for a total of 12 minutes, beginning immediately after the incubation of both substances. The increase of absorbance at 409 nm (which corresponds to ferric Mb) was recorded (see Fig. 2), and the resulting metMb concentration was again calculated using Lambert Beer's formula (see 2.2).

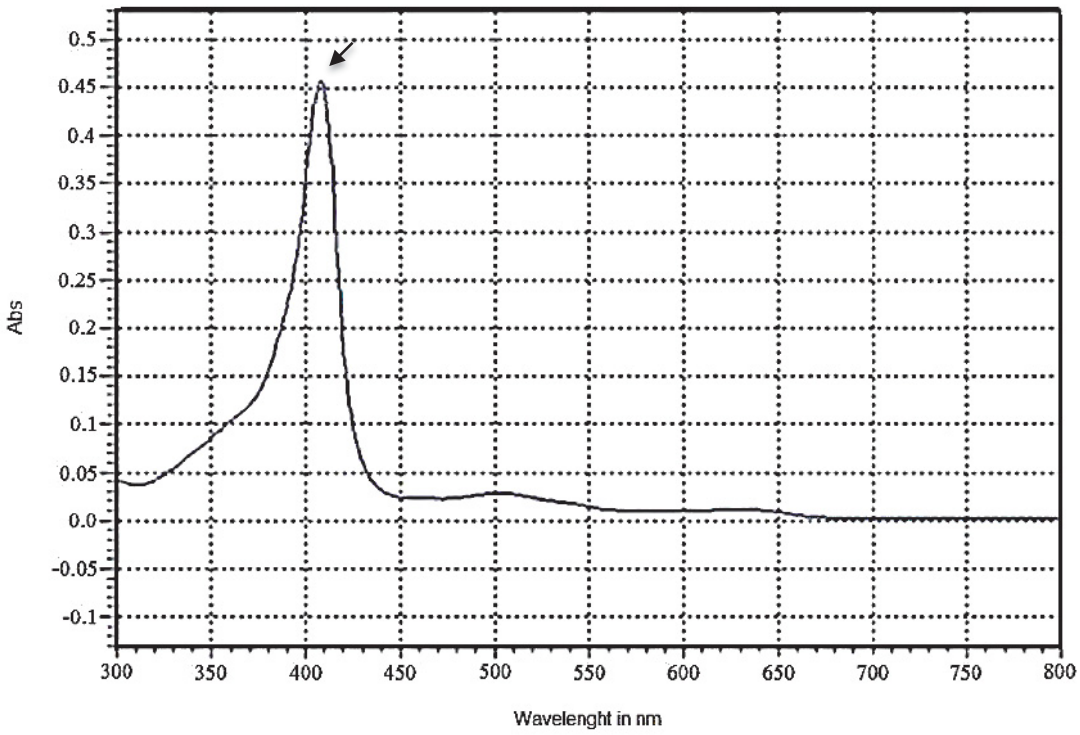

Fig. 2: **Spectra of metMb:** Original recording of metMb showing the maximal absorbance at 409 nm (arrow).

2.4 Preparation of mouse heart homogenates

For the preparation of homogenates, male Naval Medical Research Institute (NMRI) mice $Mb^{+/+}$ and $Mb^{-/-}$ - having been generated *via* the use of gene knockout

technology - were used, which were acquired from the Duesseldorf animal house. All animals were housed in standard conditions and did not significantly differ in age (12 ± 3 weeks) or weight (32 ± 6 g). The experiments were performed with permissions of the responsible ethics committee.

Prior to euthanasia, anticoagulation with heparin (0.1 ml, Liquemin 5000 IE, Roche Diagnostics GmbH, Mannheim, Germany) was established, and anaesthesia was made with urethan (1.5 g x kg^{-1}), intraperitoneally. After 10 minutes, mice were sacrificed by cervical dislocation, followed by median thoracotomy. A cannula was inserted into the thoracic aorta in order to perfuse the hearts free of blood retrogradely with ice-cold PBS for 2 minutes. The hearts were then removed from the situs, and snap-frozen in liquid nitrogen. This was followed by homogenisation in 5 x Volume ice-cold buffer, and centrifugation (10 minutes at 10.000 g, 4º C). The buffer contained a protease inhibitor in 5 ml NaCl in order to prevent protein degradation (1 tablet Complete Protease Inhibitor (Roche Diagnostics GmbH, Mannheim, Germany)).[72] Supernatant was removed with a pipette and stored on ice.

To determine the concentration of Mb in the $Mb^{+/+}$ and $Mb^{-/-}$ mouse heart homogenates, the Bio-Rad Bradford Protein Assay (Bio-Rad, Munich, Germany) was performed.[113] This is a colorimetric assay based on a differential color change of the Coomassie blue dye, which is dependent upon the concentrations of the protein to be measured. The absorbance of the protein can be quantified with the spectrophotometer at a wavelength of 595 nm. The concentration of the protein can then be calculated by comparison with a standard curve.

Using serial dilutions, eight standard solutions containing bovine serum albumin (BSA) were prepared, with increasing concentrations in a range of 0.2 to 2.0 mg/ml. Furthermore, two dilutions (1:10 and 1:20, respectively) of the samples to be assayed were performed.

The analysis of the protein content was made in a 96-well plate (UV Plate, 96 Well, Corning, NY, USA). In each well, 5 µl of the sample or standard curve, 25 µl work solution (NaCl) and 200 µl Coomassie dye reagent were added. The plate was kept at room temperature for 15 minutes, and the measurement of the absorbance

was made with a Fluostar Omega microplate reader (BMG Labtech, Ortenberg, Germany) at the wavelength of 595 nm. The standard curve was constructed by plotting the absorbance against the corresponding concentration of the standard protein. Based on the measured absorbance, the concentration of Mb in the homogenates was calculated using the equation of the standard curve: $y = ax + b$, where x is the absorbance and y is the protein concentration. Finally, the concentration of Mb in the samples was expressed as µg/ml protein, after applying the dilution factor.

2.5 NMR assessment of blood and hemoglobin-free perfusion of tissue preparation

In order to analyze the properties of human and horse Mb accurately, mouse homogenates free of blood and Hb were required. NMR was used to evaluate if there was any Hb left in the blood-perfused mouse hearts.

After perfusing the heart with a Krebs-Henseleit buffer[114], the homogenates were prepared as described in section 2.4. As control samples, mouse heart homogenates were directly homogenized without prior perfusion with Krebs-Henseleit buffer. After centrifugation, the supernatants were subjected to CO for 3-5 minutes, and then stored at 4°C until measurements were performed.[114]

NMR experiments were conducted in cooperation with Prof. Dr. Raphael Stoll from the Faculty of Chemistry and Biochemistry, Ruhr-University Bochum. The measurements were performed using a Bruker spectrometer. The parameters of acquisition included 16 k scans, 10.000 Hz spectral width, 2.048 data points and 110 ms repetition time.

2.6 Chemiluminescence for nitrite and nitric oxide species

Principle of chemiluminescence

Chemiluminescence-detection (CLD) represents one of the most sensitive methods to determine NO and its oxidative products (e.g. nitrite), as well as nitroso species.[43, 115] The general principle of chemiluminescence detection is based on measurement of the photons resulting from the reaction between NO and ozone (O_3) (Eqs. (1) and (2)).[116]

$$(1)\ NO + O_3 \rightarrow NO_2^* + O_2$$

$$(2)\ NO_2^* \rightarrow NO_2 + h\nu$$

The intensity of light is directly proportional to the concentration of NO. The reaction yields NO* in an excited state which emits a photon when it returns to its ground state. The photon is transformed into an electrical signal by a photomultiplier tube.[116]

Briefly, NO is produced from NO species (i.e. *S*-nitrosothiols, *N*-nitrosamines, iron-nitrosyls) and nitrate within a glassware system, which contains a redox solution which can reduce or oxidize these species. In order to arrive in the NO analyzer (NOA) where it reacts with ozone, the NO is carried out of the solution to the reaction chamber of NOA by forcing an inert gas (e.g. helium) into the vessel (Fig. 3).[43, 115, 116]

The redox active solution inside the reaction chamber can be either an oxidative or a reductive solution.

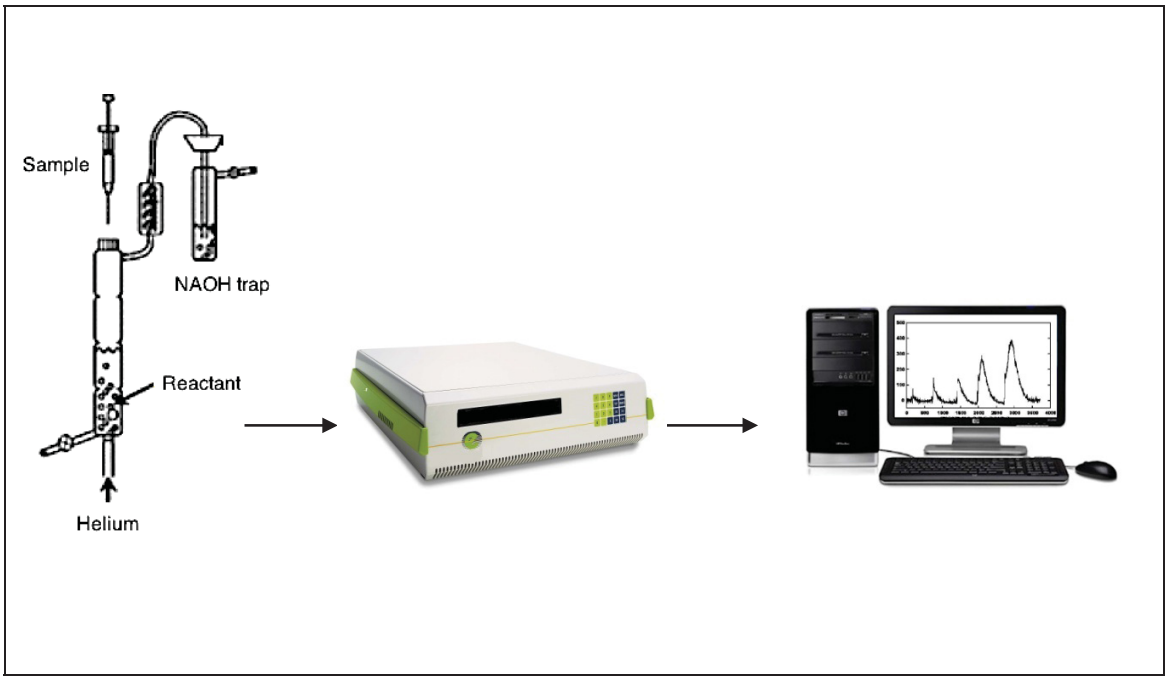

Fig. 3: Schematic view of the chemiluminescence detection: After the injection of the sample into the reaction chamber filled with reductive/oxidative solution, NO is transported through a solution of sodium hydroxide (NaOH) to eliminate volatile acids before entry into the NOA[117], where it reacts with O_3. The signal of the NOA-produced-chemiluminescence is recorded by a computer.

The ferricyanide solution can be used as an oxidative agent for NO-heme products, including Mb, Hb, and other heme proteins. Ligands of the heme form a stable compound with heme-Fe^{2+}. The bond becomes unstable *via* the oxidation of Fe^{2+} to Fe^{3+}, resulting in the release of NO (Eq (3)):

$$(3)\ [Fe^{3+}(CN)_6]^{3-} + \text{heme-}Fe^{3+}\,NO \rightarrow [Fe^{2+}(CN)_6]^{4-} + \text{heme-}Fe^{3+} + NO$$

The reduction solution consists of potassium iodide (0.81 g KI, 45 mM) and iodine (0.285 g I_2, 10 Mm), dissolved in 100 ml acetic acid. Using the reduction solution, NO from RSNOs, RNNOs, Fe-Nitrosyl products and nitrite is released (Eqs. (4), (5), (6)).[116]

$$(4)\ I_2 + I^- \rightarrow I_3^-$$

(5) $I_3^- + 2RXNO \rightarrow 3\ I^- + RS^{\cdot} + 2NO^+$

(6) $2NO + 2\ I^- \rightarrow 2NO + I_2$

Chemiluminescence is a sensitive method, which can detect not only NO, but also other nitroso compounds, with a detection limit of 1 nM.[116] To differentiate between the various nitroso compounds (RXNO), the samples can be chemically treated before application to the reaction solution.[118] In order to detect the nitrosation products (RXNO) in plasma and tissue, the sample must be preincubated with acidified sulfanilamide to convert nitrite into a diazonium cation, which is not detectable with chemiluminescence (Eq. (7)). The consequent signal represents the sum of NOx and RNO-products (Fe-nitrosyl, N-nitrosamines) in the sample.

(7) NO_2^- + sulfanilamide → diazonium cation

To determine the concentration of nitrite, the peak area representing RNO (aliquots pretreated with sulfanilamide) is subtracted from the total signal (untreated sample), which represents nitrite and RNO. To eliminate RSNO from the sample, it firstly needs to be treated with a mercuric chloride solution ($HgCl_2$), and then with the sulfanilamide solution (Eqs. (8), (9)). Incubation with $HgCl_2$ yields to S-NO bond cleavage. The peak area $HgCl_2$-resistant therefore represents the RNNO species.

(8) NO_2^- + sulfanilamide → diazonium cation

(9) $Hg^{2+} + 2RSNO \rightarrow Hg(RS)_2 + 2NO^+$

Nitrite calibration range

Before quantifying any samples, a daily calibration of the CLD is required to increase the accuracy of the chemiluminescence detection. For calibration, a freshly prepared acidic tri-iodide (I_3^-) reagent is used. This compound can reduce

nitrite, producing stoichiometric quantities of NO and iodide (Eqs. (10), (11) and (12)).

$$(10)\ I_3^- \rightarrow I_2 + I^-$$

$$(11)\ KI \rightarrow K^+ + I^-$$

$$(12)\ 2NO_2^- + 2I^- + 4H^+ \rightarrow 2NO + I_2 + 2H_2O$$

As a reducing agent, I_3^- also reacts with RSNO followed by the release of free iodine, thiol radicals - which subsequently combine to form disulfide -, and nitrosonium cations, which further react with free iodine, yielding NO (Eqs. (13), (14)).

$$(13)\ I_3^- + 2RS\text{-}NO \rightarrow 3I^- + RSSR + 2NO^+$$

$$(14)\ 2NO^+ + 2I^- \rightarrow 2NO + I_2$$

After diluting the nitrite stock solution in PBS in order to obtain the calibration standard solutions, 100 µl from every sample with 0 nM, 50 nM, 100 nM, 150 nM and 200 nM concentrations respectively, were injected three times into the CLD vessel. This resulted in the release of NO as displayed in Fig. 4. The standard curve was constructed using Origin 7.0 Software.

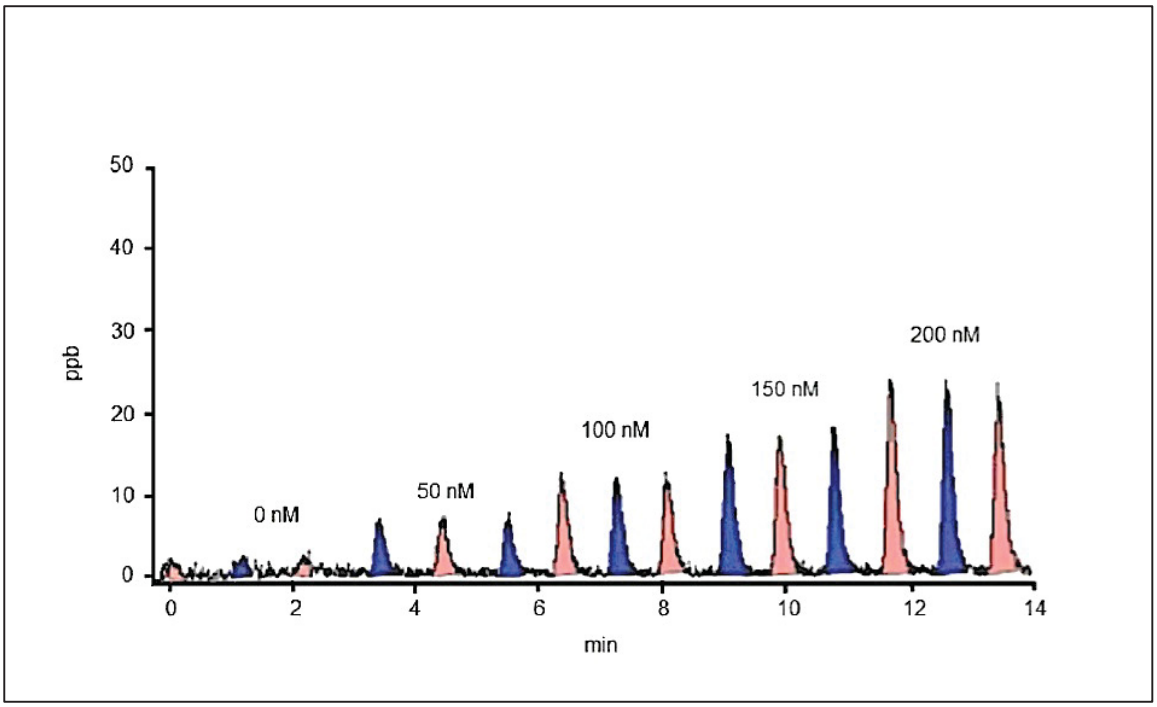

Fig. 4: Calibration peaks (example of an original recording): The peaks represent the release of NO after application of various concentration of the nitrite solution (0 nM, 50 nM, 100 nM, 150 nM and 200 nM) into the CLD reaction chamber.

To calculate the concentration of nitrite in a sample (µM), the area under its corresponding curve must be divided by the slope from the standard curve (area/pmol), and by the volume of the sample injected (µl) (Fig. 5).[116]

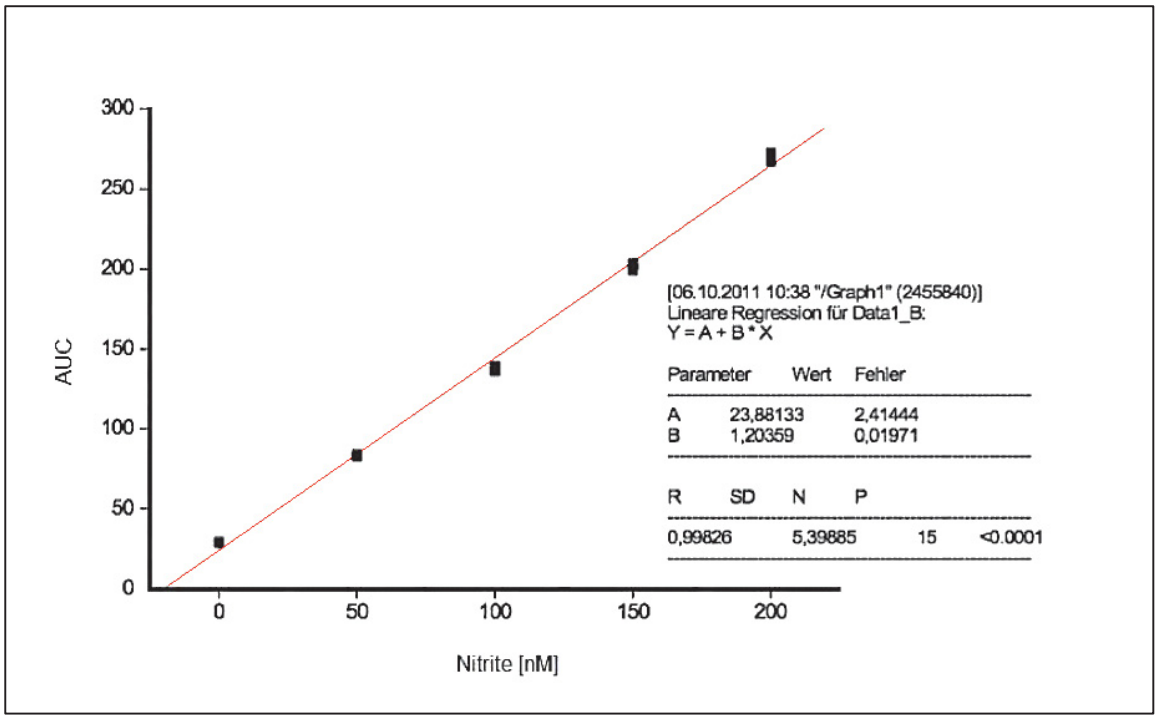

Fig. 5: Example of a standard calibration curve: The standard curve was constructed using Origin 7.0 software by plotting the area of each peak shown in Fig. 4 on the y-axis and the corresponding quantity of nitrite on the x-axis.

2.7 Nitrite reductase activity of human and horse myoglobin

Following CLD calibration, a 20 ml aliquot of the 100μM nitrite-PBS solution was added into the reaction chamber of the CLD at 37° C. Prior to protein administration, the content was deoxygenated with helium (Linde, Germany) for 20 minutes. As a result of the acidic disproportionation of nitrite, a baseline release of NO was then detected.[119] 80 μl of human Mb or horse Mb were then injected into the CLD vessel using a Hamilton syringe (Hamilton, Bonaduz, Schweiz), with a final Mb concentration of 2 μM. Aliquots of human Mb had been preincubated with 10 mM NEM in order to block the additional thiol group that human Mb exhibits, thus preventing S-nitrosation.[43]

The release of NO was measured for a total of 10 minutes. The experiment was repeated three times, with the release rates calculated per gram of protein, and per second. The NO release was determined as area under the curve (AUC) per second.

2.8 Nitrite reductase activity of mouse heart tissue

After homogenisation and centrifugation of $Mb^{+/+}$ and $Mb^{-/-}$ mouse hearts, Biorad Assay was performed to determine the corresponding volume for 300 μg protein. 20 ml of the 100 μM nitrite-PBS solution were added to the reaction chamber of the CLD at 37°C (see above). 22 μl $Mb^{+/+}$ homogenate - the corresponding volume for 300 μg protein - was injected. The release of NO was measured for 10 minutes (see 2.4). The experiment was repeated with 24 μl $Mb^{-/-}$ homogenate, the corresponding volume for 300 μg protein.

Human and horse oxyMb were prepared as described in section 2.1. Spectrophotometry was used to measure their concentration. 20 ml of 100 μM nitrite-PBS solution were injected into the vessel, followed by a 20 minute period of deoxygenation. Following this, 300 μg of $Mb^{-/-}$ mouse heart homogenate were added, and the release of NO was recorded for 10 minutes. In order to demonstrate the role of Mb in restoring the NO production in $Mb^{-/-}$ heart homogenates, 10 nmols of human and horse Mb were added into the reaction chamber containing both the nitrite solution and the homogenate of the $Mb^{-/-}$ mouse heart.

Finally, both homogenates from $Mb^{+/+}$ and $Mb^{-/-}$ were incubated with 100 μM allopurinol (final concentration), 200 μM diphenyliodonium (DPI) (final concentration) or with 250 μM L-NG-Nitroargininemethyl ester (L-NAME, Cayman, Ann Arbor, United States) (final concentration) in order to inhibit the potential alternative nitrite-dependent NO producers, such as XO or NOS.[14]

2.9 EPR assessment of the reaction between nitrite and metmyoglobin

The second aim of this study was to determine the binding affinity of nitrite to metMb by EPR spectroscopy.

Principle of EPR

EPR spectroscopy is an investigative method that can detect chemical compounds with unpaired electrons, using the magnetic properties of unpaired electrons instead of atomic nuclei as in NMR. The electrons have ”spins”, which represent an intrinsic form of rotational motion and confer them magnetic properties. The electrons can be influenced by external magnetic fields. In a magnetic field, the paramagnetic electrons can orientate themselves either parallel (lower energy level), or antiparallel (upper energy level) to its direction, causing a shift in their spin-state. In EPR experiments, this magnetic field is controlled by a preassigned frequency of microwave radiation, which leads to a transition of the electrons' spins. The microwaves provided from a source are transmitted through an attenuator and then passed on to the sample to be measured, which is located in a microwave cavity. The microwaves are then reflected back from the sample and conducted to a detector diode. The detector diode can identify changes in the level of the microwaves' energy spectrum, the resulted signal is therefore analogous to the absorption of microwaves by the sample.[120]

In vitro preparation of horse metmyoglobin and (^{15}N) Nitrite

During the following experiments, a N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer was used. It was made from 11.9 g HEPES, 3.75 g potassium chloride (KCl) and 19.65 mg diethylene triamine pentaacetic acid (DTPA) in 500 ml purified water (H_2O) to 100 mM HEPES, 0.1 M KCl and 0.1 mM DTPA (final concentrations), respectively. The solution was then adjusted to a pH of 7.4 with NaOH using a Lab 870 pH meter (Schott, Mainz, Germany).

Isotope-labeled nitrite was purchased in the form of sodium nitrite ($Na^{15}NO_2$). In order to produce a 20 mM nitrite solution, 0.00699 g sodium nitrite (molecular

weight 6.99 mg) was added to 5 ml HEPES Buffer. Subsequently, by performing serial dilutions, nitrite solutions with the following concentrations could be obtained: 1000 μM, 500 μM, 100 μM, 50 μM and 10 mM respectively.

The solution of horse heart Mb was prepared as described in 2.1 with a final concentration of 1 mM. 1 ml of 50 mM FeCN solution was added to the metMb solution (volume 1:9) to achieve the oxidation of Fe^{2+} to Fe^{3+}, in order to produce a completely oxidated Mb preparation. Chromatography was performed to collect pure metMb. Spectrophotometry was applied to determine the concentration of metMb in the stock solution. An aliquot of 10 μl metMb solution was introduced with a Glass Pipette (Typ 1710/RN, Hamilton, Bonaduz, Schweiz) in one of the cuvettes of the Beckman Spectrophotometer, which contained 2990 μl HEPES Buffer (1:300 dilution). Subsequently, a repeated wavelength scan in the range of λ 300 to 650 nm was carried out. The increase of absorbance at 409 nm, which corresponds to metMb, was then recorded (Fig. 5), and the concentration of metMb was calculated using Lambert Beer's formula, as described above (see 2.2). The volume of metMb required to prepare solutions with increasing concentration of metMb (10 to 200 μM) could be determined according to the Law of Equivalence (see 2.2).

After the preparation of both compounds, nitrite and metMb were incubated for 5 minutes at 37° C and then frozen before EPR measurements.

EPR measurement

The EPR experiments were performed in cooperation with Prof. Dr. rer. nat. Heinz-Jürgen Steinhoff and Dr. rer. nat. Johann Klare from the University Osnabruck, Department of Physics. EPR spectra of nitrite-bound metMb or metMb were measured using a Bruker Elexsys E680 spectrometer at 100 kHz (modulation frequency), 10 mV (microwave power), 5 G (modulation amplitude) with a spectral collection range between 500-5500 G. The measurements were analyzed using a Bruker WINEPR software.

2.10 Statistical analyses

Origin 7.0 Software (OriginLab Corporation, Massachusetts, USA) was used to perform the standard curve for the chemiluminescent measurement of NO. GraphPad Prism 6.0 (GraphPad Software, California, USA) for Mac OS X was used for statistical analysis. The results were expressed as means ± standard deviation (SD). A P-value of < 0.05 was considered significant.

Student's t-test was applied to compare two groups, and 1-Way ANOVA when comparing three groups. 1-Way ANOVA followed by post-hoc Bonferroni adjustment were used to measure the response to inhibitors (allopurinol, DPI, L-NAME) in both $Mb^{+/+}$ and $Mb^{-/-}$ mice.

3. Results

3.1 Human myoglobin scavenges nitric oxide

Mb plays a central role in reducing intracellular NO, thereby contributing to protection from nitrosative stress.[2] In this study, UV-Vis Spectroscopy was used to directly measure the conversion of oxyMb to metMb *via* reaction with NO. Human and horse oxyMb solutions were incubated in cuvettes that contained nitrite at pH 5.0. Under ischemic conditions, nitrite can be reduced to NO through acidic disproportionation.[12, 119] Based on this property, nitrite was used in this experiment as a source of NO. The resulting NO reacted with oxyMb leading to metMb formation. The shift of absorbance from 540 and 580 nm to 409 nm indicated the increase of metMb over time (Fig. 6). Using repetitive wavelength scan for 12 minutes, at 60 seconds intervals, a gradual increase of absorbance could be seen for human and horse metMb, respectively. The formation rate of human metMb was measured from the first wavelength scan until reaching a plateau (Fig. 7), and was found to be 107 pmol*s^{-1}, while the formation rate of horse metMb was 37 pmol*s^{-1} (Data represent mean ± SED of n = 3, ***P < 0.001.). This data demonstrated a significant increase in metMb formation by adding human oxyMb in comparison to horse oxyMb.

However, at high levels of nitrite, Mb can be directly oxidized to metMb, without prior reduction of nitrite to NO.[121] The experiments were therefore repeated using the same concentration of nitrite at pH 7.4, in order to eliminate the possibility of oxyMb oxidation by nitrite. Under these conditions metMb could not be detected. It was therefore possible to categorically state that NO production by acidic disproportionation of nitrite was responsible for the increase in metMb formation, and the oxidation of oxyMb by nitrite could be excluded.

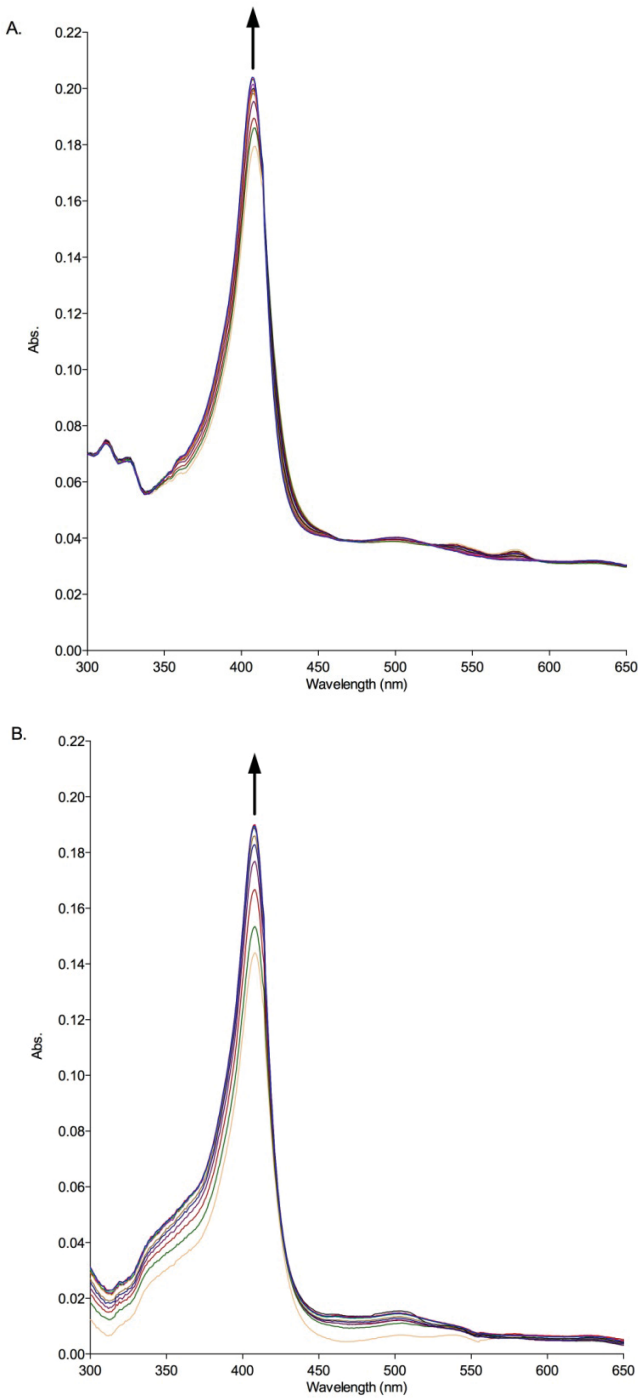

Fig. 6: Formation of horse (A) and human (B) metMb: Human and horse oxyMb were incubated with 100 μM nitrite at pH 5.0. After NO release through acidic disproportionation, it reacted with oxyMb yielding metMb. Using repetitive wavelength scan in the range of 300 to 650 nm, for 12 minutes, at 60 seconds intervals, a gradual increase of absorbance at 409 nm (see the arrows), which corresponds to metMb, could be seen for horse **(A)** and human **(B).**

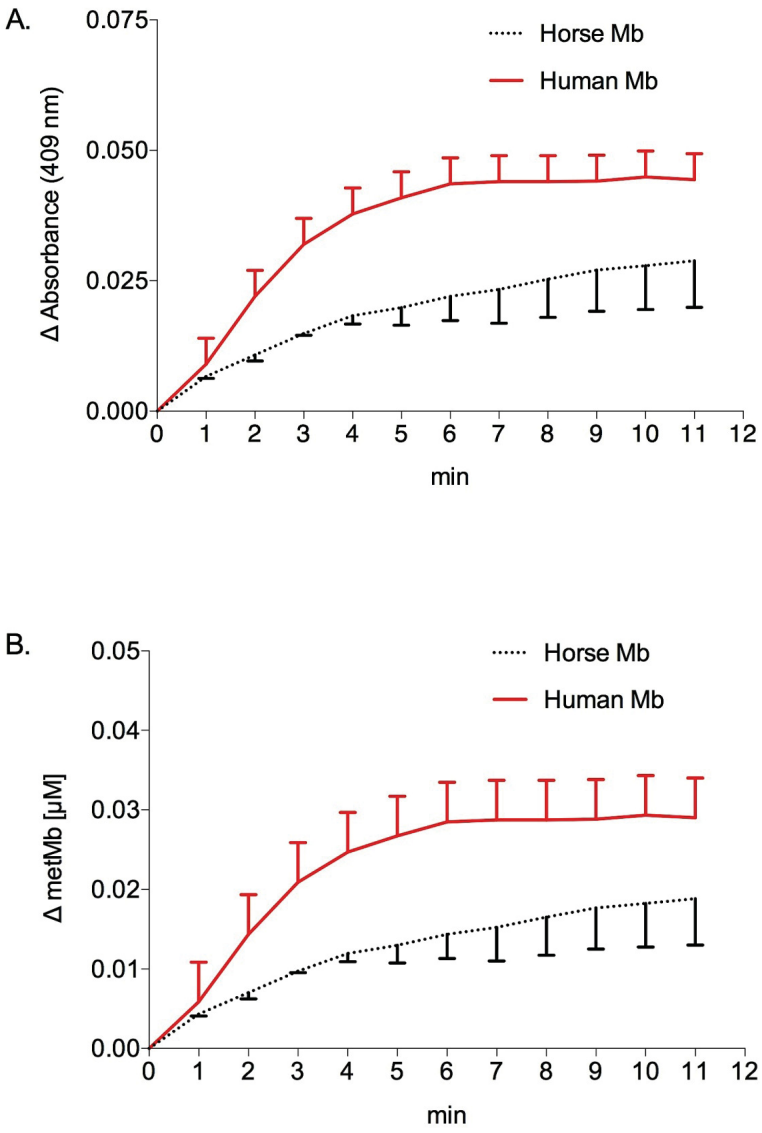

Fig. 7: Mb as a scavenger of NO: The increase of absorbance at 409 nm for both human and horse Mb was recorded from the first wavelength scan **(A)**. Quantitative analysis of human and horse metMb formation until plateau **(B)**, respectively, showed a significantly higher formation rate in human Mb (107 pmol*s^{-1}) compared to horse Mb (37 pmol*s^{-1}) (Data represent means ± SD of n = 3, ***P < 0.001).

3.2 Nitrite reductase activity of human myoglobin

The ability of deoxyMb to reduce nitrite to NO has already been reported.[14] It is widely known that both mice and horse deoxyMb can reduce nitrite to NO under hypoxic conditions, a mechanism which is responsible for the downregulation of cardiac energy status.[15] A comparable activity for human Mb has not yet been determined. To analyze the formation of NO from nitrite by human deoxyMb, a deoxygenated Mb solution and a PBS solution (pH 5.0) containing nitrite (100 μM) had been previously prepared (see 2.3). Chemiluminescence was therefore performed with the aim of detecting the formation of NO.[115]

The nitrite solution was added into the reaction chamber and deoxygenated with helium for 20 minutes in order to create anaerobic conditions. An NO signal could immediately be registered, as a direct result of the acidic disproportionation of nitrite. The chemiluminescence signal was registered until reaching a steady-state plateau (15-20 minutes). Equal amounts of oxygenated horse or human Mb were then injected into the vessel, with final concentrations of 2 μM. In a further experimental set, human Mb was incubated with 10 μl NEM (10 nM) in order to block the additional thiol group which human Mb exhibits, preventing S-nitrosation.[122] The sustained release of free NO was measured for 10 minutes.

The resulting area under the curve was calculated in relation to the 100 nM nitrite solution (see 2.6). To allow standardization of the measurements, the quantities were divided by the time of registration (nmoles per liter per unit of time). The quantitative analysis (Fig. 8) displayed a free NO release by human Mb of 38.84 $nmol*g^{-1}*s^{-1}$. In comparison, the release of free NO by horse Mb was significantly lower (21.89 ± 2.21 $nmol*g^{-1}*s^{-1}$; data represent means ± SD of n = 3, $*P < 0.05$.). By blocking the additional thiol group in human Mb, the formation rate of free NO was not significantly affected (43.15 ± 18.23 $nmol*g^{-1}*s^{-1}$; data represent means ± SD of n = 3, P = ns.).

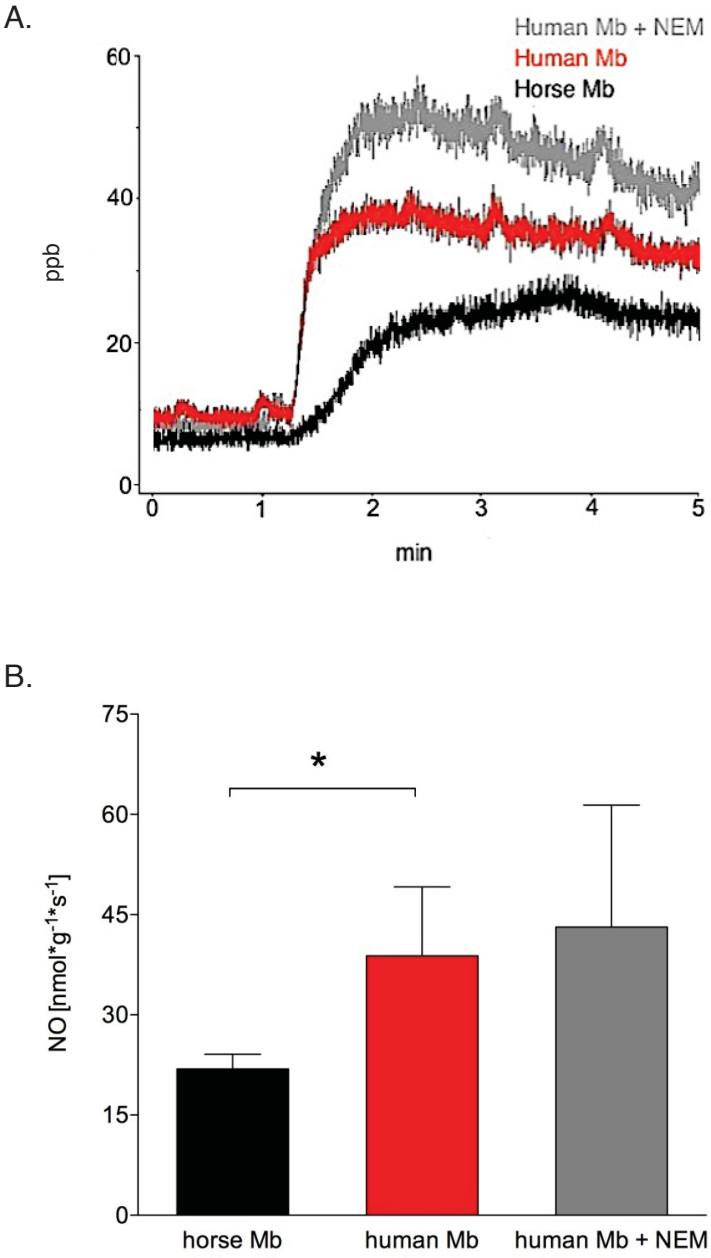

Fig. 8: Nitrite reductase activity of Mb: **(A)** Representative tracings show a higher release of NO in human Mb compared to horse Mb. Incubation of human Mb with NEM does not significantly affect the NO production. **(B)** Quantitative analysis (n = 3) of NO release after incubation of 100 μM nitrite solution at pH 5.0 with horse Mb, human Mb and human Mb + NEM, respectively, measured by chemiluminescence. By comparison, the release of NO was significantly higher in human Mb than in horse Mb (Data represent means ± SD of n = 3, *P < 0.05.). The formation rate of NO was not significantly affected after incubation of human Mb with NEM.

3.3 NMR spectra of blood/hemoglobin-free tissue preparations

In order to analyze the properties of human and horse Mb, mouse homogenate Hb-free was required. NMR was used to determine the presence of Hb in the blood-perfused mouse hearts. The ^{1}H NMR spectra for the MbCO solution extracted from the buffer-perfused mouse homogenate are displayed in Fig. 9A. The peak at -2.40 ppm, which corresponds to MbCO[114], was recorded. The control solution shows two peaks, at -1.72 and -1.92 ppm (Fig. 9B), which represent the resonances from the alpha and beta subunits of Hb, respectively. Furthermore, the control solution exhibits a peak at -2.40 ppm that corresponds to MbCO. These values are comparable to those documented in the existing published data.[114]

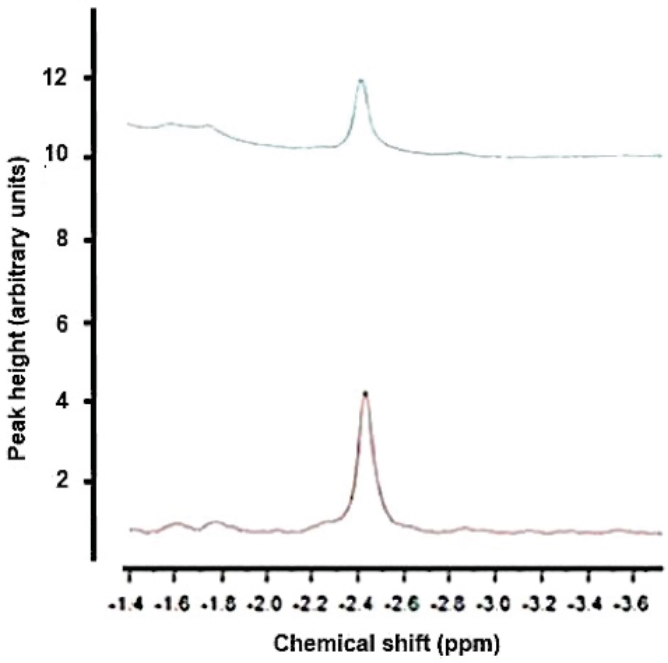

Fig. 9A: ^{1}H NMR spectra of MbCO (example of original recording): The spectrum of MbCO extracted from perfused mouse heart homogenates shows a peak at -2.40 ppm.

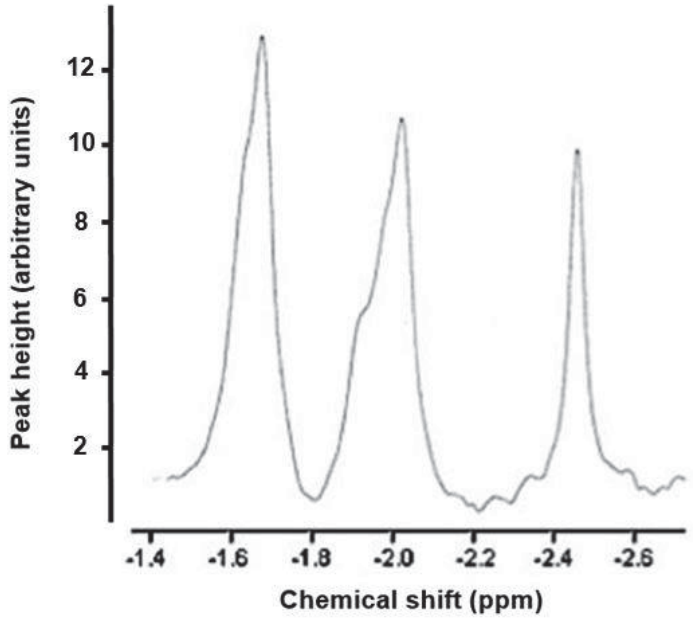

Fig. 9B: ^{1}H NMR spectra of MbCO and HbCO (example of original recording): The spectrum of HbCO and MbCO solution extracted from mouse blood, which represents the control solution, shows a peak at -2.40 ppm which corresponds to MbCO, and two peaks at -1.72 and -1.92 ppm, corresponding to the alpha and beta subunits of Hb.

3.4 Impaired nitrite reductase activity in myoglobin-knockout mouse heart homogenates is restored by myoglobin

The relationship between Mb and NO in both $Mb^{-/-}$ and $Mb^{+/+}$ mice has been shown by comparison: the release of NO was quantified in the presence and absence of Mb. The homogenates from both $Mb^{+/+}$ and $Mb^{-/-}$ mice were deoxygenated and injected into the 100 μM nitrite-containing reaction chamber. After the injection of the homogenate from $Mb^{+/+}$ mouse heart, NO was released at a rate of 11 ± 3.3 $g^{-1}s^{-1}$. In the absence of Mb (Mb homogenates from $Mb^{-/-}$ mouse hearts), NO was released at a rate of 5.4 ± 0.67 nmol $g^{-1}s^{-1}$. This process revealed a significantly higher release of NO for $Mb^{+/+}$ compared with $Mb^{-/-}$ hearts homogenates ($Mb^{+/+}$ vs. $Mb^{-/-}$; data represent means ± SD of n = 3, *P < 0.05.) (Fig. 10).

Following this, Mb was replenished by injection of human and horse Mb (Fig. 11) into the reaction chamber containing $Mb^{-/-}$ supernatant (final concentration 200

μmol/l). The rate of NO formation increased similarly with the NO formation in $Mb^{+/+}$ homogenate. Moreover, immediately after the administration of human/horse oxyMb to homogenates from $Mb^{-/-}$ mice, oxyMb acted first as an NO scavenger, thereby reducing the NO formation (Fig. 11 (a)). After complete deoxygenation, Mb changed from an NO scavenger to an NO producer (Fig. 11 (b)). Quantitative analysis of NO formation after administration of human oxyMb and horse oxyMb (Fig. 11C), respectively, showed a higher release of NO in human Mb compared to horse Mb (13.56 ± 1.1 *vs.* 9.46 ± 0.62 $nmol*g^{-1}*s^{-1}$; data represent means ± SD of n = 2, *$P < 0.05$.)

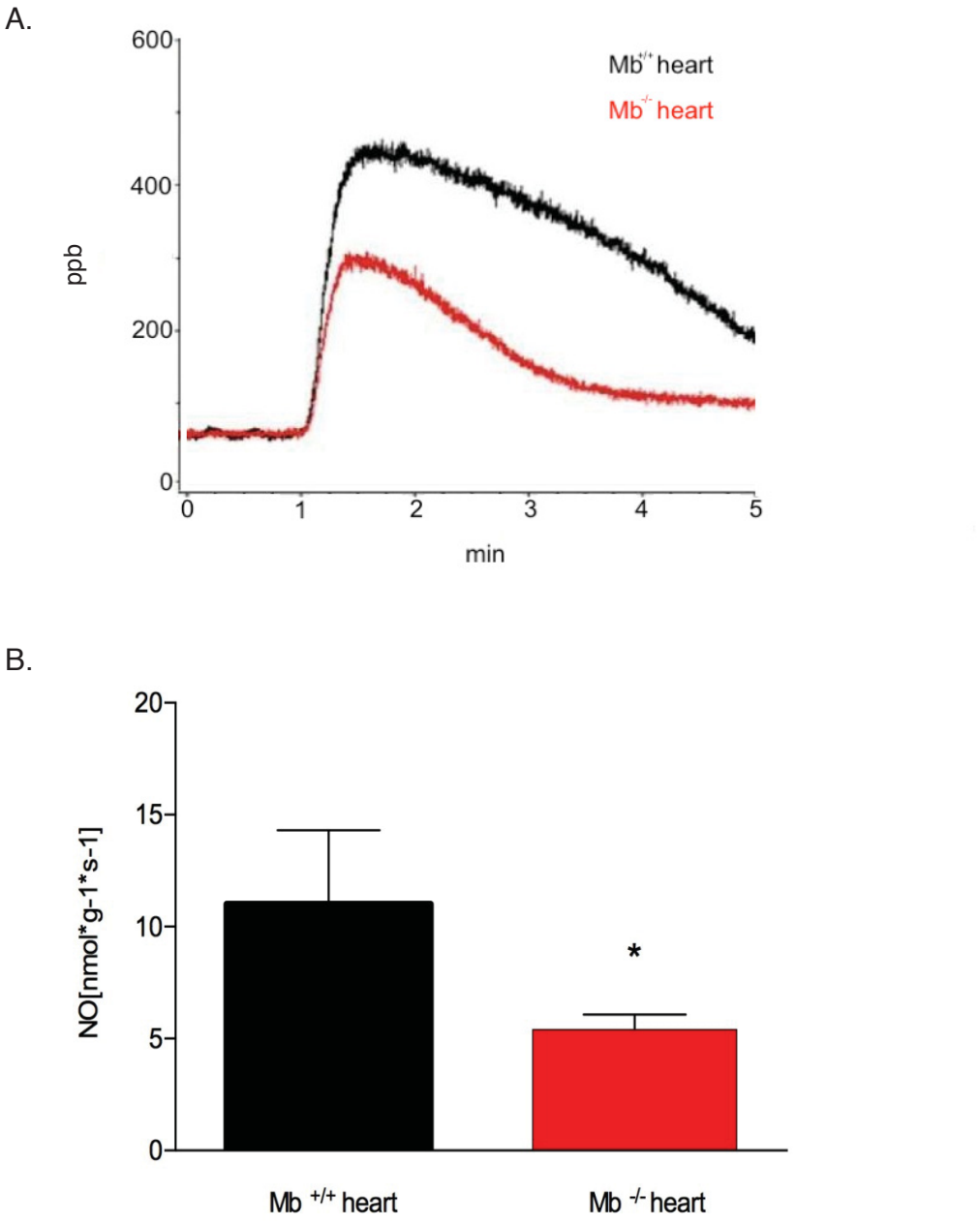

Fig. 10: Mb-dependent nitrite reductase activity in mouse heart homogenates: (A) Representative tracings show a higher release of NO when heart homogenates from $Mb^{+/+}$ mice are injected into the nitrite solution-containing reaction chamber compared with $Mb^{-/-}$ mice. **(B)** Quantitative analysis of NO production after incubation with $Mb^{+/+}$ vs. $Mb^{-/-}$heart homogenates with a 100 μM nitrite solution, measured by chemiluminescence (Data represent means ± SD of n = 3, *$P < 0.05$.).

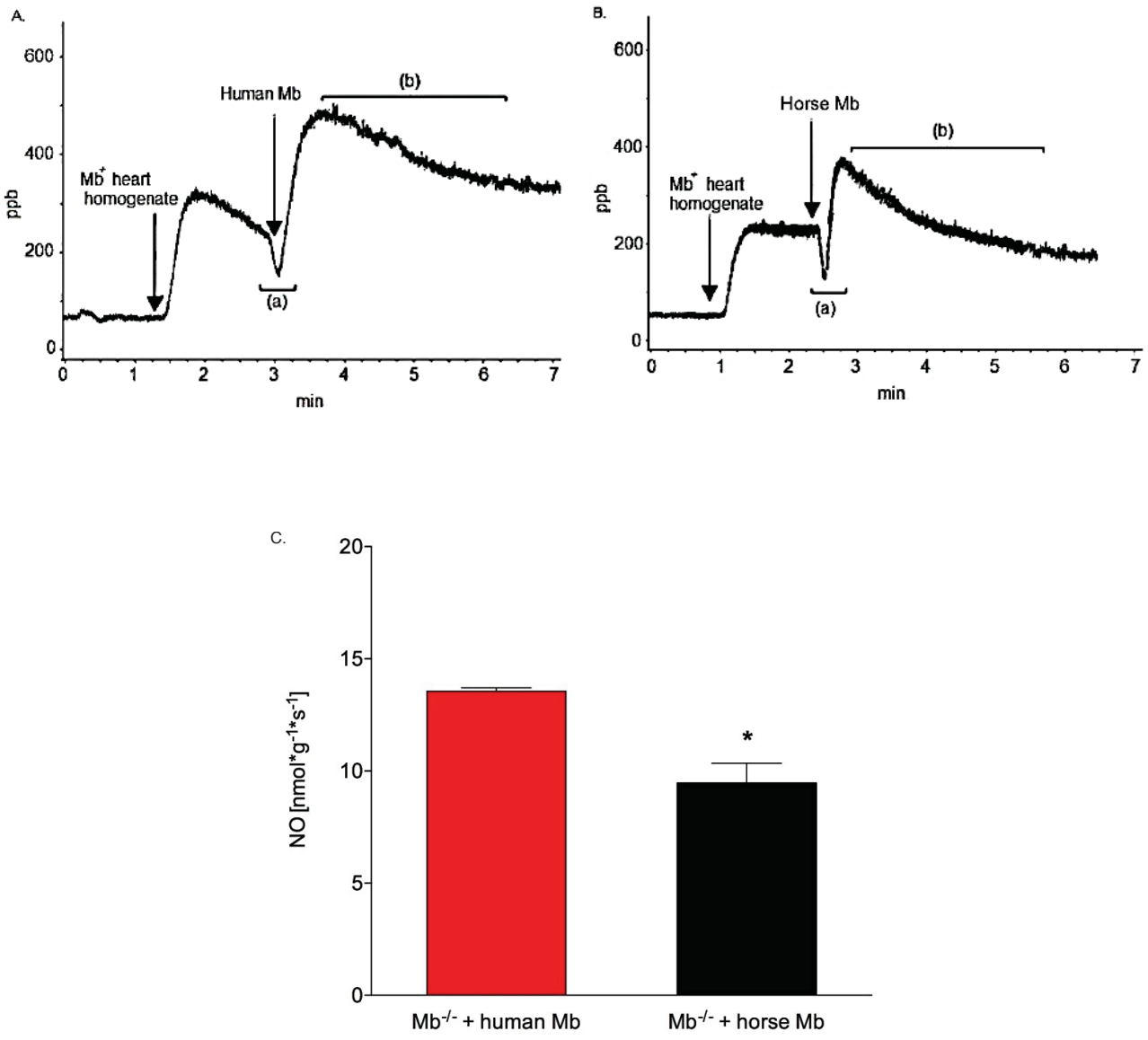

Fig. 11: Impaired NO production in $Mb^{-/-}$ mouse heart homogenates is restored by Mb: After administration of human (**A)** or horse **(B)** oxyMb, the NO release from nitrite reduction in $Mb^{-/-}$ was restored. After addition of Mb to $Mb^{-/-}$ homogenates, oxyMb first reacted with NO to nitrite and metMb **(a).** After deoxygenation, Mb became an NO-producer, thus restoring the NO production **(b).** (**C)** Quantitative analysis of NO formation after administration of human oxyMb and horse oxyMb, respectively, shows that the NO release is significantly higher in human Mb compared with horse Mb (13.56 ± 1.1 *vs.* 9.46 ± 0.62 $nmol*g^{-1}*s^{-1}$; data represent means ± SD of n = 2, *P < 0.05.).

In order to demonstrate the impact of potential alternative nitrite-dependent NO producers - such as XO or eNOS - another experimental set was performed (Fig. 12). After inhibition of XO with allopurinol and diphenyliodonium (DPI), and of eNOS with L-NAME, the release of NO was not significantly affected when compared with untreated controls (11.51 ± 2.13 vs. 9.65 ± 1.191 vs. 10.20 ± 2.68 nmol*g^{-1}*s^{-1}; data represent means ± SD of n = 3, P = ns.). In contrast, the presence of Mb significantly increased the production of NO independently of inhibitors, when compared with $Mb^{-/-}$ mice (Data represent means ± SD of n = 3, ***P < 0.001.).

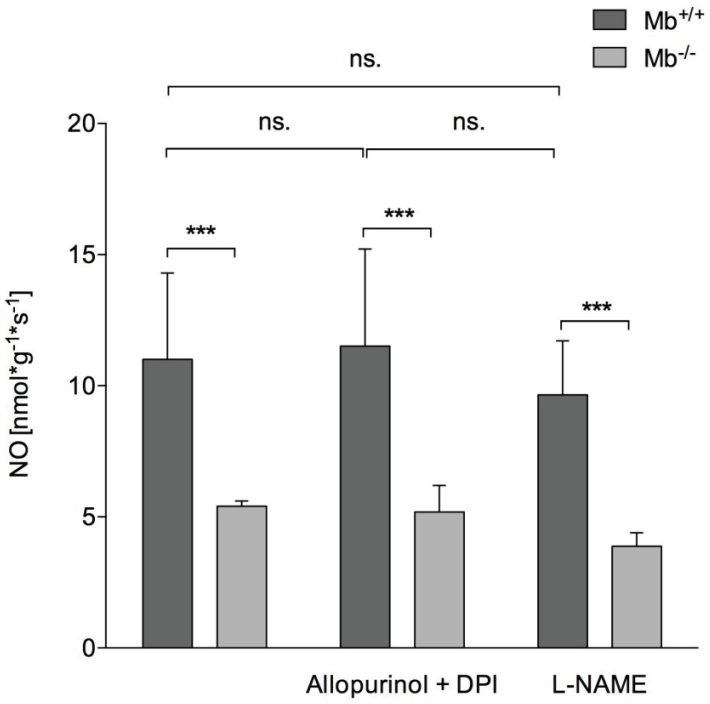

Fig. 12: Nitrite reduction to NO was independent of XO and eNOS: After incubation of both homogenates from $Mb^{+/+}$ and $Mb^{-/-}$ with 100 μM allopurinol, 200 μM DPI or with 250 μM L-NAME, the production of NO did not significantly change in either $Mb^{+/+}$ or $Mb^{-/-}$mice (Data represent means ± SD of n = 3, P = ns.). The presence of Mb significantly increased the production of NO, when compared to $Mb^{-/-}$ mice (Data represent means ± SD of n = 3, ***P < 0.001.).

3.5 EPR Study for nitrite and metmyoglobin

EPR spectroscopy was employed in order to investigate the binding affinity of nitrite to metMb. The EPR spectra of both human metMb and horse metMb are illustrated in Fig. 13 (A, B). In all of the samples, the signals at ≈1100 G (a), ≈2500 G (b) and ≈3350 G (c) could be evidenced.

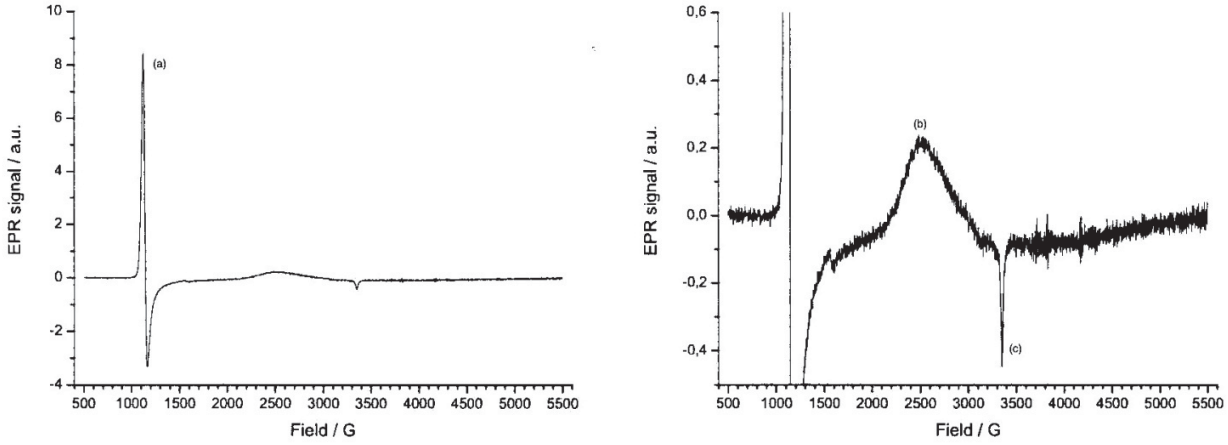

Fig. 13A: EPR spectra of horse metMb: The maximal peaks were recorded at ≈1100 G (a), ≈2500 G (b) and ≈3350 G (c), respectively.

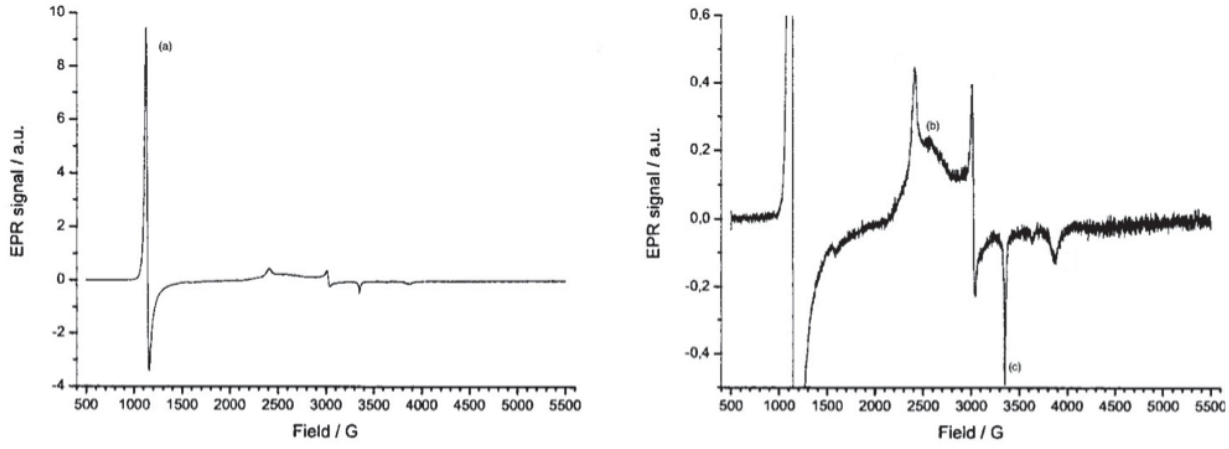

Fig. 13B: EPR spectra of human metMb: The peaks at ≈1100 G (a), ≈2500 G (b) and ≈3350 G (c), respectively, which have been evidenced in horse metMb, were also visible in human metMb,

The nitrite-metMb preparations are displayed in Fig. 14. In every sample it is clear that signal at ≈1000 G predominate (a), which, according to Svistunenko et al., is related to metMb.[123] The line-shape of the signals seen in all samples bear close similarities, their amplitude directly depending upon the concentration of the metMb in the nitrite-buffer solution. It should be emphasized at this point that the second signal (at ≈3200 G (b)), which corresponds to nitrite (as it could also be observed in the control sample - see Fig. 14 -), is highly variable and depends neither upon the concentration of nitrite nor on the presence or concentration of metMb in the respective samples.

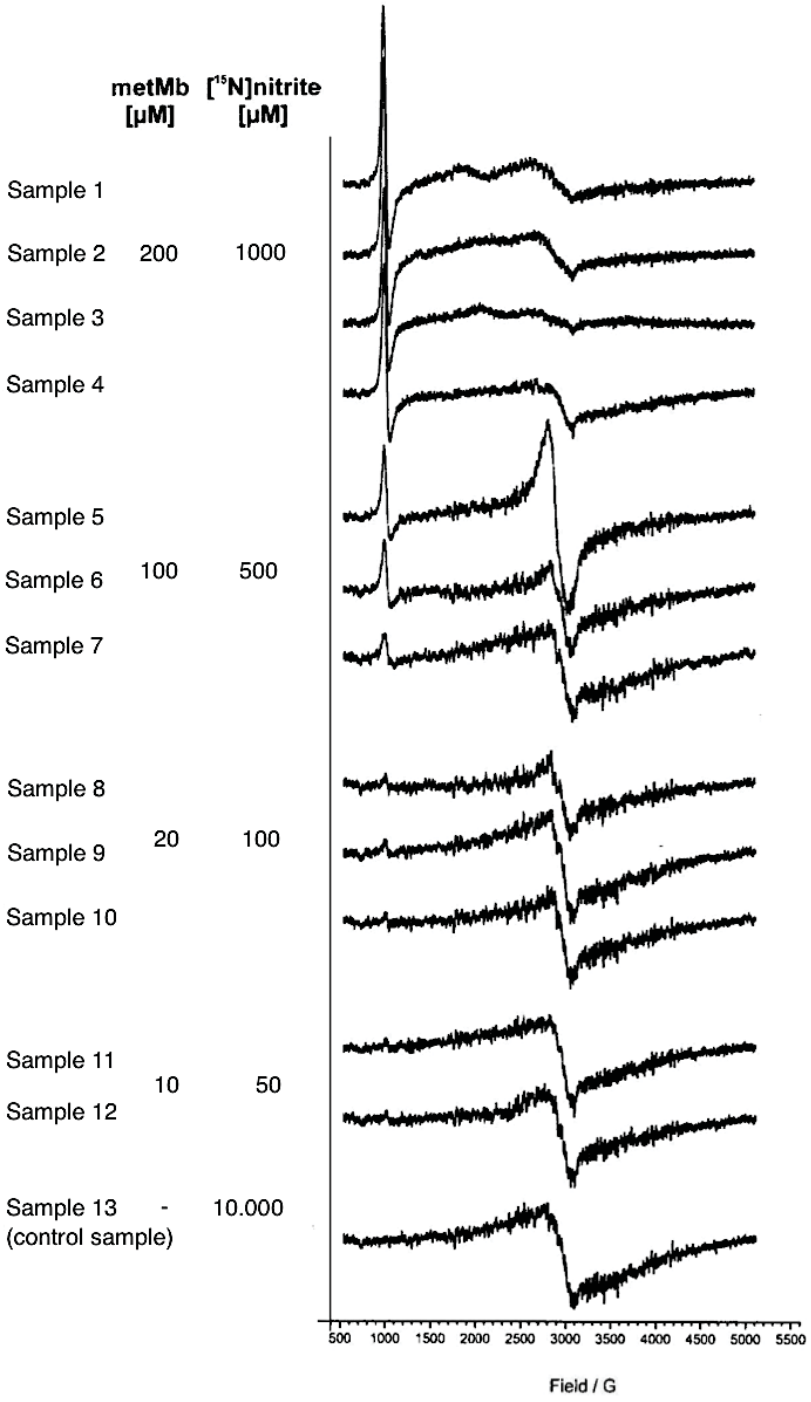

Fig. 14: EPR spectra of nitrite-metMb: Evidence of nitrite-metMb using EPR spectroscopy, with maximal peaks recorded at ≈1000 G (a), which represent the metMb signal, and at ≈3200 G, related to nitrite. In the control sample (sample 13), the signal of nitrite (at ≈3200 G) could be evidenced.

4. Discussion

The functional properties of Mb associated with O_2 homeostasis are already widely-acknowledged within the scientific literature.[1, 30] More recently it has been shown that Mb also plays an essential role in the metabolism of NO in the cardiovascular system.[2, 14, 15, 35] Mb can protect the heart both under normoxic conditions, when it scavenges NO to manage excessive detrimental concentrations[2, 7, 36, 64], as well as under hypoxic conditions, when it reduces nitrite to NO[14], thus increasing its bioavailability. These properties have already been described for both horse and mouse Mb.[14, 36] The first aim of this study was to show that Mb from the human heart regulates the NO homeostasis in the heart, acting as both NO scavenger and NO producer. Using Mb from the human heart, this study confirms that human Mb acts as an NO scavenger, and demonstrates that human Mb exhibits a nitrite reductase activity.

The reaction of nitrite reduction by deoxyMb yields NO and metMb.[14] In accordance with the properties attributed to metHb, metMb further interacts with nitrite resulting in nitrite-metMb formation.[74] The *in vitro* formation of this compound has been already described for metMb from horse skeletal muscle.[77] However, it has remained unclear whether or not heart metMb from horse and human also exhibits a binding affinity for nitrite. The interaction between horse and human heart metMb with nitrite becomes apparent in this study, as demonstrated by EPR signals.

In the following section, the property of Mb to act as a NO scavenger under normoxic conditions will be discussed (4.1). Subsequently, the nitrite reductase activity of horse and human metMb will be debated (4.2). Finally, the interaction between nitrite and metMb will be analyzed using EPR signals (4.3).

4.1 Scavenging of nitric oxide by human myoglobin

Despite its well-accepted role in O_2 transport and storage, new data show an essential role of Mb in maintaining NO homeostasis.[2, 14, 21, 36, 50] Under normoxic conditions, the role as a NO-scavenger represents a major mechanism of reducing excessive intracellular NO.[36] In mammalian biology, NO is an important molecule which regulates a wide range of physiological and pathological phenomena.[10, 39, 54, 65, 68, 93] However, high levels of NO as found in inflammatory conditions can be rather deleterious.[36] Induction of NO in excess is associated with the production of ROS (e.g. peroxynitrite, superoxide) and the induction of cytokines.[64] Superoxide anion (O_2^-) is a reactive oxygen species that further reacts with NO, resulting in peroxynitrite formation.[36] This may cause nitrosative and oxidative stress, which in turn induces apoptosis, inhibition of the respiratory chain of mitochondria and a negative inotropic effect on the myocardium.[36-38] This may finally result in structural damage, such as cardiac fibrosis and remodelling.[36] It has been postulated that iNOS has a causative role in the heart failure pathogenesis because of its properties of producing NO in the nanomolar range.[36] By using a double transgenic mouse model with iNOS overexpression and Mb-deficiency, a pathological phenotype could be identified.[36] The wild type-hearts were not associated with modifications of heart structure or function, thereby showing that Mb can attenuate the nitrosative stress.[36] In addition, by using transgenic hearts with different levels of Mb (i.e., 100%, 50%, 0%), it has been demonstrated that Mb is able to inactivate NO.[36] In 2001 Flögel et al. demonstrated that oxyMb scavenges NO in a dose-dependent manner, the conversion of oxyMb to metMb progressively increasing with the NO concentration.[2] Interestingly, it has been shown that NO also contributes to the expression of Mb gene in the vascular smooth muscle.[124] Together, these data suggest a “feedback relationship” between NO and Mb, which is responsible for the whole body NO homeostasis.

The property of oxyMb as a NO scavenger has been widely explored by investigating mice and horse Mb[2]. In 2001, Witting et al. showed that human

deoxyMb can interact with NO, forming MbNO. Furthermore, in the presence of O_2, human Mb oxidized NO to NO_3^-.[107] In this study, the role of oxyMb as a scavenger of NO in humans and horses was confirmed. UV-Vis Spectroscopy enabled the direct measurement of the conversion of oxyMb to metMb by its reaction with NO. The results were in line with previous spectrophotometric outcomes based on incubation of oxyMb with NO.[122] Additionally, this study showed that human oxyMb can scavenge NO with a significant increase of metMb formation when compared with horse Mb. Remarkably, nitrite was used as the source of NO in this study, which transformed into NO *via* acidic disproportionation. This method resembles *in vivo* chemical pathways more closely, with nitrite being a NO pool under hypoxia.[112] In mammals, nitrite is present at concentrations of 1-20 μM in tissue and 0.3 to 1.0 μM in plasma.[15, 125] In this study, 100 μM nitrite solutions were used in order to obtain a biological response, in accordance with previous investigations by other groups.[126] It is well known that nitrite can directly oxidize oxyMb to metMb.[127] In order to eliminate this possibility in the present study, the experiments were repeated with the same concentration of nitrite at pH 7.4. Under a physiological pH, formation of metMb could not be detected. Therefore, it could be stated that the NO formed through acidic disproportionation was responsible for the oxidation of oxyMb to metMb.

The formation of metMb from oxyMb can occur by means of two different reactions. One is the direct interaction of oxyMb with NO. The second is the nitrosylation of deoxyMb, resulting in MbNO. Furthermore, MbNO reacts with O_2, yielding metMb and NO_3^-.[2] It is unclear from the present experimental data whether metMb was formed through the direct or the intermediary reaction. However, given that the Mb solution used in this study was a completely oxygenated Mb preparation, it is more likely that the reaction directly occurred between NO and oxyMb and not NO and deoxyMb. Under *in situ* conditions, especially in the proximity of the mitochondria, Mb is partially deoxygenated, meaning that the second reaction is likely to be of a greater relevance.[2, 103]

Unlike Mb from other mammals, human Mb exhibits a free thiol group, which can also react with NO. It has been demonstrated that the reaction of human oxyMb

with NO results in both oxidation of NO to nitrate with metMb formation and S-nitrosation at Cys^{110}.[107] Therefore, under normoxic conditions, human Mb may inactivate NO not only by scavenging it[2], but also through binding NO to its sulfur-containing cysteine. In addition, NO may induce post-translational modifications of human Mb through S-nitrosation of the cysteine residue.[122, 128, 129] Importantly, the accessibility of the free thiol group possessed by human Mb does not depend upon the Mb O_2 saturation.[122] It is therefore logical to conclude that with the S-nitrosation of the Cys^{110} also taking place under hypoxic conditions, the enzymatic activity of human deoxyMb could be affected.

4.2 Nitrite reductase activity of human myoglobin

Mammalian Mb has been extensively studied, in order to fully understand its function in cardiac muscle under low O_2 pressures. Several experimental studies have demonstrated that Mb can react with nitrite under hypoxic conditions, forming NO.[14, 15] Analogous in-depth studies of human Mb have been delayed due to technical difficulties and ethical issues, related to the use of biopsy specimens obtained from healthy individuals for research purposes. This study reports a previously undescribed function of human Mb: the reaction of deoxyMb with nitrite to NO.

Nitrite is known to contribute to cardioprotection during I/R.[125] It is known that these protective effects occur secondary to NO formation.[9] The enzymatic generation of NO from L-arginine is widely accepted within the literature to be the primary source of NO.[9] However, under hypoxic conditions, this pathway will decrease, as the NOS enzymes require O_2, which is rapidly depleted under ischemia.[12, 69] It has been postulated that the alternative pathway of NO synthesis is the reduction of nitrite *via* NOS-independent enzymatic catalysis.[69, 112] Recently, it has been reported that the reaction of deoxyHb with nitrite contributes to anaerobic NO production.[16] Subsequently, given the structural similarity between hemeglobins (such as Mb and Hb), the activity of Mb as a nitrite reductase has been thoroughly assessed.[130, 131] Moreover, because of the significantly lower redox potential of its

heme group (i.e. a higher ability to donate electrons), it has been shown that deoxyMb reduces nitrite to NO at a faster rate than deoxyHb is able to.[103]

The mechanism of nitrite reduction to NO by Mb is dependent upon the tissue O_2 level. Mb must be at least partially deoxygenated in order to exert its nitrite reductase activity.[83, 103] This precondition implies the protective role of Mb within the myocardium when the coronary blood flow is critically reduced. Under hypoxic conditions, Mb contributes to the „short-term hibernation“, a phenomenon which is characterized by a downregulation in the O_2 consumption of the ischemic region, which improves the metabolic state of the myocardium.[14, 103, 132] Experimental studies have indicated that under hypoxia, NO is released from nitrite by deoxyMb within the vicinity of mitochondria.[83] Furthermore, it interacts with components of mitochondria (e.g. cytochrome c oxidase), and results in a reduction of O_2 consumption, thus restoring the cardiac energy balance.[14] In addition to this, it has also been demonstrated that under more severe conditions - like myocardial infarction - Mb also acts as a nitrite reductase, thereby attenuating the I/R injury.[15] Through direct inhibition of complex I of the respiratory chain, NO leads to a reduction of its activity, with subsequent limitation of ROS production and amelioration of oxidative damage.[93]

The relevance of Mb in cardiac function was shown by taking advantage of $Mb^{-/-}$ mice. It was observed that in mice with Mb, the application of nitrite led to a dramatic decrease in infarct size by more than 60% in comparison to mice without Mb.[15] However, the nitrite reductase function of Mb under hypoxic conditions is not only relevant for the heart. Recently, a 50% decrease in hypoxia-induced systemic vasodilator response in $Mb^{-/-}$ mice compared to $Mb^{+/+}$ was observed, demonstrating the essential role of Mb in systemic hypoxic vasodilation.[78]

Using the $Mb^{-/-}$ mouse model, it has been shown in this study that under hypoxic conditions, deoxyMb is required for the production of NO from nitrite. These results demonstrate that Mb deficiency reduces the production of NO from nitrite. By adding Mb from horse and human hearts, the production of NO could be successfully replenished. The reduction of nitrite by deoxyMb was more pronounced after administration of equal human Mb, when compared with horse

Mb. It should be emphasized at this juncture that immediately after the administration of human and horse oxyMb to the homogenates from $Mb^{-/-}$ mice, oxyMb acted first as an NO scavenger, producing nitrate and metMb. After deoxygenation, Mb changed from an NO scavenger to an NO producer. These results highlight the property of Mb to behave as an „oxygen sensor", being able to „shift its role from an NO scavenger to an NO producer", according to its oxygenation state. [14, 25, 35, 49, 103]

Different studies have shown that under hypoxic conditions, nitrite can be converted to NO through a reductive reaction, not only *via* deoxyHb[16] and deoxyMb[83], but also *via* a variety of different compounds including XO[72], components of the respiratory chain in mitochondria[81], or through acidic disproportionation[119]. However, this study has shown that the release of NO is not affected after inhibiting an alternative nitrite-dependent NO producer - such as XO - with allopurinol and diphenyliodonium (DPI). Experimental studies have indicated that the increase in NO concentration during hypoxia may also be due to an activation of eNOS, which reacts with and reduces nitrite to NO.[68, 133] However, the inhibition of NOS with L-NAME did not significantly reduce the formation of NO. Moreover, in order to exclude a potential reduction of nitrite to NO by deoxyHb, the isolated mouse hearts have been previously perfused free of blood. Therefore, in the present study - under acidosis and hypoxia - the production of NO can be attributed to two distinct processes: the acidic disproportionation, which represents a simple nitrite protonation (Eq. (1)), and the enzymatic reduction of nitrite by deoxyMb (Eq. (2)).

$$(1)\ NO_2^- + 2H^+ \rightarrow H_2O + NO$$

$$(2)\ NO_2^- + \text{deoxyMb} \rightarrow \text{metMb} + NO$$

As soon as NO is generated, part of it reacts with thiols, resulting in the formation of RSNOs. Human Mb exhibits a cysteine residue at position 110 of the amino acid

sequence (Cys^{110}), which can be S-nitrosated, whereas horse Mb does not.[106, 107] It is already known that proteins can be regulated by S-nitrosation of cysteine residues.[129] This study has demonstrated no significant change in NO formation rate by blocking the additional thiol group in human Mb. This suggests that after NO production, the enzymatic activity of human deoxyMb is unaffected, despite the fact that the thiol group may represent an S-nitrosation target, which can lead to NO-mediated post-translational protein modifications.

These data provide evidence for a major mechanism to increase NO bioavailability. Nevertheless, further studies are required to confirm the protective role of human Mb. In order to strengthen the validity of the study findings, *in vivo* investigations are also required.

4.3 Binding affinity of metmyoglobin for nitrite

It is already known that nitrite represents a „reservoir of bioavailable NO".[35] Various studies have shown the role of nitrite as an endogenous mediator of NO homeostasis during hypoxia.[134] Recently, the cytoprotective effects of the exogenous nitrite (including dietary administration of inorganic nitrate as a significant source of nitrite *via* the „nitrate-nitrite-NO pathway") have been verified by numerous research groups.[12, 15, 45, 135, 136] Physiologically, the majority of nitrite is produced *via* the L-arginine NOS pathway which accounts for approximately 70% of the total nitrite, the rest is acquired through diet.[45, 135, 136] Under pathological conditions, exogenous nitrite/nitrate have been demonstrated to have a wide spectrum of beneficial effects, from inhibition of platelet aggregation[137], to reduction of arterial blood pressure[138, 139], improvement of endothelial dysfunction[140, 141], amelioration of pulmonary hypertension[142], and protection against I/R injury[12, 15]. Nitrite appears to mediate these protective functions through reduction to NO. This occurs *via* a range of proteins, including deoxygenated heme-globins (Mb, Hb, neuroglobin, cytoglobin), cytochrome P450 enzymes, mitochrondrial proteins, and molybdo-flavoproteins.[69, 85]

Within the cardiomyocyte, deoxyMb reduces nitrite to NO that further modulates

hypoxic signaling.[25] As nitrite is a requisite substrate for the production of NO, it is tempting to speculate that circulating nitrite penetrates the cell membrane, and accumulates inside the cardiomyocyte. It has been suggested that nitrite is transported across the cellular membranes either through anion exchangers, or *via* diffusion - in the form of undissociated nitrous acid (HNO_2) - however, the exact mechanisms by which this is accomplished still represent a significant research challenge.[143, 144] It may be supposed that NO is readily synthesized outside the cardiomyocyte (e.g. *via* nitrite reduction by deoxyHb), and that the circulating NO traverses the cellular membrane, thus exerting cardioprotective functions. However, this supposition is unreasonable, given the fact that in $Mb^{+/+}$ mice nitrite pretreatment resulted in a reduction of myocardial infarction by 61%, whereas in $Mb^{-/-}$ mice the administration of nitrite had no protective effects.[15] This data suggests that nitrite is imported into the cardiomyocyte, where it is reduced by deoxyMb with formation of NO and metMb. Analogous to what has been already described for metHb, metMb may further react with nitrite resulting in nitrite-metMb formation.[73] Formation of this compound - nitrite-metMb - could represent a marker of the import of nitrite into the cardiomyocyte. In the case of Hb, EPR spectroscopy was employed to explore the chemistry of nitrite-metHb, and it has been shown that this compound is more stable at lower pH.[74] Given the affinity of metMb for nitrite and its electronic configuration, nitrite-metMb has also been detected by EPR spectroscopy using metMb from horse skeletal muscle.[77] However, it remains unknown whether metMb from horse and human heart also exhibits a binding affinity for nitrite.

Therefore, the second aim of this study was to detect the interaction between nitrite and horse/human heart metMb, using EPR spectroscopy. This could represent an approach to detect nitrite-metMb, as a prerequisite for the intracellular detection of the exogenously applied [^{15}N]nitrite in future studies which investigate the therapeutic potential of nitrite.

To test this hypothesis, solutions with increasing concentrations of metMb from horse or human heart (10 to 200 μM) and isotopically labeled [^{15}N]nitrite (50 to 1000 μM) were incubated for 5 minutes, and then frozen for subsequent analysis.

Using EPR spectroscopy, nitrite-metMb signals could be detected. Their amplitude was directly dependent upon the concentration of metMb in the nitrite solution. The results indicate that the reaction previously described for Hb within the red blood cell may also take place in the horse and human cardiomyocyte, i.e. that metMb reacts with nitrite yielding nitrite-bound metMb. The chemistry for this reaction was described by Wanat et al. in 2001, using an aqueous Mb solution prepared from horse skeletal muscle at a physiological pH of 7.4 (Eqs. (1), (2)), where $k_{1,2}$ represent the equilibrium constansts.[77]

$$(1)\ \text{metMb-Fe}^{3+}(H_20) \overset{k_1}{\Leftrightarrow} \text{metMb-Fe}^{3+} + H_2O$$

$$(2)\ \text{metMb-Fe}^{3+} + NO_2^- \overset{k_2}{\Leftrightarrow} \text{metMb-Fe}^{3+}\text{-}NO_2^-$$

The reaction of horse heart Mb with nitrite over a range of pH values between 5.0 and 10.8 has been already analyzed.[145] It has been shown that nitrite is able to react with Mb containing the heme in both oxidation states, depending upon the degree of hypoxia. Under severely hypoxic conditions associated with a lower pH, the nitrite reductase activity predominated with formation of NO and metMb.[145] Interestingly, under mild hypoxia, Mb - in the form of metMb - demonstrated an increased affinity for nitrite[145], which may suggest that at a lower pH, nitrite binds to metMb rather than remaining free in the cytosol. In this study, the reaction between metMb from horse and human heart with formation of nitrite-metMb was demonstrated by EPR signals. In addition, the incubation of metMb with nitrite over a wide range of concentrations resulted in a dose-dependent amplitude of signals, the intensity of this reaction increasing with the metMb concentration in the nitrite solution. It is worth noting that EPR spectroscopy was able to determine formation of nitrite-metMb even at low concentrations of nitrite and metMb, emphasizing the sensitivity of this method.

The interaction between metMb and nitrite - with formation of nitrite-metMb - could represent an important step towards the intracellular detection of exogenously

applied [^{15}N]. Based on analysis of EPR signals, an estimation of the intracellular concentration of exogenous nitrite may also be possible in the future.

5. Conclusions

In summary, the data presented in this study extend the existing knowledge with regards to the functional properties of human Mb. Using Mb from human heart, this study confirms that oxyMb acts as an NO scavenger and demonstrates that deoxyMb exhibits a nitrite reductase activity. Furthermore, it substantiates that rates of both nitrite reduction to NO and NO scavenging are more pronounced in human than horse Mb. Finally, based on the interaction between nitrite and human metMb, as demonstrated by EPR signals, this study presents an approach to detect intracellular nitrite-metMb. This is a prerequisite for further study to investigate the therapeutic potential of exogenously applied nitrite.

These results therefore provide new insights related to the properties of human Mb, and pave the way for future studies to investigate the importance of Mb for NO metabolism in humans.

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Acknowledgments

I would like to thank Prof. Dr. M. Kelm for giving me the opportunity to work in the Cardiovascular Research Laboratory of the University Heinrich-Heine Düsseldorf, for trusting me and allowing me to work alongside his team.

I also want to express my sincere gratitude to Prof. Dr. T. Rassaf, the leader of my workgroup who coordinated this project, for the insightful helpful discussions and guidance in my research.

I would especially like to thank my supervisor, Dr. Matthias Totzeck, not only for his valuable advice and guidance every step of the way, but also for his patience and thoughtfulness during the finalization of this thesis. Without his continuous support, this thesis may have never been completed. His aptitude and passion for research will always remain a source of inspiration for me.

I also want to express my special thanks to Dr. rer. nat Ulrike Hendgen-Cotta for the valuable suggestions and helpful discussions that clarified my ideas.

I owe many thanks to Dr. Eva Kehmeier, Dr. Shazia Afzal, Dr. Ralf Erkens and Dr. Ross Cunningham for their helpful and their critical comments on this work.

I want to thank Dr. Jan Balzer for first giving me the opportunity to come in the Department of Cardiology as a student in practice. I want to thank him for his encouragement and guidance over the past 4 years.

Finally, I want to express my deepest gratitude to my parents and my sister for their unconditional love and support.

Eidesstattliche Versicherung

Ich versichere an Eides statt, dass die Dissertation selbstständig und ohne unzulässige fremde Hilfe erstellt worden ist und die hier vorgelegte Dissertation nicht von einer anderen Medizinischen Fakultät abgelehnt worden ist.

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