



Published in final edited form as:

Hypertension. 2016 December ; 68(6): 1494–1503. doi:10.1161/HYPERTENSIONAHA.116.08171.

MODULATING VASCULAR HEMODYNAMICS WITH AN ALPHA GLOBIN MIMETIC PEPTIDE (Hb α X)

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Abstract

The ability of hemoglobin to scavenge the potent vasodilator nitric oxide in the blood has been well established as a mechanism of vascular tone homeostasis. In endothelial cells, the alpha chain of hemoglobin (hereafter, alpha globin) and endothelial nitric oxide synthase form a macromolecular complex, providing a sink for nitric oxide directly adjacent to the production source. We have developed an alpha globin mimetic peptide (named Hb α X) that displaces endogenous alpha globin and increases bioavailable nitric oxide for vasodilation. Here we show that, *in vivo*, Hb α X administration increases capillary oxygenation and blood flow in arterioles acutely and produces a sustained decrease in systolic blood pressure in normal and angiotensin II-induced hypertensive states. Hb α X acts with high specificity and affinity to endothelial nitric oxide synthase, without toxicity to liver, kidney, and no effect on p50 of O₂ binding in red blood cells. In human vasculature, Hb α X blunts vasoconstrictive response to cumulative doses of

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Conflict of Interest

BEI and LC are co-authors on a patent titled "Composition and methods for modulating arterial tone." The authors declare no other conflict of interest.

phenylephrine, a potent constricting agent. By binding to endothelial nitric oxide synthase and displacing endogenous alpha globin, Hb α X modulates important metrics of vascular function, increasing vasodilation and flow in the resistance vasculature.

Keywords

alpha globin; endothelium; mimetic peptide; blood pressure; endothelial nitric oxide synthase

Introduction

Endothelial nitric oxide synthase (NOS3, eNOS) production of nitric oxide (NO) is a critical step in homeostatic regulation of vascular tone and function^{1, 2}. NO is a potent vasodilatory gas, activating soluble guanylyl cyclase in smooth muscle cells to prevent constriction and decrease resistance in vessels from the aorta to arterioles. Intracellularly, eNOS is localized at a subcellular structure unique to the vasculature called the myoendothelial junction (MEJ), where endothelial cells project through the inner elastic lamina to directly contact the surround smooth muscle cells³. Localization of eNOS in the MEJ places signal generation (NO production) directly adjacent to the site of action (smooth muscle cells). Critically, many positive and negative regulators of eNOS are sequestered in the MEJ, including calmodulin and caveolin-1, respectively^{4–8}.

Previously, we published a new paradigm for regulation of NO signaling: expression of the alpha subunit of hemoglobin (alpha globin) in endothelial cells is critical for NO scavenging effects in resistance vasculature^{9, 10}. In the vasculature, alpha globin is preferentially expressed in MEJs where it can bind directly to eNOS⁹. The formation of a macromolecular complex with alpha globin and eNOS was verified by immunoprecipitation, proximity ligation assay, and immunofluorescence microscopy in both murine arteries and a vascular cell co-culture system^{9, 10}. Since alpha globin is a potent scavenger of NO, we hypothesized that the binding of alpha globin to eNOS was critical for efficient scavenging. To disrupt the macromolecular complex, and thus increase NO availability, we designed a peptide mimicking a species-conserved sequence in alpha globin¹⁰. This peptide, termed Hb α X, was shown *in vivo* and *in vitro* to have vasodilatory properties. The physiological response (blunted vasoconstriction in response to phenylephrine (PE), a norepinephrine analog) to Hb α X is similar to alpha globin depletion (via siRNA knockdown) from endothelium of *ex vivo* murine arterioles⁹. The decreased constriction is sensitive to an eNOS inhibitor, L-nitroarginine methyl ester (L-NAME), implicating eNOS/NO as the major vasodilator in the absence of alpha globin. Without alpha globin, NO is not efficiently scavenged, increasing diffusion to smooth muscle cells and activation of dilatory pathways.

However, the pharmacologic properties of Hb α X, its effect on blood flow, and its possible therapeutic effect in long-term hypertension treatment in humans is still not known. In this study, we measure hemodynamics including flow rate and oxygen saturation in murine resistance vasculature in response to Hb α X administration, and show that the mechanism of action (binding of Hb α X to eNOS to disrupt complex formation) produces a sustained

decrease in blood pressure with little toxicity. Importantly, we demonstrate that this mechanism is translatable to human microvasculature.

Methods

Murine and human subjects declaration

All mouse procedures were performed in accordance with the University of Virginia's Institutional Animal Care and Use Committee. Human studies were approved by a University of Virginia Institutional Review Board and conformed to the principles of the Declaration of Helsinki and U.S. Federal Regulations.

Photoacoustic microscopy

An established multi-parametric photoacoustic microscopy (PAM) system^{11, 12} was utilized to acquire oxygen saturation of hemoglobin (sO₂) and blood flow speed at the microscopic level *in vivo*. SKH1-Hr^{hr}- hairless mice were used in this study (n=3). Throughout the experiment, mice were anesthetized with 1.5% vaporized isoflurane, and body temperature was maintained using a heating pad. The ear to be imaged was gently pressed against the base of a water tank heated to 37°C. A bolus (20 mg/kg) of Hb α X was administered intraperitoneally without moving the mouse. A 1 mm \times 2 mm field of view was imaged prior to the injection and at 5, 60, and 120 minutes post-injection (see Supplementary Figure S1). Quantification of the sO₂ and flow in the arteriole and venule of the mice was performed as described previously¹².

Blood pressure measurement

Radiotelemetry blood pressure measurement and Angiotensin II (AngII) osmotic mini-pump insertion (Alzet, model #2006) were performed as described^{10, 13}. Briefly, the pumps were loaded with AngII with a constant infusion rate of 1000 ng/kg/min, and a 6 week maximum life span as noted by the manufacturer. Injections of saline or peptides (5 mg/kg) were performed every other day between 16:00–19:00 via IP. Control peptide was a scrambled sequence of Hb α X, (FPYFSTKLTT)¹⁰. Mice were implanted with radiotelemetry units [Data Sciences International (DSI)]. Briefly, while under isoflurane anesthesia, the catheter of the radiotelemetry unit was placed in the left carotid artery and the radiotransmitter was placed in a subcutaneous pouch on the right flank. Blood pressure was recorded for 5 minutes/hour/day for 70 days.

Monitoring of bone marrow, liver, and kidney toxicities

Submandibular punctures were performed every 15 days and approximately 200 μ L of blood was obtained for measurement of liver and kidney function by the University of Virginia blood pathology lab.

Lucigenin assay for measuring superoxide production in tissues

Measurement of tissue superoxide level was performed as previously described¹⁴. Briefly, approximately 5 mg of tissue was added to oxygenated KREBS-HEPES buffer (KREBS-HENSELEIT buffer (Sigma, K3753) plus 25 mmol/L NaHCO₃, 20 mmol/L Sodium HEPES,

2.5 mmol/L CaCl₂, pH= 7.2–7.4) and incubated at 37°C for 30 minutes. Oxygenated buffer and lucigenin reagent (*N,N'*-Dimethyl-9,9'-biacridinium dinitrate, Sigma, M8010) were added to the wells of a microplate for final lucigenin concentration of 5 μmol/L in the well. After baseline readings, tissue samples were added to wells and luminescence values were recorded. Tissue was then dried at 60–70°C overnight. Values are reported as counts/mg dry tissue.

Co-immunoprecipitation of eNOS and HbαX peptide

An eNOS/HbαX complex was visualized by immunoprecipitation and Western blotting. A full description of this protocol is available in the supplemental methods.

Red blood cell oxygen

Measurement of red blood cell oxygenation was performed as described previously¹⁵. Blood was collected from n=3 healthy human volunteers and each sample was tested in each treatment condition.

Recombinant protein expression and purification

Protocol for eNOS protein isolation adapted from Fischmann *et al.*¹⁶. See supplemental methods for a full description.

Fluorescence polarization binding assays

FITC-labeled HbαX was dissolved in a small amount of water and diluted to 5 nmol/L in 5 mL of the above purification buffer. Recombinant eNOS is titrated into the wells of a 96-well plate from a maximum of 1 μmol/L. A full description of the method is available in the supplement.

Far Western blotting

Far Western blotting followed the protocol of Wu, Li, and Chen¹⁷. However, our biotin-HbαX peptide was incubated directly on the membrane (in lieu of a target protein antibody) in combination with an anti-eNOS primary antibody. Secondary antibodies were used as described above for detection.

Human ex vivo vasoreactivity

Under IRB-HSR Study 17194, human volunteers with hypertension (elevated blood pressure above 140 mm Hg systolic) had an approximate 40 mm x 20 mm biopsy of adipose tissue removed from the abdomen, which was immediately placed in ice-cold KREBS buffer, and arterioles dissected manually within 30 minutes of removal. The arterioles (approximately 50–90 μm) were cannulated, pressurized, and subject to phenylephrine cumulative dose–response curves as extensively described by us^{10, 18, 19}.

Statistical analyses

All results are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA unless otherwise noted in the figure legends. P-values < 0.05 were considered significant and are denoted by *.

Results

An alpha globin mimetic peptide increases flow in resistance arteries

Previously¹⁰ we have shown that HbaX blunts vascular constriction to PE and decreases murine mean arterial pressure acutely. However, we were unable to determine if the peptide had a corresponding effect on blood flow. Using photoacoustic microscopy (PAM), we investigated hemoglobin concentration oxygen saturation (sO₂), and blood flow (Figure 1) in the ear microvascular bed of SKH1 hairless mice. The abnormal strain and imaging location was used so that high-resolution images and precision measurement of these hemodynamic parameters in individual vessel segments could be obtained. Although there is an increase in sO₂ in the venule after HbaX injection, we could not detect a change in arteriole sO₂ (Figure 1E). However, as a result of HbaX injection, we could detect increased blood flow in both arteriole and venule (Figure 1F). Additional images at different time points are available in the online data supplement (Supplement Figure S1).

An alpha globin mimetic peptide can sustain low blood pressure

Although the summed acute effects of an alpha globin mimetic appear to indicate a possible effect to lower blood pressure, its long-term effect on blood pressure in a chronic model of hypertension had not been determined. For this reason, we measured blood pressure over time when mice were injected every other day with 5 mg/kg HbaX. During baseline condition, (Figure 2A), mice were normotensive, but injections of HbaX decreased blood pressure after two days. This shows that HbaX has an effect in non-pathological situations. During AngII-induced hypertension, the blood pressure lowering effects of HbaX are more pronounced. The mice had AngII-induced hypertension for ten days prior to injection of HbaX. As shown in Figure 2B, chronic AngII administration resulted in hypertension, with increase systolic blood pressure from a baseline of 110 mmHg to ~170 mmHg. Injection of HbaX resulted in a significant decrease of systolic blood pressure by ~30 mmHg. It has been proposed that prolonged AngII exposure can cause superoxide (O₂^{•-}) production via eNOS dimer uncoupling, thus contributing to hypertension by causing endothelial dysfunction²⁰. In a separate group of mice, AngII was delivered via osmotic mini-pumps at 1000 ng/kg/min and HbaX or saline was injected as described above. Since AngII induces superoxide generation in the kidney and vasculature, kidney and mesenteric tissues were obtained to determine superoxide levels using a lucigenin based chemiluminescence assay. Indeed, superoxide generation was increased in both kidney (Figure 2C) and mesenteric tissues (Figure 2D) with AngII administration compared to sham. HbaX attenuated superoxide generation in the mesenteric circulation, but not in whole kidney tissues.

To test toxicity of an alpha globin peptide over such a sustained period of time, mouse blood was drawn every 15 days during the control blood pressure measurements in Figure 2A and several blood-based organ function parameters were measured. The level of hemoglobin (Figure 3A) and number of white blood cells (Figure 3B) were unchanged, suggesting that HbaX had no effect on bone marrow function. Next, we determined kidney and liver parameters and found no difference in blood urea nitrogen (BUN) (Figure 3C), serum creatinine (Figure 3F), alanine and aspartate aminotransferase enzymatic activity (ALT, Figure 3D, and AST, Figure 3E) between HbaX and control treated groups. The sum of

these results demonstrate that the HbaX peptide itself is a potent modulator of long-term blood pressure and has no detectable kidney or liver toxicities in the 60 day period during which the peptide was administered.

An alpha globin mimetic peptide and eNOS have strong affinity

Because eNOS requires homodimerization for full activity, the ability of the peptide to bind eNOS dimers was assayed with non-denaturing gel electrophoresis. In a monolayer of cultured human aortic endothelial cells (hAoECs), the majority of eNOS exists as a dimeric species (Figure 4A, Left, lane 1). Incubating a biotin-tagged HbaX peptide allowed for immunodetection of the peptide with a streptavidin-conjugated antibody, as seen in Figure 4A, middle. The colocalization of the eNOS dimer and biotin bands (Figure 4A, right) shows that eNOS and HbaX are able to form a complex when eNOS is in its active form. In order to quantify HbaX/eNOS association, we show that the peptide also binds to eNOS *in vitro* using recombinant protein. Figure 4B shows the binding of HbaX to the oxygenase domain of eNOS (residues 64 – 492). Serial dilution of the larger protein eNOS (~50 kDa) from 1 $\mu\text{mol/L}$ with a constant background of 5 nmol/L FITC-labeled HbaX (~1.5 kDa) allows us to look at the binding affinity using fluorescence polarization measurement on a microplate reader. A ligand depleted model was used to fit the data points to calculate a K_{D21} (red dashed line, Figure 4B and 4C). To confirm the binding affinity of 7 ± 2 nmol/L, the same assay was run with 2.5 nmol/L labeled and 2.5 nmol/L unlabeled HbaX. The competition for binding site is theoretically observed as a doubling of the binding affinity because only half of the ligand is observable using the wavelength for FITC fluorophore emission, and is seen experimentally as an increase to $K_D = 13 \pm 5$ nmol/L (Figure 4C). As a control, HbaX self-oligomerization was tested by serially diluting unlabeled HbaX from 1 $\mu\text{mol/L}$ in a solution containing 5 nmol/L FITC-labeled HbaX. The lack of a hyperbolic curve in Figure 4D shows that there are no observable effects of peptide oligomerization in the sample.

Another test of the specificity of HbaX for eNOS is to determine if there are possible effects on the other component of the endothelial NO regulatory system, hemoglobin. Because HbaX is distributed through the circulation, we investigated the effects of the peptide on hemoglobin in red blood cells (RBCs) to confirm that there is no disruption of O₂ carrying ability. To show that HbaX was not inducing hypoxia and initiating a vasodilatory signaling cascade upstream of eNOS, we checked the partial pressure of oxygen needed for 50% saturation of hemoglobin. Using freshly isolated human blood, the addition of HbaX or a control peptide did not significantly change the oxygen p50 of hemoglobin in RBCs (Figure 5A). To verify the effect of HbaX in endothelium, bovine hemoglobin was serially diluted in a constant 5 nmol/L FITC-HbaX solution and fluorescence polarization was measured. The lack of a hyperbolic curve in Figure 5B shows that there is no binding of HbaX to hemoglobin *in vitro*. Taken together, these results show that HbaX is predominantly binding to eNOS, and we propose that this binding is resulting in increased NO bioavailability due to reduced scavenging by alpha globin.

Effects of the alpha globin mimetic peptide in human arterioles

Finally, to demonstrate the translational potential of Hb α X, we tested the peptide *in vitro* (using recombinant human eNOS, described above) and *ex vivo* (measuring vasoreactivity of human arterioles excised from adipose biopsy samples). Figure 6A shows a Far Western blot of eNOS using Hb α X as a probe. Briefly, purified recombinant human eNOS was run in a non-denaturing, low temperature polyacrylamide gel and transferred to a PVDF membrane for blotting with biotin-Hb α X and anti-eNOS antibodies. Fluorescent secondary antibodies show the overlap of eNOS and biotin bands, indicating that Hb α X is able to bind to human eNOS. To confirm that disruption of the alpha globin/eNOS complex is relevant to human physiology, we assayed the distribution of alpha globin in the human vasculature. As previously demonstrated in mice, alpha globin is observed in the biopsied human arterioles, but not beta globin (Figure 6B). Finally, the effect of peptide administration was assayed via vasoreactivity of human arterioles excised from adipose biopsies obtained from patients with hypertension (see Supplementary Table S1). The arterioles excised from these patients were responsive to the vasoconstricting agent phenylephrine (PE). However, when Hb α X is incubated with the arteriole (5 nmol/L), the PE dose response is significantly blunted (Figure 6C). This is identical to the observed effect in peptide-treated C57BL/6 mice¹⁰, which was restored with a NOS inhibitor. This data provides initial evidence that Hb α X may alter human peripheral resistance. When summed with the murine data above, the evidence supports a potential therapeutic value of Hb α X in hypertension.

Discussion

Here, we report hemodynamic changes in mice and humans after injection of the alpha globin mimetic peptide Hb α X. We hypothesize that Hb α X acts as a vasodilator by displacing alpha globin from eNOS and disrupting its ability to scavenge NO at the source.

The result of increased NO levels by inhibiting scavenging can be seen using PAM in Figure 1. Using the dual optical absorbance wavelengths of oxy- and deoxyhemoglobin, total hemoglobin concentration and sO₂ is measured in live tissue¹². Oxygen saturation of hemoglobin in the vessels is described in Figure 1. A difference exists in the sO₂ of arteries and veins at baseline, as expected. Upon injection of the Hb α X peptide, arterial sO₂ is unchanged (red trace) and venous sO₂ increases (blue trace) after 60 minutes. The vessel-type dependent response is consistent with physiological response to increased blood flow. Arterial oxygenation is not changed because Hb α X does not change hemoglobin oxygenation dynamics as shown in Figure 5. Venous oxygenation increases due to total increases in oxygen concentration in the tissue, so that low-O₂ induced diffusion is decreased in capillary beds. This is the result of increased blood flow in the tissues, as seen in Figure 1. Administration of Hb α X increased flow in both arterioles and venules after 60 minutes. The result of higher flow is increased O₂ distribution in the tissue surrounding capillary beds. Although the effects were only studied in ear tissue, similar effects are expected across all resistance vasculature. Additionally, NO does not provide a majority of the vasodilatory signals in resistance vasculature under normal physiology, but with relative overproduction, the impact is likely increased.

Injections every other day were enough to decrease systolic pressure in mice at baseline and mice made hypertensive by constant infusion of AngII. Even without elevated blood pressure, the administration of Hb α X decreased systolic pressure by approximately 5 mmHg (Figure 2A). This shows that increasing NO has an effect in non-pathological contexts because there is constant regulation of NO availability by alpha globin, and that disrupting the macromolecular context permits increased NO signaling in smooth muscle cells. In an AngII model of hypertension, administration of Hb α X resulted in a ~30 mmHg decrease in blood pressure compared to saline and control peptide treatment that was constant until the end of pump lifetime (red line, Figure 2B). AngII has been shown to increase damaging superoxide radical formation in the tissues due to activation of NADH and NADPH oxidase through its type 1 receptor^{22, 23}. In order to address whether the large decrease in blood pressure in Hb α X treated-treated mice in AngII-hypertension is in part due to a reduction in superoxide levels, lucigenin was used to assay tissue production of O₂^{•-} in the kidney (a major target organ of AngII) and the resistance mesenteric vessels. In whole kidney tissues, AngII-induced superoxide generation was not attenuated by Hb α X injection. This could be explained by lack of sensitivity of the assay due to the presence of many other cell types, including epithelial cells that do not express eNOS; thus, any effect of Hb α X on eNOS would be difficult to detect when whole renal tissue is assessed. However, there were lower superoxide levels in the mesenteric tissue after treatment with Hb α X. This could be due to a scavenging effect of superoxide by NO, forming peroxynitrite and other nitroxide species²⁴. Increased NO availability by disruption of alpha globin/eNOS binding could lead to slightly lower superoxide levels in the resistance vasculature. Although the damage of free oxygen radicals produced by AngII-activated oxidases is not completely mitigated by this mechanism, some radical scavenging is likely beneficial to endothelial function. Although the decrease in blood pressure with Hb α X did not return to 110 mmHg as seen in the basal condition, a decrease in systolic blood pressure of 30 mmHg is clinically significant and desirable in treatment. Other mechanisms of hypertension, including aldosterone dysregulation or increased sympathetic drive, may also be sensitive to increases in NO production because the constrictive signaling pathways are distinct from those involved in NO action. The interplay of increased dilatory signals among the constrictive signals might be enough to shift the balance towards lower overall blood pressure.

Because Hb α X was administered as a peptide injection, the toxicity readouts are important before considering human treatments. There are no detectable effects on liver or kidney function when the peptide is injected for up to 60 days. Because Hb α X is a peptide chain, the breakdown products (amino acids) should be relatively inert, and cleared intracellularly or from blood using endogenous protein breakdown machinery. Critically, the injection of the mimetic peptide did not increase white blood cell count, indicating no inflammatory response to the peptide (Figure 3B). Because the sequence is found in endogenous hemoglobin, it is unlikely that antibodies would be developed against the sequence. Measures of liver and kidney function (assayed by urea, ALT, AST, and plasma creatinine levels, Figure 3, C–F) showed no difference between saline, control peptide, or Hb α X treated mice, illustrating no detectable toxicity and its potential as a novel therapeutic agent in the treatment of hypertension by increasing NO availability in endothelial cells.

To confirm the proposed mechanism of Hb α X on the disruption of the alpha globin/eNOS complex¹⁰, we assayed the binding of Hb α X and eNOS *in vitro*. In cultured ECs, Hb α X immunoprecipitates with dimeric eNOS protein (Figure 4A), showing that eNOS can remain in its active oligomeric state when the peptide is bound. An active eNOS enzyme is critical for increased NO availability with Hb α X treatment, as the peptide works to disrupt the formation of an alpha globin/eNOS macromolecular complex. Our model suggests the alpha globin mimetic peptide Hb α X competes with the full alpha globin chain for a binding pocket on the oxygenase domain of eNOS, and previous work has shown fluorescently labeled Hb α X colocalized with alpha globin in the MEJ, where a functional pool of eNOS is located in ECs^{3, 9, 10}. These results suggest proximity of the functional parts, but do not directly show binding. We use FP assays to prove that Hb α X binds to eNOS and determine the binding affinity. In our experiment, we determined that Hb α X and eNOS bind with low nanomolar affinity (Figure 4B). As validation of this result, half of the fluorescently labeled Hb α X in the ligand buffer was replaced with unlabeled Hb α X. This competition experiment effectively doubles the observed binding affinity because only labeled peptide (now at half the concentration) is able to be measured and is competing for the same number of binding sites. Roughly, a doubling of the binding affinity is observed in this mode (Figure 4C). This is not an effect attributable to Hb α X oligomerization, because there is no observable binding curve from data in Figure 4D.

There is considerable evidence that NO can also be modulated by RBCs as well as the endothelium. It has been elegantly demonstrated that NO production in erythrocytes can occur either by nitrite conversion or by eNOS in the RBCs^{25–30}. It is possible that free NO availability in the circulating cells is regulated by a similar mechanism as in ECs due to the vast amounts of hemoglobin present directly adjacent to a membrane-bound NOS enzyme. Overproduction of NO in RBCs might lead to higher levels of methemoglobin within the cells and negatively affect the O₂ carrying capacity of the red blood cells. A reduced O₂ capacity for RBCs might trigger a hypoxic response leading to the observed vasodilation *in vivo*. To determine if Hb α X application could increase NO production and higher p50 values for isolated RBCs, freshly isolated human blood was incubated with concentrations of Hb α X much higher (30 μ mol/L, Figure 5) than would be encountered with therapeutic administration. Even with this level, there was no effect on the p50 of RBC hemoglobin in intact RBCs. With this result, we believe that the effects on RBC eNOS are not the major contributor to the vasodilatory response seen in our system. However, the systems for NO bioavailability in RBCs may be inherently different in terms of a signaling mechanism, or the peptide may not have been able to penetrate the membrane of the RBC.

The translational capacity of Hb α X to disrupt the alpha globin/eNOS complex to increase NO production is shown in Figure 6. Hb α X is able to bind to the oxygenase domain of eNOS, and this interaction is physiologically relevant in human vasculature. The expression of alpha globin in arteriole ECs (but not in the aortic root) shows that disruption of the alpha globin/eNOS interaction predominantly occurs in resistance vasculature, where blood pressure is controlled. The effect of Hb α X can be observed *ex vivo* on individual arterioles using pressure myography. After incubation with Hb α X, blunted vasoconstriction to PE is observed in arterioles excised from humans with hypertension (Supplementary Table S1). Thus, in the context of systemic high blood pressure, increasing local NO signaling in the

resistance vasculature might provide therapeutic benefit. This result provides a base for novel treatments of hypertension by increasing endogenous NO production.

Other strategies exist for increasing production of NO *in vivo* by removing an inhibitory pathway for eNOS. One well-characterized inhibitory binding partner for eNOS is caveolin-1, the main coat protein of caveolae^{31–34}. Interestingly, a peptide mimicking the eNOS-binding sequence of caveolin, Cav1^{6–8}, and Hb α X share some sequence similarity. Supplementary Table S2 shows the sequences of the Cav1⁶ peptide and Hb α X¹⁰ for comparison. Of note, the peptides are composed of both polar and hydrophobic residues, which indicate both buried and solvent-exposed side chains in the binding sequence. The intracellular eNOS-binding interface of caveolin interacts with the calmodulin-binding motif between the oxygenase and reductase domains of eNOS to prevent activation and NO production³⁴. The similarity of the threonine-rich sequences with surrounding hydrophobic residues may be coincidental, but provides a direction for further study of the binding location for alpha globin/Hb α X on eNOS.

There are further questions raised by these results. Our previous work has used an eNOS inhibitor, L-nitroarginine methyl ester (L-NAME), to show that blunted response to PE is a NO-dependent phenomenon^{9, 10}. A lack of human volunteers for biopsy prevented tests of L-NAME sensitivity in human arterioles, a limitation of using human samples in research. The dynamics of the alpha globin/eNOS interaction is also of importance. The binding affinity, k_{on} , and k_{off} rates for the full alpha globin chain binding to eNOS are yet to be determined and will provide evidence for a model of static or transient interaction in the scavenging of NO by alpha globin. Having a model of the alpha globin/eNOS interaction that includes dynamics will be useful in further modification of the Hb α X peptide for better pharmacokinetics.

In conclusion, this study provides evidence for modulation of blood flow and decreasing blood pressure using an alpha globin mimetic peptide to prevent NO scavenging. The peptide, Hb α X, disrupts an alpha globin/eNOS macromolecular complex by binding to eNOS and increases blood flow and oxygen saturation *in vivo*. Hb α X does not appear to affect bone marrow, liver or kidney function. The target specificity of the peptide prevents negative interactions with endogenous hemoglobin, and enhances NO signaling, leading to vasodilatory effects to lower blood pressure.

Perspectives

This work characterizes an alpha globin mimetic peptide and its role in decreasing vascular resistance and increasing blood flow. By increasing NO bioavailability, Hb α X is a potent therapeutic both for acute hypertensive crises (by increasing tissue oxygenation and blood flow in resistance arteries) and long-term treatment of hypertension (due to its low toxicity and sustained decrease in blood pressure).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Adam C Straub (University of Pittsburgh) for helpful discussion and Linda Jahn and Lee Hartline (University of Virginia) for coordinating patient enrollment.

Sources of Funding

This work was supported by the Ivy Translational Fund, University of Virginia Patent Foundation (BEI and LC), NIH 088554 (BEI), NIH 087828 and the Cottrell Award from the Research Corporation for the Advancement of Science (LC), NIH T32GM007055 and American Heart Association Grant 16PRE31180040 (TCSK), The Hartwell Foundation and NIH EY022063 (SMP), R01GM113838 (AD), NIH DK094907 (THL), AHA 15SDG25960005 and NIH EB020843 (SH).

References

1. Ignarro LJ, Cirino G, Casini A, Napoli C. Nitric oxide as a signaling molecule in the vascular system: an overview. *Journal of Cardiovascular Pharmacology*. 1999; 34:879–886. [PubMed: 10598133]
2. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. 1980; 288:373–376. [PubMed: 6253831]
3. Looft-Wilson RC, Billaud M, Johnstone SR, Straub AC, Isakson BE. Interaction between nitric oxide signaling and gap junctions: effects on vascular function. *Biochimica et Biophysica Acta*. 2012; 1818:1895–1902. [PubMed: 21835160]
4. Garcia-Cardena G, Fan R, Stern DF, Liu J, Sessa WC. Endothelial Nitric Oxide Synthase is Regulated by Tyrosine Phosphorylation and Interacts with Caveolin-1. *The Journal of Biological Chemistry*. 1996; 271:27237–27240. [PubMed: 8910295]
5. Fulton D, Gratton JP, Sessa WC. Post-Translational Control of Endothelial Nitric Oxide Synthase: Why Isn't Calcium/Calmodulin Enough? *The Journal of Pharmacology and Experimental Therapeutics*. 2001; 299:818–824. [PubMed: 11714864]
6. Trane AE, Pavlov D, Sharma A, Saqib U, Lau K, van Petegem F, Minshall RD, Roman LJ, Bernatchez PN. Deciphering the binding of caveolin-1 to client protein endothelial nitric-oxide synthase (eNOS): scaffolding subdomain identification, interaction modeling, and biological significance. *The Journal of Biological Chemistry*. 2014; 289:13273–13283. [PubMed: 24648521]
7. Bernatchez PN, Bauer PM, Yu J, Prendergast JS, He P, Sessa WC. Dissecting the molecular control of endothelial NO synthase by caveolin-1 using cell-permeable peptides. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:761–766. [PubMed: 15637154]
8. Bernatchez P, Sharma A, Bauer PM, Marin E, Sessa WC. A noninhibitory mutant of the caveolin-1 scaffolding domain enhances eNOS-derived NO synthesis and vasodilation in mice. *The Journal of Clinical Investigation*. 2011; 121:3747–3755. [PubMed: 21804187]
9. Straub AC, Lohman AW, Billaud M, Johnstone SR, Dwyer ST, Lee MY, Bortz PS, Best AK, Columbus L, Gaston B, Isakson BE. Endothelial cell expression of haemoglobin alpha regulates nitric oxide signalling. *Nature*. 2012; 491:473–477. [PubMed: 23123858]
10. Straub AC, Butcher JT, Billaud M, Mutchler SM, Artamonov MV, Nguyen AT, Johnson T, Best AK, Miller MP, Palmer LA, Columbus L, Somlyo AV, Le TH, Isakson BE. Hemoglobin alpha/eNOS coupling at myoendothelial junctions is required for nitric oxide scavenging during vasoconstriction. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2014; 34:2594–2600.
11. Ning B, Sun N, Cao R, Chen R, Kirk Shung K, Hossack JA, Lee JM, Zhou Q, Hu S. Ultrasound-aided Multi-parametric Photoacoustic Microscopy of the Mouse Brain. *Scientific Reports*. 2015; 5:18775. [PubMed: 26688368]
12. Ning B, Kennedy MJ, Dixon AJ, Sun N, Cao R, Soetikno BT, Chen R, Zhou Q, Kirk Shung K, Hossack JA, Hu S. Simultaneous photoacoustic microscopy of microvascular anatomy, oxygen saturation, and blood flow. *Optics Letters*. 2015; 40:910–913. [PubMed: 25768144]
13. Billaud M, Chiu YH, Lohman AW, et al. A molecular signature in the pannexin1 intracellular loop confers channel activation by the $\alpha 1$ adrenoreceptor in smooth muscle cells. *Science Signalling*. 2015; 8ra17:1–12.

14. Cechova S, Zeng Q, Billaud M, Mutchler S, Rudy CK, Straub AC, Chi L, Chan FR, Hu J, Griffiths R. Loss of collectrin, an angiotensin-converting enzyme 2 homolog, uncouples endothelial nitric oxide synthase and causes hypertension and vascular dysfunction. *Circulation*. 2013; 128:1770–1780. [PubMed: 24048198]
15. Doctor A, Platt R, Sheram ML, Eischeid A, McMahon T, Maxey T, Doherty J, Axelrod M, Kline J, Gurka M, Gow A, Gaston B. Hemoglobin conformation couples erythrocyte S-nitrosothiol content to O₂ gradients. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:5709–5714. [PubMed: 15824313]
16. Fischmann TO, Hruza A, Niu XD, Fossetta JD, Lunn CA, Dolphin E, Prongay AJ, Reichert P, Lundell DJ, Narula SK, Weber PC. Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation. *Nature Structural & Molecular Biology*. 1999; 6:233–242.
17. Wu Y, Li Q, Chen X-Z. Detecting protein–protein interactions by far western blotting. *Nature Protocols*. 2007; 2:3278–3284. [PubMed: 18079728]
18. Billaud M, Lohman AW, Straub AC, Looft-Wilson R, Johnstone SR, Araj CA, Best AK, Chekeni FB, Ravichandran KS, Penuela S, Laird DW, Isakson BE. Pannexin1 regulates α 1-adrenergic receptor-mediated vasoconstriction. *Circulation Research*. 2011; 109:80–85. [PubMed: 21546608]
19. Wang L, Yin J, Nickles HT, et al. Hypoxic pulmonary vasoconstriction requires connexin 40-mediated endothelial signal conduction. *The Journal of Clinical Investigation*. 2012; 122:4218–4230. [PubMed: 23093775]
20. Yang YM, Huang A, Kaley G, Sun D. eNOS uncoupling and endothelial dysfunction in aged vessels. *American Journal of Physiology: Heart and Circulatory Physiology*. 2009; 297:H1829–H1836. [PubMed: 19767531]
21. Hulme EC, Trevethick MA. Ligand binding assays at equilibrium: validation and interpretation. *British Journal of Pharmacology*. 2010; 161:1219–1237. [PubMed: 20132208]
22. Touyz RM, Schiffrin EL. Increased generation of superoxide by angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients: role of phospholipase D-dependent NAD (P) H oxidase-sensitive pathways. *Journal of Hypertension*. 2001; 19:1245–1254. [PubMed: 11446714]
23. Rajagopalan S, Kurz S, Münzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *Journal of Clinical Investigation*. 1996; 97:1916–1923. [PubMed: 8621776]
24. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *American Journal of Physiology: Cell Physiology*. 1996; 271:C1424–C1437.
25. Cortese-Krott MM, Kelm M. Endothelial nitric oxide synthase in red blood cells: key to a new erythrocrine function? *Redox Biology*. 2014; 2:251–258. [PubMed: 24494200]
26. Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon RO, Gladwin MT. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nature Medicine*. 2003; 9:1498–1505.
27. Han TH, Hyde DR, Vaughn MW, Fukuto JM, Liao JC. Nitric oxide reaction with red blood cells and hemoglobin under heterogeneous conditions. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99:7763–7768. [PubMed: 12032357]
28. Kim-Shapiro DB, Schechter AN, Gladwin MT. Unraveling the reactions of nitric oxide, nitrite, and hemoglobin in physiology and therapeutics. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2006; 26:697–705.
29. Kleinbongard P, Schulz R, Rassaf T, et al. Red blood cells express a functional endothelial nitric oxide synthase. *Blood*. 2006; 107:2943–2951. [PubMed: 16368881]
30. Liu C, Wajih N, Liu XB, et al. Mechanisms of human erythrocytic bioactivation of nitrite. *The Journal of Biological Chemistry*. 2015; 290:1281–1294. [PubMed: 25471374]
31. Sessa WC. Regulation of endothelial derived nitric oxide in health and disease. *Memórias do Instituto Oswaldo Cruz*. 2005; 100:15–18. [PubMed: 15962093]

32. Segal SS, Brett SE, Sessa WC. Codistribution of NOS and caveolin throughout peripheral vasculature and skeletal muscle of hamsters. *American Journal of Physiology*. 1999; 277:1167–1177.
33. Murata T, Lin MI, Huang Y, Yu J, Bauer PM, Giordano FJ, Sessa WC. Reexpression of caveolin-1 in endothelium rescues the vascular, cardiac, and pulmonary defects in global caveolin-1 knockout mice. *The Journal of Experimental Medicine*. 2007; 204:2373–2382. [PubMed: 17893196]
34. Gratton JP, Fontana J, O'Connor DS, Garcia-Cardena G, McCabe TJ, Sessa WC. Reconstitution of an endothelial nitric-oxide synthase (eNOS), hsp90, and caveolin-1 complex in vitro. Evidence that hsp90 facilitates calmodulin stimulated displacement of eNOS from caveolin-1. *The Journal of Biological Chemistry*. 2000; 275:22268–22272. [PubMed: 10781589]

Novelty and Significance

What is new?

This is the first study of the effects of modulating hemodynamic factors using an alpha globin mimetic peptide to increase NO availability.

What is relevant?

These results provide evidence that an alpha mimetic peptide may be a candidate for a long-term therapeutic for blood pressure pathologies.

Summary

Hypertension affects a growing number of worldwide adults, and treatment resistant hypertension is becoming more prevalent with no definitive therapy. By increasing nitric oxide availability, we are able to induce a sustained reduction in blood pressure and increase vascular flow in animal models and blunt hyperreactive vasoconstriction in human arterioles

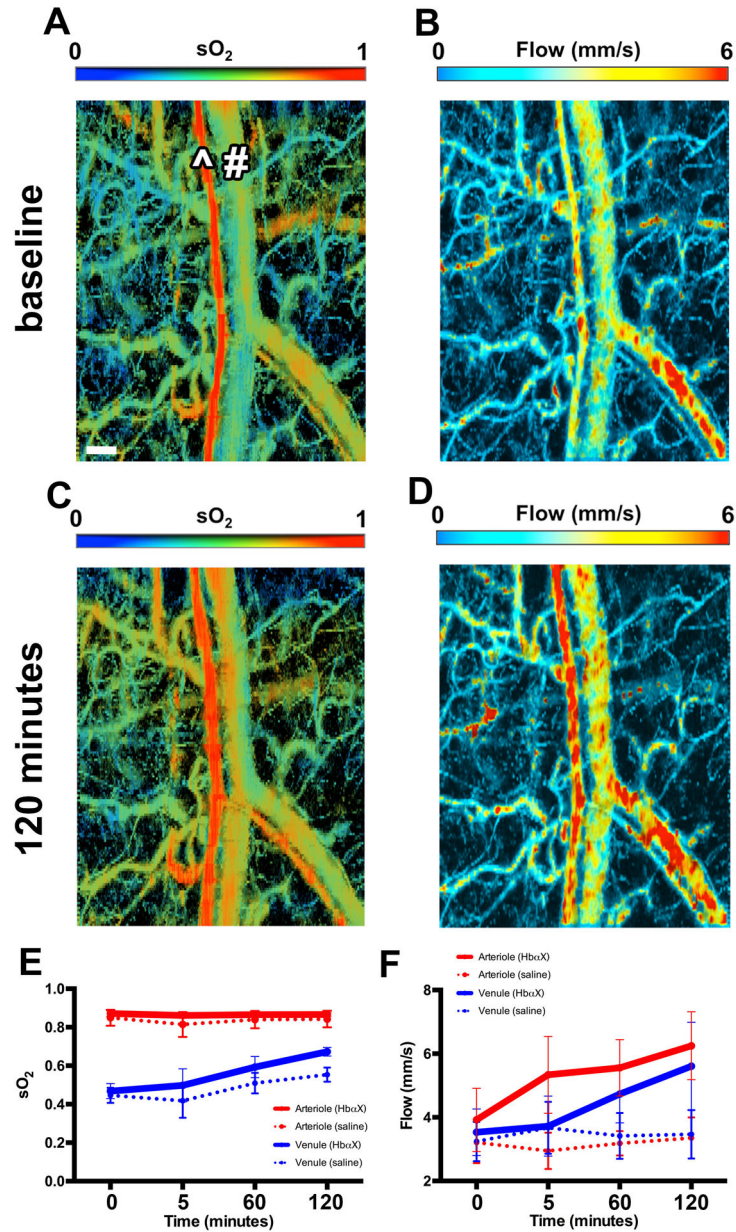


Figure 1. Acute increased O₂ saturation and blood flow in microvasculature with single injection of HbaX in vivo

(A–D) Photoacoustic microscopy images of O₂ saturation (sO₂) and blood flow from microvasculature of murine ear at baseline (A–B) and 120 minutes post HbaX injection (C–D). All images are from the same portion of the ear. The ^ symbol denotes an arteriole and # denotes a venule. Qualitatively, sO₂ increases after HbaX injection shown by warmer colors in the regions of capillary beds. Blood flow is shown in both arterioles and venules with red coloring denoting regions of highest flow. Quantification of sO₂ (E) and average flow velocity (F) from mice receiving HbaX injection (solid lines, n=3) and saline injection (dotted lines, n=3); red denotes arteriolar value, blue denotes venular. Scale bar in A is 100 μm.

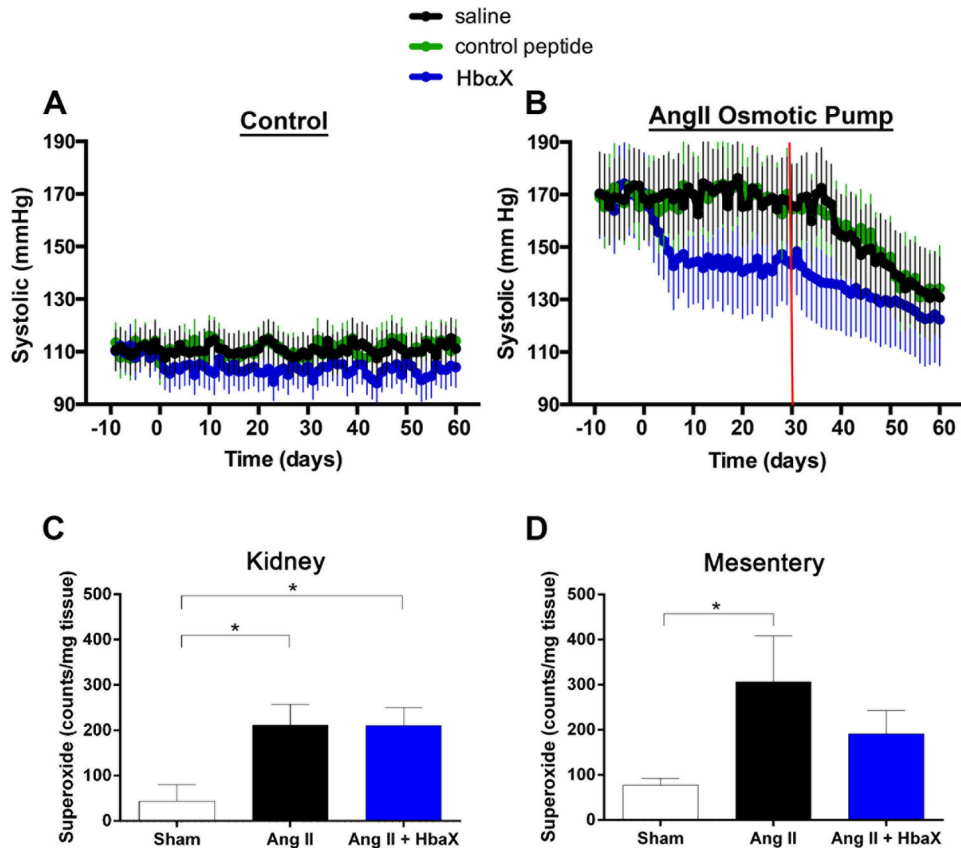


Figure 2. Sustained decrease in systolic blood pressure with HbaX

Either normotensive mice (A: N=4) or mice with osmotic pumps loaded with AngII (B: N=4) were injected every other day starting at day 0 with saline (black), a scrambled control peptide (green; 5 mg/kg) or HbaX (blue; 5 mg/kg) and systolic blood pressure via radiotelemetry was obtained daily. A baseline was measured for 10 days prior to the start of injections. Red line in B denotes approximate end of AngII infusion due osmotic pump lifetime. Superoxide production from kidney (C) and mesenteric (D) tissues was assayed from mice with AngII pumps and HbaX injections (n=3) or saline injections (n=3), or receiving sham surgery (n=4).

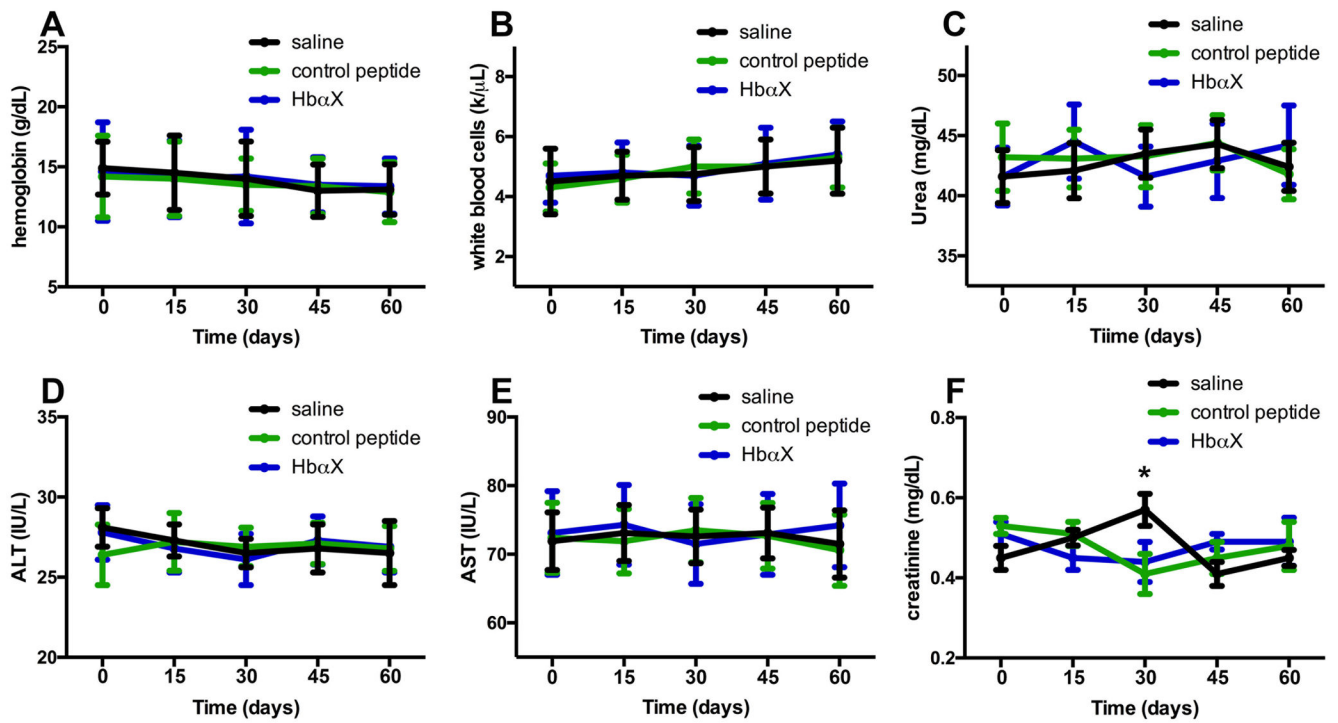


Figure 3. Prolonged injection of the alpha globin mimetic peptide does not lead to murine toxicity

Approximately 200 μ L of blood was drawn via submandibular puncture every 15 days, starting prior to the first injections on day 0 of saline (black), control peptide (green) or Hb α X (blue). The blood was subject to blood pathology laboratories at the University of Virginia to test for basic parameters that could indicate toxicity. These include a change in levels of hemoglobin (A), white blood cells in aggregate (B), urea (C), ALT (D), AST (E), and creatinine (F). Although some variability was observed, there was little significant difference using two-way ANOVA and an alpha level of either $p < 0.05$ or $p < 0.10$. $N = 3$ mice per condition.

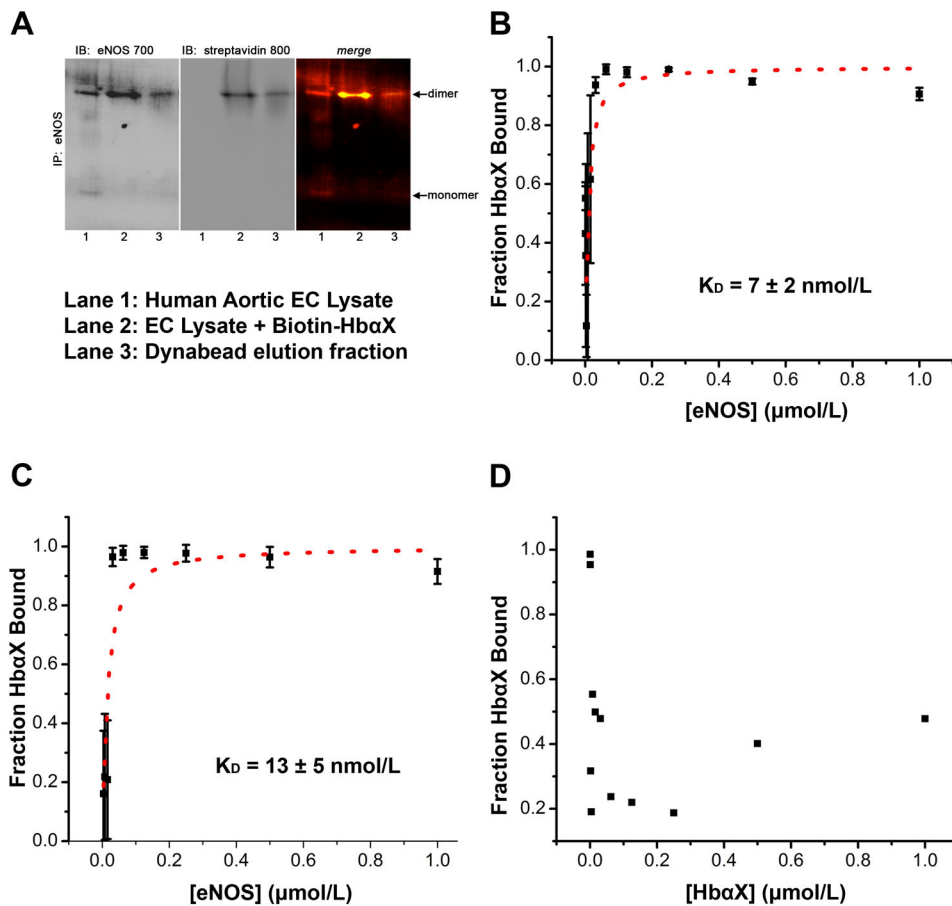


Figure 4. An alpha globin mimetic peptide and eNOS dimers bind with high affinity

(A) Co-immunoprecipitation of eNOS and biotin-labeled HbaX from hAoECs shows that HbaX binds to the dimeric form of eNOS. Non-denaturing IP and electrophoresis conditions were used to preserve the conformational state of eNOS. Lane 1 shows EC lysate as a control for eNOS species. Lane 2 is from beads incubated with EC Lysate and biotin-conjugated HbaX peptide. Lane 3 is sample eluted from dynabeads with low pH. (B) Fluorescence polarization assays show nanomolar binding affinity between fluorescein isothiocyanate (FITC) – labeled HbaX and the oxygenase domain of eNOS. The concentration of FITC-HbaX is 5 nmol/L in (B). The total concentration of peptide in (C) was held constant, but only half (2.5 nmol/L) was fluorescently tagged. This effectively will double the observed binding affinity because of the 1:1 competition for binding site. (D) shows no curve because no binding was observed between HbaX peptides. The concentration of labeled peptide was held constant (5 nmol/L) and was used to serially dilute unlabeled HbaX. In B–D, the points represent mean and bars indicate standard deviation, samples were run in triplicate in one experimental preparation.

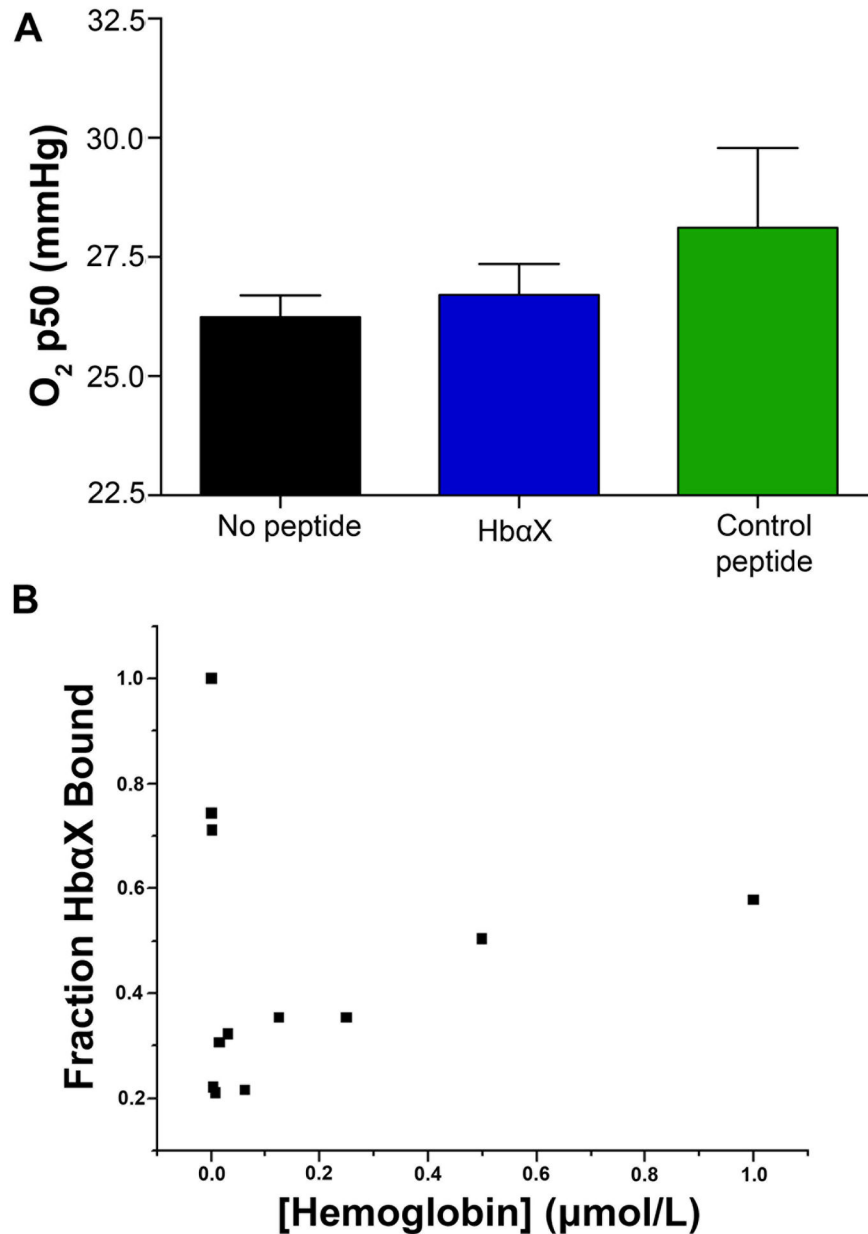


Figure 5. An alpha globin mimetic peptide does not alter the O₂ binding parameters of hemoglobin

(A) The oxygen p50 of whole human blood was measured and was found not to be altered by incubation for 5 minutes with HbaX (blue) or a scrambled control peptide (green) (n=3 experiments for each condition). (B) Fluorescence polarization of 5 nmol/L FITC-labeled HbaX incubated with bovine hemoglobin shows no concentration-dependent binding of the molecules due to the lack of a hyperbolic shape.

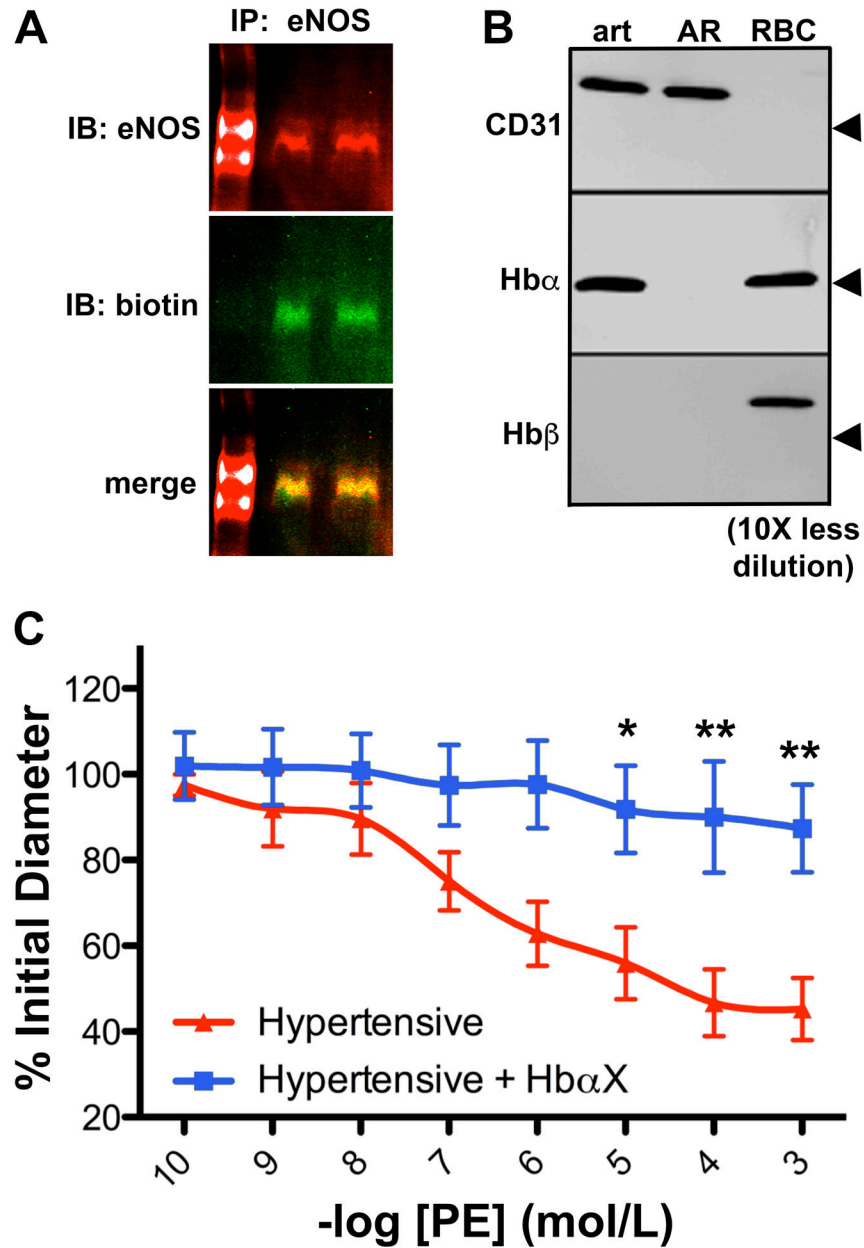


Figure 6. An alpha globin mimetic peptide significantly reduces hyperconstriction in arterioles from human patients with hypertension

(A) Far-Western blotting for recombinant human eNOS oxygenase domain on a non-denaturing gel using anti-eNOS and biotin-Hb α X probes and corresponding secondary antibodies for fluorescent detection. Left lane serves as a molecular weight marker, but it is not used to estimate molecular weight due to the non-denaturing condition of the gel. The samples were run in duplicate. (B) Western blot of the expression of alpha globin without its beta globin partner is confirmed in human arterioles, and a 10-fold dilution of RBC samples is used as a positive control for alpha and beta globin chains. Note the lack of alpha globin in aorta. Arrowheads for CD31 indicate 100 kDa, and alpha and beta globin indicate 10 kDa. (C) Resistance arterioles (approximately 100 μ m in diameter) isolated from human adipose

tissue biopsies were cannulated and subject to phenylephrine cumulative dose-response curves in the absence (black) or presence (blue) of 5 $\mu\text{mol/L}$ Hb α X. (n=4). * indicates $p < 0.05$, ** indicates $p < 0.01$ using two-way ANOVA and a Bonferroni post-test.

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