Models of unregulated nitric oxide (NO) diffusion do not consistently account for the biochemistry of NO synthase (NOS)-dependent signalling in many cell systems. For example, endothelial NOS controls blood pressure, blood flow and oxygen delivery through its effect on vascular smooth muscle tone, but the regulation of these processes is not adequately explained by simple NO diffusion from endothelium to smooth muscle. Here we report a new model for the regulation of NO signalling by demonstrating that haemoglobin (Hb) α (encoded by the HB A1 and HBA2 genes in humans) is expressed in human and mouse arterial endothelial cells and enriched at the myoendothelial junction, where it regulates the effects of NO on vascular reactivity.

Notably, this function is unique to Hb α and is abrogated by its genetic depletion. Mechanistically, endothelial Hb α haem iron in the Fe(II)/Fe(III) state permits NO signalling, and this signalling is shut off when Hb α is reduced to the Fe(II) state by endothelial cytochrome b5 reductase 3 (CYB5R3, also known as diaphorase 1). Genetic and pharmacological inhibition of CYB5R3 increases NO bioactivity in small arteries. These data reveal a new mechanism by which the regulation of the intracellular Hb α oxidation state controls NO signalling in non-erythroid cells. This model may be relevant to haem-containing globins in a broad range of NOS-containing somatic cells.

Endothelial NOS modulates blood vessel diameter in response to both vasodilators and vasoconstrictors. For example, it is known that during arterial constriction, NO from endothelium feeds back on smooth muscle to control the magnitude of the response to a vasoconstrictor (for example, phenylephrine). Phenylephrine stimulation of thoracodorsal arteries ex vivo—and of primary human endothelial cells (ECs) and vascular smooth muscle cells (SMCs) in the vascular cell co-culture (VCCC) model—reproduced classical NOS- and cyclic guanosine monophosphate (cGMP)-dependent changes in SMC biology (Supplementary Fig. 1a–d). However, NO did not diffuse into the extracellular space (Supplementary Fig. 1e–h), consistent with our previous work showing compartmentalized NO signalling at the myoendothelial junction (MEJ), the crucial EC–SMC contact point in the thoracodorsal arteries and other small arteries and arterioles.

Therefore, we studied MEJ proteins that could contribute to local regulation of NO diffusion and biochemistry. We performed a proteomic analysis of MEJs isolated from VCCCs using the isobaric tags for relative and absolute quantification (iTRAQ) system (Supplementary Fig. 2). Surprisingly, Hb α was abundant at the MEJ (Supplementary Fig. 3). Because Hb can regulate NO diffusion and biochemistry in erythrocytes and pulmonary artery endothelial cells, we proposed that it could have a similar function at the MEJ.

First, we confirmed the proteomic data using immunoblot and immunofluorescence. We demonstrated Hb α protein expression in the VCCC model, but found no expression of Hb β (Fig. 1a). There was little Hb α expression in human ECs or SMCs grown separately, and there was no Hb α in the fibroblasts or gelatin used to coat the VCCC transwells (Fig. 1a). Next, we confirmed these results in co-cultures of different types of EC and SMC in which MEJs also expressed abundant Hb α (Supplementary Fig. 4). We then studied the MEJ distribution of Hb α in situ. Gold particles labelling Hb α were abundant in the MEJ of mouse thoracodorsal arteries visualized by transmission electron microscopy (TEM) (Fig. 1b). By contrast, carotid arteries (conduit arteries that have few MEJs) expressed little Hb α as observed by TEM (Fig. 1b), immunoblot (Fig. 1c) and immunofluorescence (Fig. 1d). These data were consistent in human skeletal muscle arterioles (Fig. 1d) and throughout several tissue beds (Supplementary Fig. 5). Using en face immunofluorescence, we found punctate Hb α staining primarily at paracellular junctions of thoracodorsal, but not carotid, arteries, whereas little Hb β was observed (Fig. 1e). Chemical crosslinking analysis showed that the Hb α was monomeric in thoracodorsal arteries and in VCCCs (Fig. 1f). Next, we measured Hb α messenger RNA using real-time PCR (Fig. 1g), and established that ECs transfected with Hb α short interfering RNA (siRNA) had decreased protein expression at the MEJ (Supplementary Fig. 6a) and in the endothelial monolayer (Supplementary Fig. 6b). Loss of Hb α protein expression did not affect endothelial NOS (eNOS) expression in the EC monolayer (Supplementary Fig. 6b) or at the MEJ (Supplementary Fig. 7).

Transcripts for other globins including myoglobin, neuroglobin and cytoglobin were absent in ECs (Supplementary Fig. 8a–c). Only cytoglobin mRNA and protein were expressed in SMCs (Supplementary Fig. 8c, d), consistent with a previous report. In addition, we also found Hb α-stabilizing protein in the endothelium of thoracodorsal arteries and in VCCCs (Supplementary Fig. 9a, b). Taken together, these data show for the first time, to our knowledge, that arterial ECs express Hb α mRNA and protein, and are responsible for enriched Hb α expression at the MEJ.

To investigate the functional role of Hb α in ECs and its effect on eNOS signalling, we transfected ECs in isolated thoracodorsal arteries with Hb α or control siRNA. Knockdown efficiency was 70–80% (Supplementary Fig. 10). Loss of Hb α resulted in a marked loss in arterial reactivity after phenylephrine application in a single or cumulative doses (Fig. 2a, b) and increased reactivity to acetylcholine (ACH) (Fig. 2c), but there was no change in the response to 5-hydroxytryptamine (5-HT) (Supplementary Table 1). Half-maximum effective concentration (EC50) and maximum effect (Emax) values are listed in Supplementary Table 2. We observed no difference in basal tone (Supplementary Fig. 11a). However, after the addition of the NOS inhibitor L-NAME (L-N(G)-nitroarginine methyl ester (L-NAME)), the effect of Hb α siRNA was comparable to control conditions for both the phenylephrine and ACH responses (Fig. 2a–c). We thus proposed that eNOS, the primary isoform in the vessel wall, may be in close spatial proximity to Hb α. We tested this hypothesis using four methods: co-localization studies by immunofluorescence (Fig. 2d, g), a proximity ligation assay (Fig. 2e, f), and co-immunoprecipitations from both cell lysates (Fig. 2f, h) and purified proteins (Fig. 2i). These analyses showed that Hb α
and eNOS are in a macromolecular complex and can form a direct protein–protein interaction.

Hb α probably interacts with eNOS to regulate blood vessel tone by controlling NO diffusion through its scavenging by haem iron13,17–19. We studied the mechanism of interaction by measuring loss of NO radical in thoracodorsal and carotid arteries, and in the VCCC model. NO was lost in thoracodorsal arteries, but not carotid arteries; it was also lost in MEJ fractions, but not in EC or SMC lysates (Supplementary Fig. 12a, b). Next, we knocked down endothelial Hb α in isolated arteries (Fig. 2j) or VCCCs (Fig. 2l) using siRNA. Loss of Hb α increased NO diffusion across the vessel wall (Fig. 2k) and in VCCCs (Fig. 2m). Together, these results indicate that endothelial Hb α can regulate arterial tone through its effects on NO diffusion.

Next, we proposed that Hb α haem iron in the oxygenated Fe2+ state should control NO diffusion through a fast reaction (2.4 × 10^10 M^-1 s^-1)20 resulting in dioxigenation21,22, whereas Fe3+ state should permit NO diffusion owing to a slower reaction rate (3.3 × 10^-3 M^-1 s^-1)23. We found that Hb α haem iron resides in both states. First, using ultraviolet–visible spectroscopy, we identified a Soret peak (~420 nm) and Q bands (~540–575 nm) in isolated thoracodorsal arteries consistent with oxygen-bound Hb Fe2+, whereas there was no peak in carotid arteries (Fig. 3a). Next, we measured the oxidation state of Fe and found approximately 42% existed in the Fe2+ and 58% in the Fe3+ state (Fig. 3b). These measurements were sensitive to Hb α siRNA (Fig. 3b). Consistent with this observation, we found that carbon monoxide (CO) ligated Fe2+ haem, resulting in increased NO diffusion across isolated vessels (Supplementary Fig. 12c). When MEJ fractions were studied, we found a Soret peak (~410 nm) characteristic of the Fe3+ state (methaemoglobin) (Fig. 3c). Interestingly, pelleted membranes from MEJ fractions were dark brown, consistent with Fe3+ oxidation (Supplementary Fig. 13). We found approximately 32% of Fe existed in the Fe2+ state and 68% in the Fe3+ state (Fig. 3d), results that were also sensitive to Hb α siRNA (Fig. 3d). In addition, we observed an increase in NO diffusion in VCCCs treated with CO (Supplementary Fig. 12d).

Previous work has demonstrated that the NO–haem Fe3+ interaction results in reductive nitrosylation, a mechanism known to generate S-nitrosothiols, which we have shown to be crucial for gap-junction regulation at the MEJ14,24,25. Using N-acetylcysteine as a bait reactant on the abluminal side (Supplementary Fig. 14a, c), we also found a notable loss of S-nitrosothiol synthesis after Hb α knockdown in thoracodorsal arteries (Supplementary Fig. 14b) and in VCCCs (Supplementary Fig. 14d). Together, these results suggest that the Hb α haem oxidation state regulates both NO diffusion and bioactivation.

Next, we proposed that the mechanism regulating the Hb α oxidation state. In erythrocytes, CYB5R3, a known methaemoglobin reductase, controls the haem iron oxidation state through the reduction of Fe3+ (ref. 6). Using immunofluorescence (Fig. 4a, e (in vivo, in vitro,
Figure 2  |  Hb α regulates vessel tone, NO diffusion and associates with eNOS.  a–c, Time course effect of 50 μM phenylephrine (a), a dose response to phenylephrine (b) and a dose response to ACh (c) on thoracodorsal arteries treated with control or Hb α siRNA in the presence or absence of L-NAME. n indicates the number of arteries; value in parenthesis shows number of mice. Asterisk (*) shows significance between control siRNA and Hb α siRNA; hash symbol (#) indicates significance between Hb α siRNA and Hb α siRNA plus L-NAME; dagger (†) represents significance between control and control plus L-NAME. d, En face view of a dual immunofluorescence of a mouse thoracodorsal artery showing Hb α (red) and eNOS (green). The white box in the merge panel indicates the region of interest magnified in the far-right panel. e, Proximity ligation assay for Hb α and eNOS (red punctates) in transverse mouse thoracodorsal artery sections. Inset shows the negative control. f, Western blot (WB) analysis from samples co-immunoprecipitated (IP) for Hb α and probed for eNOS from isolated thoracodorsal and carotid arteries. g, Dual immunofluorescence for Hb α and eNOS on transverse section from a VCCC. Red indicates Hb α and green shows eNOS. h, Co-immunoprecipitation of Hb α and eNOS expression in VCCC lysates. i, Co-immunoprecipitation of purified Flag–eNOS protein probed for Hb α. j, Schematic diagram of experimental design illustrating a cannulated vessel transfected with Hb α siRNA showing NO diffusion as a readout. k, NO diffusion results from mouse thoracodorsal arteries transfected with control or Hb α siRNA (n = 5). l, Illustration of experimental setup for VCCC experiments. m, NO diffusion results from VCCCs transfected with control or Hb α siRNA (n = 4). Striped bars in k represent ex vivo data; open bars in m indicate in vitro data. Scale bars, 10 μm (d, e) and 1 μm (g). P values are shown for each comparison, by ANOVA with Bonferroni post-analysis. All error bars represent s.e.m.

Figure 3  |  The oxidation state of Hb α resides in a mixture of Fe^{2+} and Fe^{3+}. a, c, Ultraviolet–visible spectroscopy analysis of thoracodorsal arteries (a) and VCCC fractions (c). The inset in a indicates the region of interest (magenta box) of the Soret (~420 nm) and Q bands (~540–575 nm), a.u., arbitrary units. b, d, Measurement of the Hb α oxidation state calculating the ratio of Fe^{2+} to Fe^{3+} in thoracodorsal arteries (b; n = 3) and VCCC fractions (d; n = 3) with and without Hb α siRNA. Striped bars in b indicate ex vivo data; open bars in d represent in vitro data. K_{[Fe(CN)_{6}]} potassium ferricyanide. P values are indicated for each comparison, by Student’s t-test. All error bars represent s.e.m.

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and a pharmacological inhibitor of CYB5R3, propylthiouracil (PTU)\(^\text{30}\). Knockdown efficiency using mouse Cybs3 siRNA was about 70% (Supplementary Fig. 17a). We observed a decrease in arterial reactivity in thoracodorsal arteries transfected with CYB5R3 siRNA after phenylephrine stimulation with a single dose or cumulative concentrations (Fig. 4j, k), and increased arterial reactivity with increasing ACh doses (Fig. 4l). Vascular reactivity to phenylephrine or ACh in thoracodorsal arteries pretreated with PTU is shown in Supplementary Fig. 18a–c. The PTU effect was not reversible with L-thyroxine supplementation after phenylephrine stimulation (Supplementary Fig. 18b, inset). We found no change in the PTU effect after 5-HT addition (Supplementary Fig. 18c). We found no change in the PTU effect after 5-HT addition (Supplementary Fig. 18c). We found no change in the PTU effect after 5-HT addition (Supplementary Fig. 18c). We found no change in the PTU effect after 5-HT addition (Supplementary Fig. 18c).

However, after the addition of L-NAME, the effects of CYB5R3 siRNA (Fig. 4j–l) or of PTU treatment (Supplementary Fig. 18a, b) were comparable to control conditions, results that were consistent with Hb α knockdown. We found no difference in basal tone for CYB5R3 siRNA or PTU (Supplementary Fig. 11a, b). Next, we tested the effect of CYB5R3 on NO diffusion in vessels and VCCCs (Fig. 4m, n). Knockdown efficiency with CYB5R3 siRNA was ~30% at the MEJ (Supplementary Fig. 17b) and in the EC monolayer, but not in SMCs (Supplementary Fig. 17c). Both CYB5R3 siRNA and PTU treatment increased NO diffusion across isolated vessels and in VCCCs (Fig. 4n, p and Supplementary Fig. 18d–g). Note that CYB5R3 knockdown did not alter MEJ eNOS or Hb α expression (Supplementary Fig. 17d).
We conclude that EC expression of Hb  has a crucial role in the regulation of NOS-mediated signalling and in the control of arterial vascular reactivity. These results may have far-reaching implications that could influence many aspects of vascular biology and disease. For example, endothelial Hb expression may participate in blood pressure control, arteriogenesis and anti-inflammatory signalling, as well as affecting other redox signalling molecules (for example, superoxide and hydrogen peroxide). Our results correlate with diagnostic indices for human  thalassemia major (HBA1 HBA2) fetuses, who show increased cerebral blood flow during development. Furthermore, these observations may help to explain why inhibition of CYB5R3 attenuates hypertension and may suggest that CYB5R3 is a new therapeutic target for disease treatment. However, studies devoted towards understanding the mechanisms of CYB5R3 regulation and its interaction with Hb will need to be clarified. More broadly, somatic cell types as diverse as alveolar epithelial cells, macrophages, neurons and renal mesangial cells express both Hb and NOS. It is thus possible that Hb could regulate NOS signalling pathways relevant to many cell and organ systems. Taken together, these data provide evidence for a new model in which somatic cell Hb oxidation is required for NOS-dependent bioactivity.

METHODS SUMMARY

Human coronary ECs and SMCs were co-cultured and fractionated as previously described.  ITRAQ proteomic screening was used to identify and quantify proteins enriched at the MEJ as previously demonstrated. Proteins were analysed using western blot, immunofluorescence and immune-TEM. mRNA was measured using real-time-PCR. Isolated thoracodorsal arteries were cannulated, pressurized and stimulated with phenylephrine or ACh as previously shown or perfused using real-time-PCR. Isolated thoracodorsal arteries were cannulated, pressurized and stimulated with phenylephrine or ACh as previously shown or perfused using real-time-PCR. The mRNA was measured using methods found in Supplementary Methods.

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Author Contributions A.C.S. performed most of the experiments and data analysis. A.W.L. performed vessel transfactions. Vascular reactivity was executed by A.W.L. and M.B. S.R.J. carried out immunofluorescence studies and S.T.D. assisted in NO-diffusion and consumption assays. M.Y.L. and P.S.B. performed real-time PCR experiments, and A.K.B. helped with all cell culture experiments. L.C. performed the modelling experiments. B.G. helped with experimental design, provided use of the NO analyser and NO, and assisted with final manuscript preparation. B.E.I. initiated, directed and supported the work through all levels of development. All the authors discussed the results and commented on the manuscript.

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