Hemoglobin α/eNOS Coupling at Myoendothelial Junctions Is Required for Nitric Oxide Scavenging During Vasoconstriction

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Objective—Hemoglobin α (Hb α) and endothelial nitric oxide synthase (eNOS) form a macromolecular complex at myoendothelial junctions; the functional role of this interaction remains undefined. To test if coupling of eNOS and Hb α regulates nitric oxide signaling, vascular reactivity, and blood pressure using a mimetic peptide of Hb α to disrupt this interaction.

Approach and Results—In silico modeling of Hb α and eNOS identified a conserved sequence of interaction. By mutating portions of Hb α, we identified a specific sequence that binds eNOS. A mimetic peptide of the Hb α sequence (Hb α X) was generated to disrupt this complex. Using in vitro binding assays with purified Hb α and eNOS and ex vivo proximity ligation assays on resistance arteries, we have demonstrated that Hb α X significantly decreased interaction between eNOS and Hb α. Fluorescein isothiocyanate labeling of Hb α X revealed localization to holes in the internal elastic lamina (ie, myoendothelial junctions). To test the functional effects of Hb α X, we measured cyclic guanosine monophosphate and vascular reactivity. Our results reveal augmented cyclic guanosine monophosphate production and altered vasoconstriction with Hb α X. To test the in vivo effects of these peptides on blood pressure, normotensive and hypertensive mice were injected with Hb α X, which caused a significant decrease in blood pressure; injection of Hb α X into eNOS−/− mice had no effect.

Conclusions—These results identify a novel sequence on Hb α that is important for Hb α / eNOS complex formation and is critical for nitric oxide signaling at myoendothelial junctions. (Arterioscler Thromb Vasc Biol. 2014;34:2594-2600.)

Key Words: endothelial cells • endothelial nitric oxide synthase • hemoglobin α • nitric oxide

Peripheral vascular resistance, an essential component of blood pressure regulation, is tightly governed by arterial blood vessel tone. The regulation of vascular tone involves a complex set of cell–cell signaling mechanisms between the endothelium and vascular smooth muscle, and it is well documented that molecules released from the endothelium (eg, nitric oxide (NO), endothelium derived hyperpolarizing factor, prostaglandins) profoundly influence this process.1-5 For example, signals originating from vascular smooth muscle stimulate the release of endothelium-derived NO to modulate the contractile response during α1-adrenergic-mediated vasoconstriction.6,7 Thus, it is clear that a critical balance between contractile and dilatory signaling events is tightly regulated for proper maintenance of vascular tone.

Several reports have now indicated that myoendothelial junctions (MEJs) could be a key player in regulating the balance between constriction and dilatation of small resistance arteries.8-10 The MEJs are anatomical hallmarks where primarily endothelium (depending on vascular bed) breaks through the internal elastic lamina and comes into close apposition with the overlying smooth muscle cells, forming gap junctions for direct cell–cell communication (reviewed in 11). The MEJs are found in resistance arteries down to terminal arterioles, with little to no MEJs identified in conduit arteries.11 These cellular structures provide a distinct microenvironment at the interface between smooth muscle and endothelium where several proteins have been shown to be localized and enriched to influence heterocellular cross-talk in the arterial blood vessel wall. Indeed, we have found that endothelial nitric oxide synthase (eNOS) is polarized across vascular beds at MEJs,7 and more recently have demonstrated that endothelial cells in resistance arteries synthesize...
and express hemoglobin α (Hb α), which colocalizes with eNOS at MEJs, where it functions to regulate NO diffusion to vascular smooth muscle during α1-adrenergic–dependent vasoconstriction.10

Detailed biochemical analysis of the Hb α heme iron oxidation state, which is controlled by MEJ localized cytochrome B5 reductase 3, has indicated that the oxidation state of the heme iron dictates permissive NO diffusion or NO scavenging.10 From these studies, the importance of Hb α at the MEJ both in small arteries and in a vascular cell coculture was elucidated. Of particular interest, we observed that Hb α and eNOS form a macromolecular protein complex at the MEJ where they participate in protein–protein interaction as shown in small arteries, the vascular cell coculture, and purified proteins.10 These data provide a potential mechanism by which Hb α/eNOS protein–protein interaction may regulate NO signaling, acting to balance the overall constriction with relaxation to ensure tone is maintained. However, the mechanisms describing how native Hb α and eNOS associate and identification of specific protein sequences critical for this interaction remain unknown. Therefore, we hypothesized that possible disruption of the Hb α/eNOS complex at the MEJ would also disrupt the amount of NO available to smooth muscle cells.

A common and powerful method used to disrupt protein–protein interaction has been the use of mimetic peptides directed against a particular sequence where binding between 2 different proteins occurs. For example, the use of a caveolin-1 disrupting peptide has been demonstrated to have potent effects on reactivity and blood pressure.12–14 We hypothesized that the Hb α/eNOS complex, being unique to resistance arteries, would be an especially attractive target. After in silico modeling of the Hb α/eNOS macromolecular complex, our results demonstrate a highly probable and distinct region of overlap, confirmed via mutational analysis, from which a mimetic peptide with a tat tag to pass through the plasma membrane was derived (termed Hb α X). We show that this peptide disrupts Hb α/eNOS complexes from purified proteins and in the actual arterial wall, ex vivo. In addition, we found that the peptide localizes to holes in the internal elastic lamina (ie, MEJs) and inhibits PE-induced constriction, which is restored only when eNOS is inhibited. Further, we show that the peptide has no effect on conduit artery constriction or on constriction and blood pressure of eNOS−/− mice, again demonstrating the possible strong ability of the peptide to localize to MEJs in resistance arteries. To our knowledge, this data demonstrates for the first time that targeting a protein enriched at the MEJ can alter vascular reactivity, and possibly blood pressure.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Hb α X Peptide Disrupts the Interaction Between eNOS and Hb α
Previous work demonstrated that eNOS and Hb α form a macromolecular complex and can interact.10 Therefore, we used in silico modeling of the known crystal structures for the oxygenase domain of eNOS and Hb α to determine potential interactions based on geometric, electrostatic, and hydrophobic indices. From this analysis, we found a discreet Hb α sequence (LSFPTTKYFPHFDLHGSA) that interacted with eNOS (Figure 1A). Sequences were subjected to homology analysis

Figure 1. Identification of a conserved sequence of hemoglobin α (Hb α) that interacts with endothelial nitric oxide synthase (eNOS). A. In silico modeling of the protein data bank crystal structures for eNOS (gray; 3NOS) and Hb α (orange; 1Y01) using GRAMMMX server. The magnified image on right shows the region of Hb α (ribbon structure) that is modeled to interact with eNOS (dark gray region). The identified Hb α sequence is depicted below and was blasted against other mammalian species, showing conserved sequences highlighted in yellow. B, Western blot analysis of coimmunoprecipitations experiments from HEK 293 cells overexpressed with eNOS, Flag-Hb α, or Flag-Hb α mutants. C, Western blot analysis of Flag-eNOS input, Hb α input, and Hb α precipitated with Flag-eNOS (n=3 separate runs). D, Quantification of precipitated Hb α with P<0.05; all error bars represent SEM. E, Proximity ligation assay for eNOS and Hb α (red punctuates) on transverse sections of a mouse thoracodorsal artery. Green shows internal elastic lamina autofluorescence, blue is 4′,6-diamidino-2-phenylindole, indicating nuclei. Scale bar is 10 μm. F, The graph on right shows quantification of red punctuates from the proximity ligation assay with P<0.05; all error bars represent SEM (n=3 mice).
among several mammalian species, revealing a conserved peptide fragment (Figure 1A). To confirm if this sequence specifically can bind eNOS, we performed mutational analysis followed by coimmunoprecipitation. Because the proline in position 38 and the phenylalanine in position 44 have been show to be unstable, we mutated 2 portions of Hb α, LSF and TTKTY. Our results reveal that TTKTY of Hb α is essential for binding to eNOS (Figure 1B). Next, we synthesized the peptide (LSFPTTKTYF) linked to an HIV tat sequence along with a scrambled control (FPYFSTKLIT). The peptides were named Hb α X and Scr X, respectively.

To determine if these peptides competitively inhibited eNOS and Hb α binding, we incubated Flag-eNOS with tat only, Scr X or Hb α X, followed by the addition of purified Hb α chains. Complexes were precipitated and subjected to Western blot analysis, demonstrating that only Hb α X peptide significantly disrupted the eNOS/Hb α interaction (Figure 1C) and quantified in Figure 1D). To test this ex vivo, we incubated thoracodorsal arteries (TDAs) with peptides and measured colocalization of eNOS and Hb α on transverse sections using a proximity ligation assay (Figure 1E). These studies demonstrate a significant loss of protein–protein association between eNOS and Hb α in the presence of Hb α X (Figure 1E–1F). Next, we perfused fluorescein isothiocyanate–tagged Hb α X peptide into the lumen of pressurized TDAs followed by fixation and immunolabeling for Hb α (Figure 2A). Analysis of the holes in the internal elastic lamina revealed that both the fluorescein isothiocyanate–tagged Hb α X peptide and Hb α protein localized to these distinct regions and that the peptide and the protein were found to be in the same holes the majority of the time observed (Figure 2B). This data provided evidence at the protein level that the Hb α X peptide can localize to the MEJ and disrupt eNOS/Hb α interactions.

**Hb α X Peptide Alters NO Signaling in the Blood Vessel Wall**

After α1-adrenergic receptor stimulation, a rise in endothelial cell calcium and activation of eNOS occurs, resulting in increased NO production that can diffuse back to the smooth muscle cell and active soluble guanylyl cyclase, resulting in increased cyclic guanosine monophosphate (cGMP) production.6,7,10,15 To determine the effects of Hb α X peptide on cGMP accumulation during α1-adrenergic–dependent vasoconstriction, TDAs were incubated with Scr X or Hb α X peptides and stimulated with the α1-adrenergic receptor agonist phenylephrine. We observed a significant increase in cGMP accumulation in arteries treated with Hb α X as compared with the Scr X peptide, which was reversed on eNOS inhibition with the NO synthase inhibitor, -NG-nitroarginine methyl ester (Figure 3A). Our previous work demonstrated that a monolayer of endothelial cells, in the absence of contact with smooth muscle cells, do not express Hb α.16 Therefore, we tested whether Scr X or Hb α X altered eNOS activity in the absence of endogenous Hb α. Treatment of human microvascular coronary endothelial cell monolayers with Hb α X or Scr X showed no effect on eNOS phosphorylation at the activating serine 1177 site (Figure I in the online-only Data Supplement) or on the accumulation of the NO metabolite, nitrite (Figure I in the online-only Data Supplement). In addition, there was no change in nitrite accumulation between Scr X and Hb α X treatments after stimulation of human microvascular coronary endothelial cells with bradykinin, an agonist that stimulates eNOS activity in endothelial cells (Figure I in the online-only Data Supplement), indicating that the functional effects of Hb α X on NO signaling are specific to the eNOS/Hb α signaling axis. Finally, Hb α X had no effect on NO-induced dilation in aorta using acetylcholine dose-response (Figure I in the online-only Data Supplement). These results provide evidence that the Hb α X peptide applied ex vivo alters cGMP levels by disrupting the interaction between Hb α and eNOS, preventing NO scavenging by Hb α, resulting in increased NO diffusion to the smooth muscle cell layer.

Next, we performed vasoreactivity to determine the functional effect of the Hb α X peptide on vasoconstriction to phenylephrine. In TDAs, phenylephrine dose response curves

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**Figure 2.** Hemoglobin α (Hb α) X peptide localizes to holes in the internal elastic lamina of thoracodorsal arteries. In A, en face immunofluorescence of Alexa hydrazide 633 (grey), fluorescein isothiocyanate (FITC)–labeled Hb α X-FITC (green), and Hb α (red) on mouse thoracodorsal arteries. Blue boxes indicate enlarged images underneath (10×10 μm²). Scale bar is 10 μm. In B, graphs show quantification of colocalized FITC-labeled Hb α X and Hb α in holes of the internal elastic lamina (IEL). * indicates significance (P<0.05) between conditions and all error bars represent SEM.

**Figure 3.** Hemoglobin α (Hb α) X peptide increases nitric oxide signaling in the vessel wall of wild-type but not endothelial nitric oxide synthase (eNOS−/−) mice. A, Measurement of cGMP accumulation after phenylephrine stimulation in thoracodorsal arteries pretreated with Scr X or Hb α X peptide in the presence or absence of -NG-nitroarginine methyl ester (L-NAME; n=3). B, Dose response to phenylephrine on arteries treated with Scr X or Hb α X in the presence or absence of L-NAME. C, Cumulative dose response curve on thoracodorsal arteries from eNOS−/− mice with Scr X or Hb α X. In B and C, * indicates significance between Scr X vs Hb α X; ^ indicates significance between Hb α X vs Hb α X + L-NAME analyzed using 1-way ANOVA. All error bars represent SEM.
in vessels treated with the Hb α X peptide revealed a significant decrease in constriction as compared with untreated arteries, which was reversed with l-NG-nitroarginine methyl ester (Figure 3B), with basal tone being unchanged (Figure II in the online-only Data Supplement). Differences are also presented as a change in initial inner diameter measured in micrometers (Figure II in the online-only Data Supplement). The Scr X peptide showed no difference from control constriction. Both the EC$_{50}$ and E$_{max}$ are shown in Table I in the online-only Data Supplement and demonstrate a significant difference only in the presence of Hb α X. Previous work from different laboratories has demonstrated that conduit arteries (eg, aorta and carotid) do not express Hb α.$^{10,16}$ Therefore, we examined the effect of the Hb α X peptide on isolated abdominal aortic rings by wire myography, which showed no significant change in phenylephrine dose response curves compared with untreated aortas or aortas treated with Scr X (Figure III in the online-only Data Supplement). Finally, because we have shown that the Hb α X peptide disrupts Hb α/eNOS interaction, we proposed that eNOS$^{-/-}$ mice should not have an altered phenotype when Hb α X is applied. Indeed, when eNOS$^{-/-}$ mice were treated with Hb α X, there was no alteration of the magnitude of phenylephrine-induced constriction in TDA

### Hb α X Peptide Acutely Alters Systemic Blood Pressure

Our results above indicate that the Hb α X peptide has a confined and significant effect on small artery NO signaling, but not in conduit arteries. This provided initial evidence that the peptide could possibly also alter blood pressure through a change in peripheral vascular resistance. Therefore, we implanted radio transmitters into C57Bl/6 mice to monitor blood pressure in real time and elucidate the physiological effect of Hb α X on systemic blood pressure. Administration of bolus Hb α X peptide $>$1 mg/kg into C57BL/6 mice induced a significant decrease in systolic, diastolic, and mean arterial blood pressure, an effect that was absent in mice injected with saline or Scr X peptide (Figure 4A). This effect was sustained after daily single bolus injections for 7 continuous days (Figure 4B). A representative tracing after Scr X and Hb α X is shown in Figure IV in the online-only

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![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Hemoglobin α (Hb α) X peptide decreases systemic blood pressure. **A,** Radio telemetry measurements of systolic, diastolic, and mean arterial blood pressure from mice injected with various doses of Scr X or Hb α X peptide (0.5–20 mg/kg) in C57Bl/6. **B,** Time course of systolic, diastolic, and mean arterial blood pressure from C57BL/6 mice injected with a single bolus of 5 mg/kg Scr X or Hb α X daily. **C,** Systolic, diastolic, and mean arterial blood pressure from eNOS$^{-/-}$ mice injected with saline, 5 mg/kg Scr X, or Hb α X. * indicates significant differences using a t test (A and B) and ^ shows significance using a 2-way ANOVA (A). In C, statistical differences were analyzed using a 1-way ANOVA followed by a Bonferroni’s post hoc test. n=4 mice for all conditions; error bars indicate SEM.
Data Supplement. There was no observable change in the heart rate of the mice (data not shown). A previous report demonstrated that eNOS is expressed in RBCs and could possibly regulate systemic blood pressure. After exposure to Hb αX altered NO release from RBCs showed no difference compared with Scr X (Figure V in the online-only Data Supplement). The over or under expression of eNOS protein significantly contributes to systemic blood pressure regulation, and because of this, we tested whether Hb αX could alter blood pressure in eNOS−/− mice. In parallel with a lack of effect on vasoreactivity in eNOS−/− mice, there was no alterations in blood pressure in eNOS+ mice injected with saline or either of the peptides (Figure 4C; MAP of eNOS mice was 114 ±2 mm Hg). Using an angiotensin II mouse model hypertension (Figure 5A), we tested if an increased association between Hb α and eNOS occurred. Shown in Figure 5B, proximity ligation assay demonstrates augmented association between Hb α and eNOS, which was reversed with Hb α peptide. Finally, injection of Hb αX reversed angiotensin II–induced hypertension in C57Bl/6 mice (Figure 5C). Together, these results provide in vivo evidence that disruption of eNOS/Hb α interactions permit excessive NO diffusion implicated in vasorelaxation, hypotension, and ablation of Angiotensin II–induced hypertension.

Discussion

Fluctuations in peripheral vascular resistance and systemic blood pressure are governed by highly orchestrated heterocellular signaling cascades between endothelial and smooth muscle cells comprising the arterial wall. The known mechanisms regulating resistance arterial tone involve a multifaceted palate of inputs, including vasodilators, such as endothelial-derived hyperpolarizing factor, prostaglandins, and NO. The recent discovery that endothelial cells in small resistance arteries express Hb α, which functions as a key regulator of NO diffusion to smooth muscle, has provided critical insight into how small arteries modulate NO signaling networks during vasoconstriction. The work presented herein builds on this initial observation, including (i) the identification of a conserved sequence in the Hb α protein that is modeled to interface with eNOS, (ii) identification of specific amino acids critical for eNOS- and Hb α-binding, (iii) the development of a novel mimetic peptide that disrupts the eNOS/Hb α protein–protein interaction, (iv) the identification of a novel mechanism by which coupling of eNOS/Hb α is critical for NO scavenging and vascular reactivity, (v) the first line of evidence suggesting that the eNOS/Hb α interaction at the MEJ is critical for physiological blood pressure regulation, (vi) evidence that disruption of eNOS/Hb α reverses hypertension, and (vii) targeting proteins polarized to the MEJ can significantly alter vascular function. The aggregate of these results offers new mechanistic insight into how Hb α regulates NO signaling in the resistance arterial wall.

Based on our previous work, we hypothesized that the strong association and complex formation between Hb α and eNOS may be crucial for the functional role of Hb α as an NO scavenger. Our first step to test this hypothesis was to perform an in-depth protein–protein interaction analysis using in silico modeling of the known crystal structures for Hb α and eNOS. One limitation of this analysis is that eNOS, comprising both an oxygenase and reductase domain, only has the oxygenase domain crystalized, thereby constraining the modeling to this region. Despite this restriction, we identified a sequence in Hb α that is highly conserved across multiple mammalian species and models to an interaction interface between Hb α and eNOS. This predicted sequence was confirmed using site-directed mutagenesis and prompted the development of a mimetic peptide to this sequence (Hb αX) for use in disrupting the eNOS/Hb α complex. Studies using purified Hb α and eNOS protein as well as ex vivo resistance arteries show >90% inhibition of eNOS/Hb α–binding in the presence of the Hb X peptide, confirming the in silico modeling and the mutational analysis. Interestingly, there are 2 known human single nucleotide polymorphisms in the conserved Hb α interaction sequence: position 41, K→M and position 42, T→S. However, it remains to be determined whether these mutations play any functional role in blood pressure regulation or in the development of cardiovascular disease. Future studies will be required to identify the specific amino acid(s) on eNOS that are critical for binding to Hb α.

Functionally, it was shown that Hb α X disrupts NO-dependent signaling as shown in cGMP and vasoreactivity studies. This work provides the first line of evidence demonstrating the importance of the protein–protein interaction between Hb α and eNOS, possibly similar to the mechanism by which caveolin-1 regulates eNOS. We ruled out the possibility of nonspecific effects of Hb α X assessed by basal phosphorylation of eNOS S1177, NO release measured by nitrite accumulation in basal and stimulated conditions,
the lack of effect in abdominal aortas (where Hb α is not expressed), and in eNOS+ animals. Even though the functional effects of the peptide are apparent, it is still unclear at this point how the Hb α/eNOS complex assembles. The complex may be preconstructed and assembled similar to that of NADPH oxidase subunits. Based on previous work and this study, it is tempting to speculate that caveolin-1 maintains eNOS inactive until stimulation, where eNOS then dissociates and recruits met-Hb α and possibly cytochrome B5 reductase 3 to form a macromolecular complex allowing tight NO regulation. Future work dedicated to dissection of the cell biology regulating the spatial and temporal assembly of this complex will be required to elucidate this aspect.

This article for the first time describes a pharmacological approach to targeting proteins that are polarized to the MEJ. We show that the Hb α X peptide localizes specifically to holes in the polarized location of both Hb α and eNOS, where together they form a macromolecular complex. We also show that the Hb α X peptide increases cGMP after PE-induced constriction and prevents the artery from constricting. The interpretation of this work is that Hb α, acting directly at the MEJ, can regulate delivery of NO to smooth muscle. By extension, this work also provides a first-line of evidence that MEJs could play an important role in blood pressure regulation (see below). Possibly the more immediate ramifications for this work in regards to MEJs is the ability to begin to tease apart the functional roles for these structures. Indeed, the presented work underscores and builds on the plethora of data pointing to a key role for the MEJ in regulation of arterial vasoreactivity (eg, see Refs. 8, 9, 27, 29–35).

Studies demonstrating a significant effect of the Hb α X peptide on physiological and pathophysiological blood pressure control places our purified protein studies and in vitro and ex vivo experiments into an important physiological context, where NO signaling plays an important translational role. An exciting observation is that we can acutely lower blood pressure in normotensive animals, where blood pressure is tightly controlled. In the hypertensive mice, the effect was even more dramatic. With the Hb α X peptide, the observed hypotension is conceivably achieved by increasing the amount of NO available to smooth muscle cells to increase cGMP and reduce the ability of resistance arteries to constrict, lowering the overall peripheral resistance. However, more work is needed to test this hypothesis. Although the data presented is in line and correlates with our vascular reactivity results and studies of blood pressure in eNOS+ knockout animals, it does not take into account the other cell types that regulate blood pressure. These other cell types, known to express somatic Hb, include neurons in the brain and renal mesangial cells. Of note, there was no increase in NO when applied to red blood cells; this does not indicate a lack of eNOS in the cells, merely that in these cells, eNOS and Hbα do not likely interact. In addition, although there was no effect on the heart rate, we cannot at this point rule out the acute effect of the Hb α X peptide on changes in cardiac output. Blood pressure regulation is multifaceted, but the sum of the results demonstrated here both in terms of vasoreactivity and blood pressure provide a basis for further work on the potential role of this peptide in blood pressure regulation.

Finally, this discovery of a conserved Hb α sequence and the development of a novel Hb α mimic inhibitor provide important initial steps for understanding the basic physiological mechanisms that arterial blood vessels use to regulate NO signaling. These observations provide a basis for future studies to dissect the molecular and cellular mechanisms of Hb α/eNOS biology in the endothelium. This work may provide a platform for strategic development of small molecule inhibitors to treat hypertension and possibly other related cardiovascular diseases.

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Disclosures

None.

References


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15. Hu J, Zou R, Venema VJ, Venena RC. The peptide prevented phenylephrine-induced constriction, presumably because of excessive nitric oxide release. Injection of the peptide disrupted Hb α/endothelial nitric oxide synthase complexes in the vasculature and lowered blood pressure acutely and chronically, both in normotensive and hypertensive mice. This work demonstrates that a therapeutic-type molecule can be targeted to sites of protein–protein interaction at the myoendothelial junctions.
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Supplemental Table

Supplemental Table I |  \(E_{\text{max}}\) and \(EC_{50}\) comparisons for vascular reactivity dose response curves. \(E_{\text{max}}\) is expressed as % initial diameter and \(EC_{50}\) is the [PE] producing half of the maximum effect, expressed in \(\mu\text{mol/L}\).

<table>
<thead>
<tr>
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<th>Control</th>
<th>Hb α X</th>
<th>Scr X</th>
<th>Hbα X + L-NAME</th>
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<tbody>
<tr>
<td>(E_{\text{max}})</td>
<td>44.0 ± 7.6</td>
<td>65.5 ± 5.6</td>
<td>38.3 ± 2.4</td>
<td>43.1 ± 9.9</td>
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<td>(EC_{50})</td>
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Supplemental Figure Legends:

Supplemental Figure I | Hb α X peptide does not change eNOS phosphorylation, NO release in untreated and treated coronary endothelial cells or intact aortas. a, Western blot analysis of pS1177 eNOS and total eNOS from human coronary endothelial cells incubated with Scr X or Hb α X (n=3). b and c, Nitrite measurements from unstimulated and stimulated (10µM bradykinin, 5 minutes) human coronary endothelial cells treated with Scr X or Hb α X. In a and b n=3 and in c n=6. In d, abdominal aorta’s had no effect on acetylcholine (Ach) dilation capacity in response to addition of Hb α X, a process almost completely mediated by endothelial NO production. All error bars represent s.e.m.

Supplemental Figure II | Basal tone measurements following Scr X or Hb α X and dose response to phenylephrine presented as a change in microns. a, shows the resting basal tone generated following 20 min peptide treatment. b, shows a dose response curve to phenylephrine with Scr X and Hb α X peptides expressed as diameter change in microns. n indicates the number of arteries; value in parenthesis shows number of mice. All error bars represent s.e.m.

Supplemental Figure III | Effects of Hb α X peptide on wildtype abdominal aortas. Cumulative dose response curve to PE from murine C57BL/6 abdominal aortic rings treated with control, Scr X or Hb α X. All error bars represent s.e.m.

Supplemental Figure IV | Representative tracing of MAP following Hb α X injection. Radiotelemtery recording of MAP following a single bolus injection of 5 mg/kg of Scr X or Hb α X.

Supplemental Figure V | Hb α X does not alter NOx levels in RBCs. Isolated RBCs were treated with 5 µmol/L Scr X and Hb α X for 30 minutes. All error bars represent s.e.m.
Supplemental Figure II

(a) Basal tone

(b) Change in Diameter (microns)

Legend:
- Control n=5 (4)
- Hb α X n=6 (6)
- Scr X n=4 (4)
- Hb α X + L-NAME n=4 (4)
C57BL/6 abdominal aorta

Supplemental Figure III

Force, mN

PE (M)
Supplemental Figure IV

Representative Trace 1

MAP (mmHg)

Hb α X
Scr X

Time (min)

pre-injection

post-injection
MATERIALS AND METHODS

In silico modeling and peptide generation: Based on the model of Hb α (PDB number 1Y01) and the oxygenase domain of eNOS (PDB number 3NOS) previously described1 a region of Hb α (residues 35 – 44 of Hb α; LSFPPTKTYF) at the eNOS interface was chosen for multiple sequence alignment with mammalian species and peptide synthesis. Peptides analogous of Hb α (LSFPPTKTYF; Hb α X) or a scrambled peptide (FPYFSTKLT; Scr X) were synthesized with an N-terminal HIV-tat tag (YGRKKRRQRRR) for plasma membrane permeability (AnaSpec). For internalization studies, a fluorescein isothiocyanate (FITC) tagged Hb α X peptide was purchased (AnaSpec).

Site-directed mitogenesis: A Flag-pCMV6-Hb α construct was purchased from Origene and the amino acids LSF or TTKTY in the Hb α sequence were mutated to VTY and AARAF respectively, using the QuikChange Lightning Site-Directed Mutageneration Kit (Agilent) according to manufactures directions. Primers used to create Flag-pCMV6-Hb α –VTY were 5’-cccctgagagatgtacctatccaccaagacca-3’ and 5’-taggtcttggtggtggagtaggtcaagacatctctccaggg-3’ and Flag-pCMV6-Hba1-AARAF were 5’-gtctaaggtcgggaagaagccttctctccagggacacagga-3’ and 5’-gtttcttgcttcctccgccccgaccttagtc-3’.

Co-immunoprecipitation studies: HEK 293 cells were transfected with 1.5ug of pCMV6-Hb α, Flag-pCMV6-Hb α –VTY or Flag-pCMV6-Hba1-AARAF and 0.5ug of pcDNA3.1-eNOS (gift from Michael P. Bauer, University of Pittsburgh) using Lipofectamine 2000 (Invitrogen) according to manufactures directions. Forty-eight hours after transfection, cells were lysed in 220ul ice-cold RIPA buffer with protease inhibitors and homogenized using a douncer. Immunoprecipitation was performed by incubating anti-Flag nickel beads (Sigma) with lysates for 1 hr at 4°C. Beads were washed 3x with ice-cold RIPA buffer and protein complexes were eluted from beads by boiling beads for 5 min at 95°C in 1x Lamelli buffer. Proteins were then subjected to Western blot analysis as previously described18.

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REFERENCES


Supplemental Table

Supplemental Table I | \( E_{\text{max}} \) and \( EC_{50} \) comparisons for vascular reactivity dose response curves. \( E_{\text{max}} \) is expressed as \% initial diameter and \( EC_{50} \) is the [PE] producing half of the maximum effect, expressed in \( \mu \text{mol/L} \).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>( \text{Hb} \alpha \ X )</th>
<th>( \text{Scr} \ X )</th>
<th>( \text{Hb} \alpha \ X + \text{L-NAME} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E_{\text{max}} )</td>
<td>44.0 ± 7.6</td>
<td>65.5 ± 5.6</td>
<td>38.3 ± 2.4</td>
<td>43.1 ± 9.9</td>
</tr>
<tr>
<td>( EC_{50} )</td>
<td>2.1 ± 0.8</td>
<td>26.7 ± 12.5</td>
<td>2.0 ± 1.3</td>
<td>1.9 ± 0.2</td>
</tr>
</tbody>
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Supplemental Figure Legends:

Supplemental Figure I | Hb α X peptide does not change eNOS phosphorylation, NO release in untreated and treated coronary endothelial cells or intact aortas. a, Western blot analysis of pS1177 eNOS and total eNOS from human coronary endothelial cells incubated with Scr X or Hb α X (n=3). b and c, Nitrite measurements from unstimulated and stimulated (10 µM bradykinin, 5 minutes) human coronary endothelial cells treated with Scr X or Hb α X. In a and b n=3 and in c n=6. In d, abdominal aorta’s had no effect on acetylcholine (Ach) dilation capacity in response to addition of Hb α X, a process almost completely mediated by endothelial NO production. All error bars represent s.e.m.

Supplemental Figure II | Basal tone measurements following Scr X or Hb α X and dose response to phenylephrine presented as a change in microns. a, shows the resting basal tone generated following 20 min peptide treatment. b, shows a dose response curve to phenylephrine with Scr X and Hb α X peptides expressed as diameter change in microns. n indicates the number of arteries; value in parenthesis shows number of mice. All error bars represent s.e.m.

Supplemental Figure III | Effects of Hb α X peptide on wildtype abdominal aortas. Cumulative dose response curve to PE from murine C57BL/6 abdominal aortic rings treated with control, Scr X or Hb α X. All error bars represent s.e.m.

Supplemental Figure IV | Representative tracing of MAP following Hb α X injection. Radiotelemetry recording of MAP following a single bolus injection of 5 mg/kg of Scr X or Hb α X.

Supplemental Figure V | Hb α X does not alter NOx levels in RBCs. Isolated RBCs were treated with 5 µmol/L Scr X and Hb α X for 30 minutes. All error bars represent s.e.m.
C57BL/6 abdominal aorta

Force, mN

PE (M)
In silico modeling and peptide generation: Based on the model of Hb α (PDB number 1Y01) and the oxygenase domain of eNOS (PDB number 3NOS) previously described, a region of Hb α (residues 35 – 44 of Hb α; LSFPTTKTYF) at the eNOS interface was chosen for multiple sequence alignment with mammalian species and peptide synthesis. Peptides analogous of Hb α (LSFPTTKTYF; Hb α X) or a scrambled peptide (FPFYSTKLTT; Scr X) were synthesized with an N-terminal HIV-tat tag (YGRKKRRQRRR) for plasma membrane permeability (AnaSpec). For internalization studies, a fluorescein isothiocyanate (FITC) tagged Hb α X peptide was purchased (AnaSpec).

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