Nitric oxide stimulates myoglobin gene and protein expression in vascular smooth muscle

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Mb (myoglobin) is a haemoprotein present in cardiac, skeletal and smooth muscle and is primarily responsible for the storage and ‘facilitated transfer’ of molecular oxygen from the cell membrane to mitochondria. Also, Mb plays a role in regulating *NO (nitric oxide) homoeostasis through (i) binding *NO (Mb–NO complex); (ii) oxidation of *NO to nitrate; and (iii) formation of vasoactive 3-nitroso-Mb [Rayner, B.S., Wu, B.-J., Raferty, M., Stocker, R. and Witting, P.K. (2005) J. Biol. Chem. 280, 9985–9993]. Pathological *NO concentrations affect mitochondrial function and decrease cell viability through inducing apoptosis. Treatment of cultured rat VSMCs (vascular smooth muscle cells) with cumulative doses (0.1, 1 or 10 μM) of *NO from the donors diethylamineNONOate or spermineNONOate (N-[2-aminoethyl]-N-[2-hydroxy-3-nitrosohydrazine]-1,2-ethelenediamine) yielded a time-dependent increase in Mb gene expression. Concomitant transcriptional activation increased the concentration of Mb within cultured rat or primary human VSMCs as judged by Western blot analysis and indirect immunofluorescence microscopy. Cell viability did not decrease in these cells at the *NO doses tested. Importantly, sub-culturing isolated rat aortic segments for 7 days in the presence of L-arginine at 37 °C stimulated *NO production with a parallel increase in Mb in the underlying VSMCs. Overall, exposure of VSMCs (either in cell culture or intact vessels) to pathological *NO promotes an up-regulation of the Mb gene and protein, suggesting a feedback relationship between *NO and Mb that regulates the concentration of the potent cell signalling molecule in the vessel wall, similar to the role haemoglobin plays in the vessel lumen.

Key words: endothelial function, haem protein, nitric oxide metabolism, vascular smooth muscle, vasodilation.

INTRODUCTION

Vascular endothelium-derived *NO (nitric oxide) plays a vital role in vessel dilation [1,2] by regulating peripheral vascular resistance and ultimately circulating blood pressure. To elicit vessel dilation, *NO binds to, and activates, sGC (soluble guanylyl cyclase) within vascular smooth muscle, which in turn catalyses the conversion of guanosine-5′-(3-thiotriphosphate) into cGMP [3]. Synthesized cGMP activates a cascade of effector proteins that initiate vessel dilation.

However, *NO is generally considered to be a highly reactive molecule. Within the vascular lumen, millimolar concentrations of circulating oxygenated haemoglobin are primarily responsible for reactions with *NO [4,5]. Within cardiac, skeletal and smooth muscle [6,7], Mb (myoglobin) potentially plays a greater role in regulating the tissue content of *NO [8,9]. Thus oxygenated Mb rapidly oxidizes *NO (k ~ 10−4–10−3 M−1 s−1) to yield higher order nitrogen oxides [10]; ferrous and ferric Mb yield a stable haem–NO complex (Mb–NO) (dissociation constant Kd ~ 10−5 M) [11] and Mb from at least some different species [12,13] contain a free cysteine residue that can be nitrosylated to yield 3-nitroso-Mb, which is a store of vasoactive *NO capable of eliciting vascular relaxation. In strong support for the idea that Mb plays a critical role in controlling physiological *NO, oxy-Mb is demonstrated to increase the concentration of *NO in cardiac myocytes [14]. Regulation of myocardial *NO is crucial for maintaining coronary blood flow, cardiac contractility and normal heart function.

In the present study, we investigated the relationship between pathological concentrations of vascular *NO and the response to Mb within VSMCs (vascular smooth muscle cells). Our data demonstrate that *NO stimulates a increase in Mb gene and protein expression in VSMCs through a feedback regulatory mechanism. The physiological implication of this mechanism is discussed in relationship to Mb’s ability to modulate *NO concentrations within the vessel wall, particularly with reference to pathologies that may elicit uncontrolled production of the potent vasodilator.

EXPERIMENTAL

Materials

Sodium nitrite, trypsin, fetal bovine serum, L-glutamine, penicillin, streptomycin, copper(II)sulfate, acrylamide, ammonium persulfate, SDS, ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] and spermineNONOate (N-[2-aminoethyl]-N-[2-hydroxy-3-nitroso-hydradine]-1,2-ethelenediamine) were obtained from Sigma. DeaNO (diethylamineNONOate) was obtained from Cayman Chemicals. The *NO trap DAF-FM (4-amino-5-methylamino-2′-7′-difluoro fluorescein diacetate) was from Molecular Probes. All solvents and chemicals employed were of the highest quality available.
Cell culture

Primary RASMCs (rat aortic VSMCs) (Cell Applications) and human VSMCs (A.T.C.C.) were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F12 (Sigma) containing 10% serum, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37 °C in a humidified atmosphere of 5% CO₂. All VSMCs were cultured to 80-90% confluence and used between passages 8–12. Prior to use, VSMCs were washed in HPSS (Hepes-buffered physiological salt solution, pH 7.4) [15], containing 22 mM Hepes, 124 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.5 mM CaCl₂, 0.16 mM HPO₄⁻, 0.4 mM H₂PO₄, 5 mM NaHCO₃ and 5.6 mM D-glucose. Where required, the cell protein concentration was determined using a commercial kit (bicinchoninic acid assay, Sigma) and the values were used to normalize all biochemical parameters to account for potential variability in cell density.

*NO treatment of VSMCs

The *NO donors DeaNO and spermineNONOate were prepared in 0.1 M NaOH and diluted in HPSS immediately before use. Cultured VSMCs were washed twice in HPSS and treated with the *NO-donor as indicated in the legends to the Figures. In the case for DeaNO, the *NO-donor was administered to yield cumulative concentrations of *NO (final concentration 100 nM, 1 or 10 μM). These doses of DeaNO corresponded to rates of *NO release of 0.6, 6 or 60 nM·s⁻¹ respectively, as determined from the half-life (t₁/₂) = 2 min at 37 °C indicated by the manufacturer. In the case for spermineNONOate, a final *NO concentration of 100 nM (corresponding to a rate of *NO release of 0.21 nM·s⁻¹; t₁/₂ = 39 min at 37 °C) was employed. These two *NO-donors were selected on the basis of different rates of decomposition to release *NO, thereby mimicking the exposure of VSMCs to rapid or prolonged exposure to *NO. After *NO treatment, cells were re-cultured in complete media for a further 2, 5 or 24 h. To assess whether *NO entered the cultured VSMCs, experiments were performed with cells pre-loaded with DAF-FM (Sigma). After 1 h exposure to *NO, cells were imaged with an inverted fluorescence microscope (Zeiss Axiovert 200) fitted with a 60× objective. Images were captured with an MRC 200 digital camera (Zeiss).

Gene regulation in cultured VSMCs

First strand cDNA synthesis

Cultured VSMCs were harvested and total RNA was extracted with a commercial kit (Sigma). First-strand cDNA was generated via reverse transcriptase by Oligo(dT) priming (Bioline, Sydney, Australia) as described previously [16].

RT–PCR (reverse transcription–PCR)

Primers specific for rMb (rat Mb), MNF (myocyte nuclear factor), iNOS (inducible nitric oxide synthase) and the house-keeping genes GAPDH (glyceraldehyde phosphate dehydrogenase), β-actin and ribosomal S29 RNA, were synthesized by Proligo (Lismore, NSW, Australia). Primer sequences for rMb were 5′-CTTGGGATGC-3′ and 5′-TCCACCACCCTGTTGCTGA-3′ and for MNF, 5′-GAGCAGTCTGGTCCG-3′ and 5′-TAATCCACATGCCCATG-3′ and for iNOS, 5′-GACCTGCAGGATTGTG-3′ and 5′-ACCACACTGTGCAAGTGC-3′ and for β-actin, 5′-GGATGTTGAGGCCTGTCC-3′ and 5′-ACCACACTGTGCAAGTGC-3′ and for GAPDH, 5′-GAGCAGTCTGGTCCG-3′ and 5′-ACCACACTGTGCAAGTGC-3′. Total RNA was isolated from VSMCs (Biometra, Gottingen, Germany) with normalizing against viable cell number or total homogenate protein prior to between treatment comparisons.

Western blotting

Cell lysates, from *NO-treated VSMCs or aortic homogenates (see below), were centrifuged (13000 g, 10 min) and the supernatant subjected to immunoprecipitation with GammaBind G Sepharose (Amersham Biosciences, Uppsala, Sweden). Isolated beads were boiled and the supernatant was subjected to SDS/PAGE, transferred on to a nitrocellulose membrane and Mb bands visualized with a human Mb monoclonal antibody (1:1000 v/v, Sigma, Australia) and enhanced chemiluminescence detection (Amersham Biosciences). Densitometric analysis of Western blots was carried out using a BioDocAnalyze system (Biometra, Gottingen, Germany) with normalizing against viable cell number or total homogenate protein prior to between treatment comparisons.

Immunofluorescence studies

For the in situ visualization of cellular Mb, human VSMCs were grown on to coverslips, washed and overlayed with HPSS containing 10 μM DeaNO or vehicle alone (control) and treated as described above. After incubation, cells were fixed in 4% (w/v) paraformaldehyde, permeabized with 0.1% (v/v) saponin, and non-specific binding was blocked with 1% (w/v) BSA in PBS (pH 7.4), before being probed for Mb using a polyclonal anti-human Mb antibody (Sigma; final dilution 1:250, v/v) and a secondary anti-rabbit IgG–FITC conjugate (Amersham; final dilution 1:1000, v/v). Fluorescent images (excitation: 488; emission: 525 nm) were captured using an Olympus Fluoview FV500 confocal microscope (Olympus) fitted with a 100× objective.

Assessing holo-Mb within VSMCs

Apomyoglobin is able to bind a haem prosthetic group in a non-covalent fashion to yield holo-Mb. To determine whether exposure of VSMCs to *NO resulted in the formation of holo-Mb or the Mb apoprotein that is deficient in haem, we purified cell lysates to isolate fractions containing Mb by liquid chromatography as described previously [18]. Briefly, isolated cell pellets were treated with phosphate buffer (50 mM, pH 7.4) containing 1% (v/v) Triton X-100, protease inhibitor cocktail (Roche), 1 mM EDTA and 10 μM butylated hydroxytoluene. The metal chelator and low-molecular-mass antioxidant were added to inhibit post-translational oxidative modification to cell proteins including Mb. Next, the protein content of the lysate was determined and samples containing 25 pg of total protein were injected on to a C₈ column (25 cm, diameter 4 mm, particle size 5 μm) and cell proteins were separated using a linear gradient over 35 min starting at 100% solvent A [0.1% TFA (trifluoroacetic acid) in water] and ending at 100% solvent B (0.09% TFA in 65% aqueous acetonitrile) with monitoring at 210 nm. Samples of authentic horse heart Mb (Sigma) were employed to identify the retention time for Mb. Importantly, the
column was maintained at 18°C using a column oven. Under these conditions, Mb eluted between 19–21 min with a majority present as the holo-protein [18]. Note, at higher temperatures, the protein unfolds and haem is displaced from the apoprotein [18].

Next, we employed Mb-mediated oxidation of ABTS as an independent assessment of whether holo-Mb protein eluted from the VSMC lysates. Thus Mb-containing fractions, isolated from the lysates of cultured VSMCs exposed to *NO by liquid chromatography as described above, were employed in peroxidase activity assays. Pooled samples were resuspended in 50 mM phosphate buffer, pH 7.4, supplemented with 1 mM ABTS. Next, the samples were treated with 100 μM H₂O₂ and the rate of ABTS oxidation was monitored at 420 nm using a Hitachi UV-Vis spectrophotometer (over 30 min) as a surrogate for Mb peroxidase activity [19]. In some experiments, the pooled Mb-containing fractions were pre-treated with 50 μM sodium cyanide (Sigma) prior to use in ABTS oxidation studies. Cyanide is a ligand for haem in Mb and binds strongly to the iron centre and inhibits peroxidase activity of the protein.

Measurement of VSMC viability

The membrane integrity of VSMCs was assessed by Trypan Blue exclusion [20]. The extent of apoptotic and necrotic cell death was determined by dual-staining with FITC–annexin V (FITC-conjugated annexin V), with PI (propidium iodide) counterstaining as previously described in [16].

Measurement of *NO

The accumulation of *NO in the media bathing the aortic segments was monitored by a *NO-selective electrode (ISO-NO MII) coupled with a DUO-18™ data recorder (World Precision Instruments, v1.55) as described previously [21]. Media samples were treated with a mixture of nitrate reductase and NADPH to quantitatively reduce nitrate to nitrite, which was then chemically reduced to regenerate *NO for monitoring with the *NO-selective electrode as described previously [22]. The area under the peak response curve was estimated using integration software supplied with the system and the *NO concentration determined by comparison with a standard curve [13]. Finally, evolved *NO was normalized to total homogenate protein prepared from the corresponding pooled aortic segments (see below). In the absence of tissue, the levels of *NO, derived from nitrite in the media, were <2 pmol/mg of protein. All values of *NO determined in the samples of media that bathed the isolated rat aortic segments were corrected for this basal level of *NO/nitrite.

Animals

All animal work was performed with approval from the Sydney South West Area Health Services Animal Ethics Committee (approval number 2006-008A). All studies were performed by a team of experienced researchers that strictly adhered to the appropriate local ethical procedures for the handling of animals for experimental procedures. Male Sprague–Dawley rats (0.4–0.6 kg) were obtained from the ARC facility (Western Australia) and housed in pairs for the entire study period. Initially, rats were acclimatized to the local environment (1 week minimum). Standard rodent chow and water were provided ad libitum for 6–8 weeks prior to the harvest of the entire aorta for biochemical studies. Prior to harvest of aorta, animals were administered with isoflurane using a metered anaesthetic machine with medical grade oxygen as the carrier gas (2% v/v in oxygen, 1.5 l/min). Next, the unconscious animals were injected i.p. (intraperitoneally) with ketamine (50 mg/kg of weight) and xylazine (10 mg/kg of weight). After 5 min, animals were tested for reflex action and if judged sufficiently anaesthetized, blood was harvested by direct cardiac puncture and finally the aorta was isolated following a thoracotomy procedure. Animals did not recover from this procedure. When required, the descending aorta was cut into 5 mm sections and carefully cleaned of extravascular tissue. Segments were then pooled in groups of 10 and immersed in complete media and incubated at 37°C in a humidified atmosphere of 5% CO₂. To test whether *NO stimulated Mb gene and protein expression in vascular tissues, pooled aortic rings were incubated in complete media containing 100 μM L-arginine for periods of up to 7 days. Following incubation, aortic sections and media were harvested after 0, 1, 3, 5 and 7 days of incubation and frozen immediately and stored at −80°C.

Preparation of aortic homogenates

Where required, frozen samples of aortic rings were thawed, placed into 2 ml of phosphate buffer (50 mM, pH 7.4) containing 1% (v/v) Triton X-100, protease inhibitor cocktail (Roche), 1 mM EDTA and 10 μM butylated hydroxytoluene and then homogenized with a rotating piston and matching teflon-lined tube as described in [23,24]. The total homogenate was split into two samples and one sample was then centrifuged (16 g) further and the supernatant collected for analysis with Western blotting. The other sample was employed for iNOS gene analysis with total mRNA extracted using a commercial kit (Sigma).

Statistical analyses

One-way ANOVA analyses with the Newman–Keuls post-hoc test were performed to determine significant changes between data sets. In all cases, statistical significance was accepted at the 95% confidence interval (P < 0.05).

RESULTS

Intracellular fluorescence increased in a dose-dependent manner in DAF-FM-loaded rat VSMCs following addition of 0.1, 1 or 10 μM *NO (derived from DeaNO) (Figure 1A), but did not lead to any significant increase in cell death over the 24 h period studied (Figures 1B and 1C): a marginal increase in Trypan Blue uptake (Figures 1B) was detected, although this did not reach statistical significance (P = 0.06; n = 6 samples). Together, these data suggest that the added *NO was capable of penetrating the cell membrane, but did not significantly affect cell viability.

To determine whether *NO was capable of altering the intracellular content of Mb, Mb gene expression was determined in cultured rat VSMCs exposed to vehicle (control), DeaNO or spermineNONOate as chemical sources of *NO (Figure 2). Irrespective of the donor, the gene encoding for Mb increased markedly 2 h after exposure to the *NO-donor, was sustained at 5 h and thereafter returned to basal levels 24 h after stimulation relative to the housekeeping gene (Figures 2A–2C). Notably, VSMCs treated with diethylamine (at a final concentration corresponding to the highest concentration of DeaNO employed) showed no change in Mb gene expression (Figure 2B), indicating that the residual organic component of the donor had no affect on Mb gene expression. Despite detecting the increased gene expression of Mb in VSMCs as early as 2 h after *NO-treatment, MNF was not detected in the cells at any time point or even in control cells in the absence of *NO (results not shown).

Next, we determined whether Mb gene regulation induced an accumulation of Mb protein in primary human VSMCs (Figure 3). Western blot analysis indicated an increase in total cellular
Mb commencing as early as 5 h post-treatment with the *NO donor and was sustained at increased levels over the 24 h assay period (Figure 3A). Similarly, fluorescent imaging of VSMCs treated with 10 μM DeaNO demonstrated an increase in immunoreactive Mb present in the cell cytoplasm relative to cells exposed to the vehicle (control) when measured 24 h after treatment (Figure 3B). Parallel quantitative measurement of intracellular Mb fluorescence using flow cytometric analysis demonstrated a significant increase in the population of cells containing Mb when measured 24 h post-exposure to 10 μM DeaNO (Figure 3C).

Each Mb molecule contains a single haem prosthetic group that is crucial for binding molecular O2, and is directly involved in at least two pathways of Mb regulation of *NO (see the Introduction). Accumulation of Mb within VSMCs can be derived from a haem-containing protein (holo-Mb) or simply a Mb apo-protein (devoid of haem), both of which remain immunoreactive to antibodies raised against the Mb protein and therefore cannot be distinguished by immunoassays such as those shown in Figure 3.

To assess whether holo-Mb accumulated in the *NO-treated cells, we fractionated lysates of pooled rat VSMCs using reversed-phase HPLC 24 h after exposure to *NO (Figures 4A and 4B). Fractions eluting from the column between 19–21 min contained Mb as a shoulder peak (Figure 4B, arrow) as confirmed by comparing the retention time against a sample of horse heart Mb (results not shown). The presence of Mb in this fraction was confirmed by Western blotting where pooled samples of eluting Mb from VSMCs showed immunoreactivity similar to a standard of authentic horse heart Mb (Figure 4C). Importantly, the rate of ABTS oxidation increased significantly when exposed to a sample of the Mb fraction isolated from rat VSMCs relative to the control in the absence of *NO-treatment (Figure 4D). As anticipated, pre-incubation of Mb fractions isolated by chromatography with sodium cyanide significantly inhibited ABTS oxidation. Taken together, the enhanced oxidation of ABTS and its inhibition by cyanide is indicative that haem is incorporated into the protein,
and that holo-Mb has accumulated in VSMCs following treatment with *NO.

Next, we assessed whether *NO elicited an increase in Mb protein within intact rat aortic tissues incubated in the presence of L-arginine. Initially, the media content of nitrate/nitrite was negligible (Figure 5A, complete arrow indicating addition of the sample). As anticipated, studies with addition of sodium nitrite (serving as a positive control, broken arrow) elicited a marked increase in the current response (Figure 5A, broken arrow). Next, media samples from the isolated aortic segments incubated for 2, 5 and 7 days were tested and shown to yield increased *NO accumulation in the presence of L-arginine, as judged by the time-dependent increase in nitrate/nitrite measured with a *NO-electrode (Figures 5B–5D, complete arrow). Notably, nitrate/nitrite increased to 10–15 pmol after 5 days of culture and remained constant thereafter, indicative of a time-dependent increase in the production of *NO in these tissue segments (Figure 5E). Together, these results indicated that isolated rat aortic vessels produced significant levels of *NO during culture for up to 1 week.

To explore a potential source of *NO in the aortic segments we assessed the tissue expression of the *iNOS* gene. Results shown in Figure 6(A) indicate that expression of the gene encoding for *iNOS* showed a trend for increased expression when measured at days 1 and 3 compared with basal levels in the vessel, suggesting that *iNOS* may be responsible for the increased accumulation of *NO oxidation products* in the media. Finally, we determined whether the cultured aortic segments contained elevated aortic content of Mb in response to increased production of *NO*. Consistent with the cell culture studies, incubation of aortic segments with L-arginine over 3–5 days significantly increased total aortic Mb as judged by Western blots from samples of vascular homogenate (Figure 6B). Notably, repeated immunoprecipitation of the same sample resulted in no positive Mb bands in Western blots, indicating that the majority of the protein was efficiently removed in the first immunoprecipitation process (results not shown).
DISCUSSION

The synthesis of vasoactive NO is the first step in a cascade of events that leads to endothelium-dependent vessel dilation through activation of sGC to produce the intracellular effector molecule cGMP in vascular smooth muscle. Regulation of NO bioavailability affects vascular tone and, therefore, mechanisms have evolved to tightly regulate the concentration of this potent cell signalling molecule in the vasculature and peripheral tissues [25]. In the present study we have identified a biochemical feedback mechanism that involves interplay between NO and Mb in VSMCs underlying the vascular endothelium. Although NO regulation in the vasculature is largely credited to the relatively high concentrations of haemoglobin (4–5 mM depending on higher oxidation states such as nitrite or nitrate [10]. Therefore the site within the circulatory tree [26]), the role for Mb in regulating vascular NO levels within the vessel wall has not been previously established. Results obtained in the present study with cultured VSMCs and isolated aortic segments have demonstrated that pathological concentrations of NO can stimulate an increase in Mb gene expression with resultant accumulation of the holo-protein in the cell cytoplasm. This may result in the enhanced elimination of NO poisoning through limiting the available NO can...
it is possible that Mb can be involved in a self-regulating loop process that involves evolution of *NO in tissues exposed to low-oxygen tension ([28], [29], but see [29a]), whereas under normoxic conditions changes in Mb content within VSMCs are not required.

Parallels between the interactions between haemoglobin/*NO and Mb/*NO are readily drawn on to explain how *NO concentrations are regulated in vivo and under different pathological conditions, albeit these proteins exist largely in separate biological compartments. Within cardiac and skeletal muscle, Mb is one of the more prevalent cellular proteins, with concentrations ranging from 0.1 to 0.4 mM, and its primary role in facilitating oxygen transport is well established [6]. In addition to oxygen transport, a number of research groups have contributed to extending our understanding of the role for Mb in regulating nitrosative stress. Thus increased expression of iNOS in the myocardium is implicated in decreased consumption of molecular oxygen [30], impaired myocyte contractility and bioenergetics [31] and increased cardiac myocyte apoptosis [32]. This body of evidence taken together with a study that indicates an insufficiency in the translational diffusion of cardiac Mb to fully account for oxygen transport in myocytes under normal oxygen tension [33] supports the conclusion that Mb probably plays several roles in heart muscle. Thus, in addition to facilitating oxygen transport and acting as a storage reservoir for dioxygen, Mb also plays a role in scavenging *NO and thereby regulating its bioactivity and bioavailability [34].

Although a role for Mb in scavenging *NO is now established in Mb-rich tissues such as the heart and skeletal muscle, a similar role for Mb within VSMCs underlining the vascular endothelium has not been previously revealed, despite the documented presence of Mb in smooth muscle [9]. Moreover, the ability for *NO to elicit Mb gene and protein expression in VSMCs underpins the tight relationship between these two components of the vasculature. For example, mice deficient in Mb exhibit multiple compensatory mechanisms that increase capillary density and enhance oxygen diffusion into major organs such as the heart [35]. Furthermore, Mb overexpression can impair angiogenesis in an animal model of hind-leg ischaemia [36]. Nitric oxide and the molecular pathways regulated by this potent signalling molecule play an integral role in the development and maintenance of microvascular networks in various tissues [37,38]. Therefore results from these animal studies can be taken as strong support for the idea that Mb regulates *NO concentrations in vivo.

The molecular mechanism for *NO regulation of Mb gene and protein expression in VSMCs detected in the present study is not clear. Stimulation of Mb gene expression occurs during myogenesis through the transient expression of MNF [39]. Increased Mb gene expression has also been documented in skeletal muscle during endurance training under hypoxic conditions [40]. Multiple transcriptional control elements have been identified for muscle-specific expression of the human Mb gene [41,42], with a CCAC box sequence and a MEF2 (myocyte enhancer factor-2)-like consensus sequence linked to protein transcription by the native Mb promoter. In addition, overexpression of vascular endothelial growth factor in skeletal muscle prior to experimental ischaemia elicits Mb gene expression [43], and the Mb protein concentration in skeletal muscle shows some dependency on the bioavailability of iron [44]. Whether the transcriptional elements that control Mb gene expression are affected directly by *NO is not presently known, and we were unable to detect MNF expression in VSMCs exposed to *NO at any time. Further studies are warranted to identify the molecular mechanism that explains *NO-mediated increases in Mb concentration within VSMCs.

The ability of *NO to reversibly inhibit respiration by binding to both haem iron (competitively) and copper (non-competitively) centres in cytochrome c oxidase [45] has been suggested as a mechanism that regulates mitochondrial function and in turn the cellular pool of ATP. On this basis, the simplest interpretation for the ability of VSMCs to enhance *NO clearance by increasing their cytosolic content of Mb appears to be a protective biochemical feedback mechanism that acts to maintain mitochondrial respiration at times of increased nitrosative stress. However, interactions between cytochrome c oxidase and *NO are complicated by changes in activity induced by *NO that are not simply limited to complete inhibition of electron transport (referred to as *NO poisoning). Thus it is known that *NO can control cytochrome c oxidase activity [46], and this may be dependent on the local cellular concentration of dioxygen and the flux of electrons flowing through the respiratory chain. Thus during periods of normal mitochondrial function, oxidation of *NO by cytochrome c oxidase may inhibit electron flow and inhibit ATP production. On the other hand, inhibition of cytochrome c oxidase is also important in limiting the excessive build-up of mitochondrial membrane potential that in turn promotes the generation of reactive oxygen species under pathological conditions [47]. This form of mitochondrial dysfunction may involve S-nitrosylation of proteins that comprise mitochondrial complex 1 through interactions with low-molecular-mass S-nitrosothiols [48]. Therefore the precise level of cellular *NO needs to be carefully regulated so as to maintain *NO-dependent control of mitochondrial electron transport. In view of this, the expression of Mb in VSMCs exposed to *NO may be seen as an additional level of regulation that is involved in strictly maintaining *NO homeoeostasis and therefore the balance of intracellular nitrosative stress.

The pathophysiological relevance of the present study depends on the likelihood that VSMCs are being exposed to the *NO concentrations used here. Under normal physiological conditions, *NO concentrations range up to a maximum of 1 μM at any given time in the vasculature tree (for a review, see [49]). However, as *NO is generated in a paracrine fashion to act locally at sites within the vessel wall, local *NO concentrations are estimated to be significantly lower, ∼0.01 nM [50]. Importantly, *NO concentrations increase significantly in severe cases of inflammation, such as that measured in animal models of allograft rejection [51] and sepsis in humans [52,53]; reportedly *NO can achieve up to ∼10 μM concentration in the vasculature as measured directly using a *NO-sensitive electrode. In addition, *NO is freely diffusible, and it is therefore plausible that VSMCs are exposed (locally) to relatively high micromolar concentrations of *NO in the setting of severe inflammation. Under these conditions, *NO-mediated increases in Mb gene and protein expression is feasible.

In summary, we have demonstrated that VSMCs respond to nitrosative stress by induction of the Mb gene and a parallel increase in holo-Mb accumulation in the cytoplasm of these cells. This regulation occurs in cultured VSMCs and, importantly, is recapitulated within isolated aortic vessels. This relationship between *NO and Mb may represent a novel biochemical feedback loop that regulates the concentration of *NO in the vessel wall and may ultimately protect VSMC mitochondria from *NO-induced stress and damage.

**AUTHOR CONTRIBUTION**

Benjamin Rayner is a Ph.D. student under the supervision of Paul Witting, and carried out the majority of the work in the present study with guidance from the senior researcher. Together with Paul Witting, Benjamin Rayner was involved in formulating
a draft manuscript. Benjamin Rayner performed most of the cell studies and biochemical assays outlined in the manuscript. Tharani Sabarenam was responsible for the fluorescent imaging and presentation of the image data. Susan Hua performed additional experiments requested by the reviewers following assessment of the original manuscript. All authors were involved in reviewing and updating the text associated with the revised manuscript.

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