Human S-Nitroso Oxymyoglobin Is a Store of Vasoactive Nitric Oxide

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Nitric oxide (NO) regulates vascular function, and myoglobin (Mb) is a heme protein present in skeletal, cardiac, and smooth muscle, where it facilitates O2 transfer. Human ferric Mb binds NO to yield nitrosyl-heme and S-nitroso (S-NO) Mb (Witting, P. K., Douglas, D. J., and Mauk, A. G. (2001) J. Biol. Chem. 276, 3991–3998). Here we show that human ferrous oxy-myoglobin (oxyMb) oxidizes NO, with a second order rate constant k = 2.8 ± 0.1 × 107 M−1 s−1 as determined by stopped-flow spectrophotometry. Mixtures containing oxyMb and S-nitrosoglutathione or S-nitrosocysteine added at 1.5–2 moles of S-nitrosothiol/mol oxyMb yielded S-NO oxyMb through trans-nitrosation equilibria as confirmed with mass spectrometry. Rate constants for the equilibrium reactions were kforward = 110 ± 3 and kreverse = 16 ± 3 m−1 s−1 for S-nitrosoglutathione and kforward = 293 ± 5 and kreverse = 20 ± 2 m−1 s−1 for S-nitrosocysteine. Incubation of S-NO oxyMb with Cu2+ ions stimulated NO release as measured with a NO electrode. Similarly, Cu2+ released NO from Mb immunoprecipitated from cultured human vascular smooth muscle cells (VSMCs) that were pre-treated with diethylaminononoate. NO release was observed from VSMCs treated with vehicle alone or immunoprecipitates obtained from porcine aortic endothelial cells with and without diethylaminononoate treatment. Importantly, pre-constricted aortic rings relaxed in the presence of S-NO oxyMb in a cyclic GMP-dependent process. These data indicate that human oxyMb rapidly oxidizes NO and that biologically relevant S-nitrosothiols can trans-(S)nitrosate human oxyMb. Furthermore, S-NO oxyMb can be isolated from cultured human VSMCs exposed to an exogenous NO donor at physiologic concentration. The potential biologic implications of S-NO oxyMb acting as a source of NO are discussed.

Endothelium-derived NO, generated through the action of nitric-oxide synthase(s) on L-arginine, plays a vital role in blood vessel dilation and thereby in the regulation of peripheral vascular resistance and ultimately circulating blood pressure (1, 2). To elicit vessel dilation, NO binds to and activates its molecular target, soluble guanylyl cyclase (3), within vascular smooth muscle cells (VSMCs), which in turn catalyzes the conversion of guanosine-5′-(3-thiotriphosphate) to cyclic GMP (cGMP) (4). Synthesized cGMP activates a cascade of effector proteins that initiates VSMC relaxation and thereby promotes vessel dilation.

The heme protein myoglobin (Mb) is present in cardiac, skeletal, and human smooth muscle (5, 6). In cardiac muscle, the concentration of Mb ranges from 0.3 to 0.5 mM (5), whereas the precise concentration of Mb in smooth muscle is not known. The role of intracellular Mb is generally accepted as that of a passive di-oxygen storage protein that facilitates di-oxygen transfer from the extra- to intracellular space. However, in vitro studies have shown that oxygenated ferrous Mb (oxyMb) also rapidly reacts with dissolved NO gas (k = 107 M−1 s−1) to yield higher order N-oxides such as nitrate (7). In addition, both ferrous deoxy and ferric Mb form stable heme-NO complexes (Mb-NO) with dissolved NO gas (dissociation constant Kd = 10−5 M) (8). Together, these chemical reactions have the potential to effectively eliminate NO within its expected lifetime in biological systems, suggesting that Mb could play an active role in maintaining NO homeostasis. Indeed, there is support for this notion. For example, Mb limits the extent of NO-induced inactivation of cytochrome c oxidase (9). Also, conversion of ferrous to ferric Mb regulates the myocardial concentration of NO (10), which is crucial for maintaining overall heart function, coronary blood flow, and contractility, processes that are more severely affected by NO in mice lacking Mb compared with wild-type animals (10). Thus, the focus for Mb has shifted from O2 transport to a central regulatory role in NO homeostasis (11).

An intriguing feature of human Mb is that it possesses a reactive cysteine residue (Cys110) (12, 13). Under aerobic conditions, Cys110 reacts with NO to yield S-NO Mb (14), similar to S-NO Hb (15). Unlike Hb, however, for which nitrosation is dependent on the allosteric (R-T) transition (16), the degree of Mb oxygen saturation does not affect accessibility of the Cys110 for S-nitrosation, as judged by comparable x-ray crystal structures of ferric and ferrous Mb bound to a diatomic ligand (see Fig. 5 in Ref. 17).

Under physiologic conditions, cytochrome b5 reductase main-

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1 The abbreviations used are: VSMC, vascular smooth muscle cell; cGMP, cyclic GMP; CYS-NO, S-nitrosocysteine; DeaNO, diethylenemnononate; DTT, dithiothreitol; DTPA, diethylenetriaminepentacetic acid; ESI-MS, electrospray ionization mass spectrometry; GSH, reduced glutathione; GS-NO, S-nitrosoglutathione; HPSS, HEPES-buffed physiologic salt solution; Mb, myoglobin, oxyMb, ferrous oxy-myoglobin; PAEC, porcine aortic endothelial cell; SNP, sodium nitroprusside; S-NO, S-nitroso; RS-NO, S-nitrosothiol; HPLC, high performance liquid chromatography.
contains Mb in the reduced state for oxygenation to yield oxyMb (18). It is not clear at present whether S-nitrosation of ferrous oxyMb is feasible under physiologic conditions, although one would expect oxyMb to rapidly oxidize 'NO based on the high rate constant for the reaction of oxyMb and dissolved 'NO gas (19). Herein we demonstrate that Cys\textsuperscript{110} S-nitrosation of human ferrous oxyMb (the predominant physiologic form of the protein) occurs through trans-nitrosation equilibria reactions with low molecular mass S-nitrosothiols (RS-NO), that 'NO released from S-NO oxyMb can dilate pre-constricted vessels similar to authentic endothelium-derived relaxant factor, and that Mb immunoprecipitated from VSMCs pre-treated with an 'NO donor can release 'NO.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trypsin, Triton X-100, phenylephrine, urea, EDTA, DTPA, trypثن, copper(Il)sulfate, acrylamide, ammonium persulfate, sodium dodecylsulfate, reduced glutathione (GS\textsubscript{H}), sodium nitroprusside (SNP), cysteine, and yeast extract were obtained from Sigma. Dithiothreitol (DTT) was obtained from Fisher Scientific. Dithiylamino-noreactone (DeaNO); a chemical source of 'NO and H\textsubscript{1}-\textsubscript{2},4,4-oxadiazole-2-ylidene (azo-dye) were obtained from Cayman Chemicals (Ann Arbor, MI), and authentic 'NO gas was obtained from BOC Gases (Sydney, Australia). Buffers were prepared from Nanopure water and stored over Chelex-100 (Bio-Rad) at 4 °C to remove containing transition metals (20). Except for isolated vessel studies, DTPA and EDTA (final concentrations, 100 \muM) were added to all reaction mixtures to minimize the possibility of metal-mediated decay of RS-NO (21). Solvents and all other chemicals employed were of the highest quality available.

**Animals**—New Zealand White rabbits (2.5–3 kg) were obtained from a commercial farm (Wauchope, New South Wales, Australia) and housed individually for the entire study period. Rabbits received normal chow with feed and water provided ad libitum for an acclimation period of 2 weeks. Local ethics committee approval was obtained before commencing the study.

**Cell Culture**—Cultured primary human VSMCs (American Type Culture Collection) and porcine aortic endothelial cells (PAECs; Cell Applications) were maintained in Dulbecco's modified Eagle's medium/ Ham's F-12 (JRH Biosciences) and M199 media, respectively. All culture media preparations were supplemented with 10% fetal bovine serum (Sigma), 100 mg/ml streptomycin, 100 units/ml penicillin, and 1 \mug/ml gentamicin. All media were replaced every 3–4 days and media were changed for an acclimation period of 2 weeks.

**Preparation of Recombinant Wild-type and C110A Variant Human Mb**—DNA manipulations were performed as described previously (22). Purification of Mb (23) and Mb sequence confirmation were performed by DNA sequence analysis before protein expression in bacteria. The sequence was confirmed, the BamH\textsubscript{I}-HindIII fragment from the amplified DNA, which also contained the murine Mb coding, was ligated to the BamH\textsubscript{I}-HindIII fragment from the pMb3 vector to yield the expression vector (12) that was later transformed to the appropriate cell line for protein expression as described previously (23). Preparations of purified wild-type and C110A variant of human Mb were snap-frozen in liquid nitrogen and stored at ~80 °C before use. All preparations exhibited a $A_280/A_350$ ratio of peak absorbance of 5 (data not shown), indicative of the purity of the protein preparations.

**Preparation of S-NO oxyMb**—Recombinant human Mb was prepared by chemical reduction of the recombinant human ferric Mb with a 2-fold excess of DTT and stirring under an atmosphere of air for 10 min. Residual DTT was removed from the preparation by three successive gel filtration columns (PD-10 pre-packed column; Amersham Biosciences). Formation of oxyMb was confirmed by electronic absorbance spectroscopy. For samples designated for mass analyses, S-NO oxyMb was purified with simultaneous change of buffer to Nanopure water containing 100 \muM DTPA (high salt concentrations yield protein adducts that affect mass determinations). Finally, S-nitrosation of oxyMb was confirmed by an increased absorbance at $A_{357}$ ($\epsilon_{357}$ mm$^{-1}$ cm$^{-1}$) and verified unambiguously by electrospray ionization mass spectrometry (ESI-MS) (see below). The preparation of S-NO oxyMb were maintained in the dark and used within 5 min of purification.

**Stopped-flow Kinetic Measurements**—Where required, saturated solutions of authentic 'NO gas were prepared, and the concentration of dissolved gas was standardized as described previously (14). Kinetic determinations for the reaction of oxyMb with dissolved 'NO gas were performed with an Applied Photophysics SX-17 MV stopped-flow spectrophotometer as described previously (23). Typically, 250 time-dependent spectra (logarithmic time base; integration, 2.56 ms; dead time, ~2 ms; $A_\lambda$ = 350–750 nm; resolution, 1 nm) were collected at 25 °C. Kinetic data were processed using Pro-Kinetist global analysis software (Pro-Kinetist version 4.1; Applied Photophysics, Leatherhead, UK), as values used previously (25). Apparent rate constants ($k_{app}$) were then determined by linear regression.

**Mass Analyses**—Where required, molecular mass was measured routinely for native and modified Mb by ESI-MS as described in detail elsewhere (14). Briefly, mass spectra were acquired using a hybrid tandem mass spectrometer (Applied Biosystems, Foster City, CA). Samples (~10 pmol, 1 \muL) were dissolved in water:acetonitrile (~20:80) and loaded onto the trans-nanospray LTQ instrument as a solution (~10 mm from the orifice). Nitrogen was used as curtain gas, and a potential of +800 V was applied to the needle. Next, time-of-flight scan was achieved with $m/z$ 50–2000, 1 s and accumulated for ~1 min into a single file. These conditions favor the detection of Mb apoprotein due to unfolding of the tertiary structure and loss of the heme prosthetic group (26). Mass accuracy of the system was tested routinely before use. Mass values were obtained by standard fitting analyses of the various $m/z$ distributions.

**Measurement of NO from RS-NO**—Release of NO from a range of low molecular mass or proteinaceous RS-NO was monitored by a NO-selective electrode (ISO-NO MII; World Precision Instruments Inc.) coupled with a DUO-18TM data recorder (v1.55; World Precision Instruments Inc.). Briefly, the electrode was pre-equilibrated in phosphate buffer (50 mM, pH 7.4) containing 100 \muM copper(II)sulfate (Cu\textsubscript{2+}) under an atmosphere of argon gas. Authentic RS-NO (or immunoprecipitated from VSMCs or PAECs) was added to the solution, and the time-dependent increase in current was monitored until the current stabilized. Area under the peak response curve was estimated with integration software supplied with the data recording system. The amount of NO liberated from the various RS-NO was then compared with a standard calibration curve generated using authentic GS-NO (solution concentration standardized with $\epsilon_{366}$ mm$^{-1}$ cm$^{-1}$) prepared as described previously (24). Standard curves were generated before commencement of individual experiments to verify electrode function and to account for the day-to-day variation in electrode response factor.

**Assessment of Vessel Relaxation**—Vessel bioassays were performed with an Isometric Tension Instrument, Australia. Vessels were suspended in a bath, with a vessel myobath (WPI, Coherent, Australia) as described in detail elsewhere (27). Briefly, rabbit aortic rings were suspended in organ chambers and incubated in Krebs buffer with constant degassing (Carbogen gas mixture, 5% CO\textsubscript{2}, 95% O\textsubscript{2}) for 30 min. After equilibration, rings were pre-constricted with a dose of phenylephrine to half the maximal constriction force value. Vessel relaxation was assayed in response to SNP (positive control), S-NO oxyMb (prepared by trans-nitrosation with CYS-NO), and the oxyC110A variant of human Mb pre-treated with CYS-NO in identical fashion to wild-type oxyMb (negative control), and dilution was expressed as a percentage of the pre-constriction force value. In some studies, vessels were denuded of the endothelium by gently applying the blunt end of a surgical tweezer to the inner surface of the aortic ring. This procedure had no effect on constriction to phenylephrine, although it eliminated the vessel response to the endothelium-dependent agonist acetylcholine (data not shown).

**Preparation of Aortic Homogenates for cGMP Assessment**—To determine tissue cGMP, aortic rings were first incubated at 37 °C in Krebs buffer containing 5 \muM 3-isobutyl-1-methylxanthine and Com
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**Complete® protease inhibitors mixture added as per the manufacturer’s instructions.**냥

**DTPA, tryptone, copper(Il)sulfate, acrylamide, ammonium persulfate, sodium dodecylsulfate, reduced glutathione (GS\textsubscript{H}), sodium nitroprusside (SNP), cysteine, and yeast extract were obtained from Sigma. Dithiothreitol (DTT) was obtained from Fisher Scientific.**냥

**Absorption spectroscopy with characteristic absorptions at 543 and 680 nm. Samples of recombinant human oxyMb were then treated with either S-nitrosogluthathione (GS-NO) or S-nitrosocysteine (CYS-NO) (final concentration of added NO < 2 \mumol/\muL) dispersed in phosphate buffer (50 mM, pH 7) and then left to equilibrate in the dark at 20 °C. Stock solutions of GS-NO or CYS-NO were prepared immediately before use as described previously (24). After 60 min of equilibration, excess low molecular mass RS-NO was removed by repeated size exclusion chromatography. For samples designated for mass analyses, S-NO oxyMb was purified with simultaneous change of buffer to Nanopure water containing 100 \muM DTPA (high salt concentrations yield protein adducts that affect mass determinations). Finally, S-nitrosation of oxyMb was confirmed by an increased absorbance at $A_{357}$ ($\epsilon_{357}$ mm$^{-1}$ cm$^{-1}$) and verified unambiguously by electrospray ionization mass spectrometry (ESI-MS) (see below). The preparation of S-NO oxyMb were maintained in the dark and used within 5 min of purification.
instruction (Roche Applied Science). The tissue was then homogenized with a rotating piston and matching Teflon-lined tube as described previously (28). Samples of homogenate (50 μl) were removed for protein determination (BCA assay; Sigma), and the remainder was employed for tissue cGMP determinations using a commercial kit (Cayman Chemical).

**Monitoring Accumulation of the Native Human Mb and Its Homodimer**—Where required, reaction mixtures employed for NO evolution studies were subsequently analyzed for the accumulation of a Mb disulfide dimer (expected mass, 34,107 atomic mass units) by SDS-PAGE and after staining with Coomassie Blue as described previously (13). Where required, selected samples were also pre-incubated with DTT to reduce disulfide cross-links.

In some experiments, confluent cultured human VSMCs or PAECs (3×10^6 cells) were washed thoroughly with HPSS buffer as described previously (29), overlaid with HPSS, and treated without (vehicle alone, control) or with DeaNO administered at final concentration of 100 nM or 10 μM (corresponding to a rate of NO release of 0.6 or 60 nmol min⁻¹, respectively, as determined from the half-life (τ/2) = 2 min at 37 °C indicated by the manufacturer). Control and NO-treated cells were harvested after a 5- or 60-min incubation, washed thoroughly with phosphate-buffered (50 mM, pH 6.5) lysis solution containing 1% (v/v) Triton X-100, a Complete® mixture of protease inhibitors (Roche Applied Science), and 100 μM DTPA. Next, cells were lysed by repeated (3×1) freeze-thaw and centrifuged (15,000 rpm), and the supernatant was treated with monoclonal anti-human Mb antibody (final dilution, 1:500(v/v); Sigma) followed by addition of G-protein-linked Sepharose (Sigma) to yield immunoprecipitates of cytosolic Mb. The presence of Mb in isolated immunoprecipitates was confirmed by SDS-PAGE and Western blotting and tested for stored NO using the ‘NO-selective electrode in the presence of 100 μM Cu²⁺. Suitable controls included generation of immunoprecipitate samples with PAECs and G-protein-linked Sepharose in the presence and absence of Mb antibody.

**Kinetics of Human oxyMb Trans-nitrosation by Added RS-NO**—The consumption of GS-NO or CYS-NO and concomitant accumulation of their corresponding reduced thiol forms were monitored time-dependently in the presence of human oxyMb using high performance liquid chromatography (HPLC) as described previously (30), with minor modifications. Briefly, 25 μM oxyMb was treated with 2-fold molar excess of GS-NO or CYS-NO in the presence of 100 μM DTPA, and the reaction mixture was equilibrated in the dark at 20 °C. Reaction mixtures were sampled at regular intervals over 60 min as indicated in the figures. Samples were then treated with trichloroacetic acid (final concentration, 4% (v/v)) to precipitate the protein and centrifuged, and the supernatant was analyzed using a LC-18 column (5 μM, 25×0.46 cm) eluted at 0.6 ml/min with a 10 mM sodium acetate buffer (pH 5.5) containing 50 μM DTPA and monitored at 214 nm for RS-NO and reduced thiols. Retention times for the various analytes were as follows: GSH, 5.1 min; GS-NO, 7.4 min; cysteine, 4.9 min; and CYS-NO, 5.8 min. The peak absorbance with retention time 7.8 min was present in all preparations of GS-NO and showed no increase in area in the presence of human oxyMb (data not shown). Reduced thiols were standardized using authentic commercial samples (GSH and cysteine), whereas corresponding RS-NO was generated and standardized as described previously (24). Where required, rate constants were determined by data fitting with a curve generated as described previously (31) and using Prism software version 3.0 (GraphPad Software).

**Electronic Absorption Spectra**—Where required, steady-state concentrations of ferric Mb, oxyMb, and nitrosyl-Mb solutions prepared from recombinant human Mb in 50 mM phosphate buffer (pH 7.4) were quantified using the appropriate extinction values (ε_max = 153 (32), ε_Mn = 14.4, and ε_HI5 = 10.5 mm⁻¹ cm⁻¹ (19, 33), respectively). Quantitative absorption spectroscopy was performed with a multi-well plate reader (Model 550; Bio-Rad) or with a UV-visible spectrophotometer (UV-1601; Shimadzu).

**Statistical Analyses**—Statistical differences in relaxation response obtained from the vessel studies were determined with one-way analysis of variance analyses. Student’s t tests were performed to determine significant changes between data sets, with Welch’s correction employed for unequal variances where appropriate. In all cases, statistical significance was accepted at the 95% confidence interval (p < 0.05).

**RESULTS**

The second order rate constant for the reaction of oxyMb with dissolved NO gas has been determined for a various mammalian Mb, and ranges between -0.3 and 4.4 × 10⁷ M⁻¹s⁻¹ (7, 19, 34). Consistent with these studies, mixing recombinant human oxyMb with increasing concentrations of NO resulted in the rapid, dose-dependent conversion of ferrous oxyMb to ferric Mb (Fig. 1, A and B). Global simulation of the data afforded estimates for the observed rate constant of 2.8 ± 0.1 × 10⁻⁷ M⁻¹s⁻¹ with a linear fit of R² = 0.98 (Fig. 1C).

Parallel steady-state product analyses indicated a rapid and near stoichiometric conversion of oxygen Mb to ferric Mb with increasing dose of the chemical NO donor (DeaNO), as estimated by electronic absorbance spectroscopy (Fig. 2). Interestingly, ferrous nitrosyl-Mb (Mb(NO)) formed in relatively minor yields at ≤1 mol of the chemical NO donor per mole of oxyMb (Fig. 2). In contrast to this rapid conversion of oxyMb to ferric Mb by DeaNO, addition in the dark of GS-NO or CYS-NO (data not shown) to human oxyMb at <2 mol RS-NO/mol oxyMb did not promote significant oxyMb oxidation (Fig. 2). Notably, Mb(NO) was not detected (limit of detection, ~0.1 μM) over the dose range of RS-NO employed. Increasing the ratio of added RS-NO to >2 mol RS-NO/mol oxyMb significantly increased the extent of oxyMb oxidation, indicating that free NO was released from RS-NO despite the presence of the metal chelating agents EDTA and DTPA. Alternatively, RS-NO may be oxidized directly by ferrous deoxyMb, present in preparations of oxyMb, in analogy to the oxidation of GS-NO by ferrous deoxyhemoglobin (35). In contrast, addition of the corresponding concentration of GSH or cysteine alone did not affect rates of oxyMb autoxidation (data not shown). Subsequently, all trans-nitrosation reactions were performed with RS-NO in low concentrations.
excess relative to oxyMb (−1.5−2 mol RS-NO/mol oxyMb), to optimize the yield of S-NO oxyMb.

Parallel mass analyses of the apoprotein in reaction mixtures containing oxyMb and NO (derived from DeaNO) at molar ratios of 2 indicated that protein mass remained unchanged, suggesting that S-nitrosation had not occurred (data not shown). Electrospray ionization mass analyses were restricted to Mb apoprotein to avoid the possibility of any ambiguities derived from nitrosylation of the heme prosthetic group. Notably, S-NO oxyMb was formed through trans-nitrosation equilibria by reaction of oxyMb with physiologic low molecular mass RS-NO. Thus, reaction of recombinant human Mb (17,084 ± 4 atomic mass units, mean ± S.D.; n = 3) with GS-NO or CYS-NO gave S-NO oxyMb (17,084 ± 4 atomic mass units, mean ± S.D.; n = 3) as verified unambiguously by ESI-MS spectrometry (Fig. 3). Table I shows the percentage of conversion of human oxyMb to the corresponding S-NO oxyMb as determined from the extent of S-NO oxyMb accumulation relative to the native unmodified parent protein in the same sample (Fig. 3, inset). It was noted that all preparations of S-NO oxyMb contained unmodified parent protein that was not separated from the S-nitrosated protein. Assuming that the distribution of native and modified Mb accounts for all the human Mb in the reaction, trans-nitrosation reactions with CYS-NO consistently afforded higher yields of S-NO oxyMb than that obtained with GS-NO (Table I). This difference in yield (CYS-NO versus GS-NO) reflects the relative steric bulk of glutathione relative to cysteine that imparts a greater stability to the corresponding low molecular mass S-nitroso-educt (36) and results in a lower yield of S-NO oxyMb.

Trans-nitrosation is an equilibrium reaction occurring spontaneously under physiological conditions (31). To determine the respective second order rate constants, the time-dependent consumption of GS-NO (Fig. 4A) and CYS-NO (Fig. 4B) in the presence of human oxyMb was monitored together with the accumulation of GSH and cysteine. Using conditions identical to those employed to produce samples for mass analyses, the reaction profiles for trans-nitrosation of human oxyMb with a 2-fold molar excess of RS-NO were established (see the insets in Fig. 4, A and B). Consumption of RS-NO resulted in near stoichiometric accumulation of the corresponding reduced thiol that is a surrogate for S-NO oxyMb accumulation and at equilibrium closely matched the yield of S-NO Mb determined by mass spectrometry. This observation strongly supports the idea that native and S-nitrosylated Mb detected by mass spectrometry accounted for all Mb in each trans-nitrosation reaction, with no other significant protein modifications evident. Fitting the data shown in Fig. 4 to a second order process (31) afforded estimates of the values for the forward ($k_{\text{forward}}$) and reverse ($k_{\text{reverse}}$) rate constants for the various equilibria (Table I). Equilibrium constants ($K$) were then determined from the relationship $K = k_{\text{forward}}/k_{\text{reverse}}$ (Table I). Notably, the 2-fold difference in the $K$ values determined for trans-nitrosation with CYS-NO and GS-NO also reflected the relative yields of S-NO oxyMb estimated by mass spectrometry and quantitative HPLC analyses (Table I), indicating that S-nitrosylation of oxyMb is favored in the presence of low molecular mass RS-NO.

Both capture and the subsequent release of NO from endothelium-derived relaxant factor (e.g., RS-NO) are important to the preservation of NO bioactivity in vivo (21). At present, it is generally accepted that Cu(II) catalyzes the decomposition of RS-NO to NO and the corresponding disulfide dimer (37–39). To assess whether S-NO oxyMb was capable of releasing NO, we therefore exposed various RS-NO to Cu(II). In the case of GS-NO, this yielded a time-dependent release of
oxidation may be facilitated by protein-bound di-oxygen. Analyses of the reaction mixtures containing S-NO oxyMb and Cu²⁺ ions with SDS-PAGE indicated the presence of a Mb dimer sensitive to DTT (Fig. 5B, inset). Significantly, the extent of this homodimer formation reflected the yield of both S-nitrosated protein (Table I) and 'NO (Fig. 5C).

Next, we determined whether S-NO oxyMb is a potential source of bioactive 'NO using a biological model system that assesses vascular function (Fig. 6). Addition of S-NO oxyMb to pre-constricted rabbit aortic vessels caused an immediate and dose-dependent relaxation of magnitude comparable with that observed with the corresponding concentration of SNP (Fig. 6A). Vessel relaxation determined in response to S-NO oxyMb was independent of the presence of an intact endothelium, inhibited by 1H-(1,2,4)oxa-diazole(4,3-a)quinoxalin-1-one (Fig. 6A), and caused an increase in the tissue concentration of cGMP (Fig. 6B). In contrast, the C110A variant of oxyMb pre-treated with CYNO failed to both elicit vessel relaxation (Fig. 6A) and increase tissue concentrations of cGMP (Fig. 6B). Together, these findings suggest that S-NO oxyMb elicits vessel relaxation through the activation of soluble guanylyl cyclase.

Finally, we assessed whether intracellular S-NO oxyMb is formed in human VSMCs exposed to 'NO (Fig. 7). The physiologic concentration of 'NO ranges from 0.01 to 1 µM in vascular (41, 42) and myocardial tissues (43), with higher concentrations (~13 ± 4.3 µM) detected in an animal model of allograft rejection (44) using an 'NO-selective electrode. In these studies, the corresponding rates of 'NO release, estimated from the time to reach maximal 'NO concentration, were 2 and 140 nM·s⁻¹ for coronary (42) and internal mammary arteries (43) stimulated with bradykinin and acetylcholine, respectively, and 325 nM·s⁻¹ for cardiac allografts. We therefore exposed VSMCs to 'NO doses in this pathophysiologic range. Thus, confluent VSMCs were exposed to 'NO generated at ~1 and 60 nM·s⁻¹ derived from the decomposition of added DeaNO (corresponding to final 'NO concentrations of 0.15 and 15 µM in the media) and cultured further for 5 or 60 min. These times were chosen to correspond to a time required for the complete decomposition of DeaNO (τ½ = 2 min, 37 °C) and a time at which trans-nitrosation of oxyMb by intracellular RS-NO would be expected to reach equilibrium (see Fig. 4), respectively. Next, Mb was immunoprecipitated and assessed for 'NO release induced by Cu²⁺ (Fig. 7) and SDS-PAGE with Western blotting (Fig. 7C, inset). As expected, and independent of 'NO pre-treatment, Mb was detected in VSMCs but not in PAECs (Fig. 7C, inset). Isolated Mb immunoprecipitates obtained from VSMCs exposed to 10 µM DeaNO and incubated for 5 or 60 min consistently yielded 'NO in the presence of Cu²⁺ (Fig. 7, A and B); increased 'NO release was detected in samples incubated for 1 h after DeaNO treatment. By contrast, immunoprecipitates from VSMCs exposed to vehicle (control) or PAECs pre-treated with 'NO did not yield measurable 'NO (Fig. 7, C and D). Also, Mb immunoprecipitates obtained from VSMCs treated with 100 nM DeaNO failed to yield measurable 'NO independent of the incubation time (data not shown). Together, these data support the notion that human Mb can yield a stable protein RS-NO in VSMCs, at least when exposed to 'NO produced at relatively high concentrations and rate of release from DeaNO.

**DISCUSSION**

There is growing evidence to support the idea that protein-bound forms of 'NO act as stores of relaxing factor for VSMCs (36, 45). For example, isolated rat aortic vessels incubated with 'NO donors release a labile, relaxing, and soluble guanylyl
cytose-activating factor that is associated with protein thiols (46). However, identification of the specific protein(s) responsible for the NO bioactivity-enhancing factor in the vessel wall has proven elusive. Here we demonstrate for the first time that recombinant human oxyMb yields an S-nitroso adduct. This is achieved by a relatively small (~2-fold) molar excess of low molecular mass RS-NO and occurs at Cys\textsuperscript{110} on human oxyMb via trans-nitrosation equilibria. In contrast, direct S-nitrosation by NO under aerobic conditions is excluded as a mechanism due to the rapid rate of oxyMb-mediated oxidation of NO. The yield of S-NO Mb observed via trans-nitrosation is dependent on the steric constraints of the donor RS-NO. Similar to S-NO ferric Mb (47), the NO stored in the form of S-NO oxyMb can be released by Cu\textsuperscript{2+}, as demonstrated directly using a NO-sensitive electrode, and in the absence of added Cu\textsuperscript{2+}, S-NO oxyMb can relax constricted blood vessels in vitro. In the vascular function studies described here, S-NO oxyMb was added to isolated vessels, whereas in vivo, any S-NO oxyMb formed would be expected to be present in VSMCs. Therefore, whether the vessel relaxing activity of S-NO oxyMb has biological significance and precisely what intracellular concentration of S-NO oxyMb accumulates in VSMCs under different conditions remain to be established.

Myoglobin is present in skeletal and cardiac muscle at relatively high concentration. For example, in the cytoplasm of cardiac myocytes, the Mb concentration is estimated to be ~350 μM (5, 48). More recently, Mb has been localized to human smooth muscle (6), although the precise concentration in this tissue is not known. Within the sarcoplasm of smooth or skeletal muscle, translational diffusion of oxyMb, balanced by a reverse flow of ferrous deoxyMb, is believed to support a flux of oxygen from the sarcoplasma (closest to the capillary) to the mitochondria (48). The proportion of cardiac ferrous deoxyMb to oxyMb determined in situ is at least 10% under resting conditions (49). The high translational (50, 51) and virtually unimpeded rotational diffusion (51) of Mb suggests that the protein is capable of moving rapidly from the sarcoplasma boundary through the cytoplasm and on to the mitochondrial target to support oxidative phosphorylation. In this process, Mb is responsible for local dissipation of oxygen near the capillary and establishing a shallow oxygen gradient within the sarcoplasm (52). Indeed, the high motility of Mb within muscle cells coupled with the high rate constant for oxidation of NO by oxyMb is taken as evidence to support the notion that oxyMb regulates intracellular concentrations of NO (48). For example, NO partitioning in the mitochondrial membrane impacts upon mitochondrial respiration (9, 53) through binding to the binuclear heme center of cytochrome c oxidase (54). The observation that vascular NO catabolism decreases with a decreasing oxygen gradient extending away from the capillary (55) and subsequently increases again at the sites of mitochondria (56)

![Inset A](image1.png)

**Fig. 5.** Nitric oxide stored in the form S-NO oxyMb is released in the presence of Cu\textsuperscript{2+} ions and detectable with an NO-selective electrode. A, addition of authentic GS-NO (~10–200 nM) to an NO electrode pre-equilibrated in argon-gassed phosphate buffer (50 mM, pH 7.4) containing 100 μM Cu\textsuperscript{2+} yields a dose-dependent current response (arrows indicate time of GS-NO addition). Inset shows the linear correlation between [GS-NO] and area under the peak response curve ($R^2 = 0.99$). B, peak response of the NO electrode to the addition of two independent preparations of S-NO oxyMb obtained by trans-nitrosation with (1) CYS-NO and (2) GS-NO that yield ~85% and 20% conversion to the S-nitrosated protein, respectively (Table 1). Inset shows a representative SDS-PAGE gel illustrating the accumulation of Mb disulfide dimer (~34 kDa) in the Cu\textsuperscript{2+}-containing buffer after addition of the following: lane 1, molecular mass markers expressed in kDa; lane 2, wild-type recombinant human Mb; lane 3, S-NO Mb obtained from trans-nitrosation with CYS-NO; lane 4, S-NO Mb obtained from trans-nitrosation with GS-NO; lane 5, same as lane 4, except that the sample was pre-incubated with DTT before loading onto the gel. Proteins were visualized with Coomassie Blue staining before video capture with a BioDoc Gel Analyzer (Biometra Biomedicine Analytical Group). C, the concentration of NO liberated from S-NO oxyMb prepared by trans-nitrosation with CYS-NO or GS-NO and of authentic GS-NO was determined by monitoring NO release with an NO-selective electrode and by peak area comparison to the standard curve. *, significantly different ($p < 0.002$) from yield the of NO determined for wild-type or C110A variant Mb controls.
cytosol within cardiac muscle cells or VSMCs through transnitrosation reactions at the cell membrane, in analogy to that reported for the cell surface protein disulfide isomerase (57). Furthermore, low molecular mass thiols are present in cells and thought to store bioactive NO (21), although for steric reasons they are less stable than S-nitroso proteins (33, 58, 59). Protein S-nitrosation is emerging as a fundamental post-translational protein modification that plays a key role in modulating NO bioavailability. It is possible that proteins represent a biologic sink for bioactive NO and that S-nitroso proteins represent a subsequent source of bioactive NO that ultimately contributes to NO bioavailability in human vessels. Our data support both ideas because Mb immunoprecipitates obtained from NO pre-exposed VSMCs released NO. These findings are consistent with a recent study by Janero et al. (60) indicating the presence of S- and N-nitroso adducts together with metal nitrosyls in organs of rats exposed to glyceryl trinitrate. Notably, although not a major target for nitrosylation, the vasculature contained S- and N-nitrosoylated products that were differentially localized in venous (90% RS-NO) and aortic (30% RS-NO) vessels (60).

The estimated values for $k_{\text{forward}}$ and $k_{\text{reverse}}$ for transnitrosation of oxyMb are similar to those of S-nitrosylation of bovine serum albumin (31), and S-NO serum albumin is formed in vivo (61). They are ~1000-fold greater than the $k_{\text{forward}}$ value for S-nitrosylation of ferrous deoxy- or oxyhemoglobin (7, 16, 62), yet S-NO Hb is also formed in low nanomolar concentrations in vivo (63). Therefore, it appears likely that S-NO oxyMb is formed at least in the myocardium,
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**Scheme 1.** Potential mechanisms for human Mb-mediated regulation of NO availability in human cardiac myocytes or VSMCs. Schematic represents cardiac myocytes or VSMCs underlying vascular endothelial cells (EC). Human Mb (a) facilitates O\textsubscript{2} transport from the capillary to mitochondria and eliminates excess EC-derived NO by (b) oxidizing it to nitrate, (c) capturing it as a nitrosyl heme (MbNO), and (d) via formation of S-NO oxyMb through trans-nitrosation reactions with low molecular mass RS-NO. Both authentic NO and NO stored as S-NO oxyMb (e) activate soluble guanylyl cyclase to produce cGMP from GTP, ultimately stimulating (cardiac or vascular) smooth muscle cell (SMC) relaxation through an NO-dependent pathway.

where Mb concentrations approach millimolar levels. Our data indicate that it may also be formed in VSMCs, at least under conditions in which NO is produced at a moderate rate (60 nM s\textsuperscript{-1}), keeping in mind that the physiologic rate of NO production ranges from 2 to 140 nM s\textsuperscript{-1} (42–44) and is dependent on both the type of vascular bed and the vaso-stimulus applied. The facile reaction between oxyhemoglobin and NO is increasingly viewed as a competitive reaction that severely limits the physiological relevance of S-nitroation of Hb (64). By analogy, the high rate of NO oxidation by oxyMb may also limit the physiologic relevance of S-NO Mb.

Overall, available data suggest that Mb plays a multifaceted role in NO homeostasis in skeletal, cardiac, and possibly human smooth muscle (summarized in Scheme 1) via elimination of NO via both oxidation and formation of Mb-NO complexes, as well as the potential for NO preservation through Mb S-nitrosation. Thus, the sarcoplasmic concentration of endothelium-derived relaxant factor in humans may well depend on the balance between the rates of oxyMb reaction with NO, the recycling of ferric Mb to oxyMb, and the formation and subsequent decomposition of S-NO oxyMb. Importantly, the presence of a reactive cysteine sulfhydryl group in Mb isoforms obtained from rat heart (65, 66) and tuna (67) indicates that regulation of vascular NO through formation of S-NO oxyMb may be important in species other than humans. A better understanding of the mechanisms of NO regulation by intracellular Mb may help elucidate the processes by which NO chemistry interfaces with its biological function and warrants additional studies on elucidating the precise physiologic role of S-NO oxyMb in both the myocardium and vasculature in humans.

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