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 described[1] 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 (FPYFSTKLTT; 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 mitagenesis: 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 Mutatgenesis Kit (Agilent) according to manufactures directions. Primers used to create Flag-pCMV6-Hb α –VTY were 5'-ccctggagaggatgttcgtgacctatcccaccaccaagaccta-3' and 5'-taggtcttggtggtgggataggtcacgaacatcctctccaggg-3' and Flag-pCMV6-Hba1-AARAF were 5'-gtcgaagtgcgggaagaaggccctggcggcggggaaggacaggaac-3' and 5'-gttcctgtccttccccgccgccagggccttcttcccgcacttcgac-3'.

Co-immunoprecipitaion 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 described[18].

Purified eNOS and Hb α protein interaction studies: Purified Flag-eNOS was purchased from Origene and isolated Hb α chains were generated as previously described[1]. Co-immunoprecipitation studies were performed by incubating 1 μg of Flag-eNOS with 5 μmol/L of each peptide (*tat*-only, Scr X or Hb α X) for 30 minutes at 37°C while shaking. Then, 1 μg of isolated Hb α chains were added to the Flag-eNOS/peptide complex for an additional 30 minutes while shaking at 37°C. Anti-flag nickel beads, blocked with 1% bovine albumin serum for 1 hour, were added to each binding reaction for an additional hour with agitation. Proteins were washed 3x with PBS for 15 minutes and purified protein-protein complexes were precipitated using a strong magnet. The nickel beads were incubated with 5x Laemmli sample buffer to elute proteins off the beads. Samples were then subjected to Western blot analysis to determine peptide-induced disruption of Hb α and eNOS binding.

Mice: Male C57BL/6 or eNOS$^{-/-}$ male mice between the ages of 10-12 weeks were purchased from Taconic Farms or Jackson Labs and were used according to the University of Virginia Animal Care and Use Committee guidelines.

Coronary endothelial cell culture and stimulation: Primary human microvascular coronary endothelial cells (Lonza) were cultured on plastic 6-well dishes as previously described[1]. For studies involving basal NO release, endothelial cells were incubated with 5 μmol/L of Scr X or Hb α X for 20 minutes followed by medium collection and nitrite measurements as described below. For bradykinin studies, coronary endothelial cells were grown to confluence followed by serum starvation overnight in a cocktail of Lonza EGM-2 medium supplemented with EGM-2 bullet kit and Opti MEM reduced growth medium using a ratio of 1:9 respectively. The next day, endothelial cells were incubated with Scr X or Hb α X peptide for 20 minutes followed by the addition of 10 μM bradykinin (Sigma) for 5 minutes followed by medium collection for nitrite measurements as described below.

Visualization of Hb α X-FITC peptide and Hb α in the holes of the internal elastic lamina: Thoracodorsal (TD) arteries were isolated from C57BL/6 mice and immediately placed in Krebs-HEPES buffer. Each artery was cannulated in the chamber of a pressure myograph (Danish Myo Technology) containing Krebs-HEPES and the lumen was perfused and pressurized at 80 mmHg with Krebs-HEPES containing 1% BSA. After a 30 minute equilibration period, the lumen was perfused with the Hb α X-FITC peptide (5μmol/L) for 20 minutes. The lumen was then washed with calcium-free Krebs-HEPES and fixed with 4% PFA for 30 minutes. After washing with Krebs-HEPES, the lumen was perfused for 30 minutes with blocking solution (5% goat serum, 0.5% BSA, and 0.25% Triton-X100 in PBS) while the pressure myograph chamber was filled with the same blocking solution. A rabbit anti-Hb α primary antibody (Abcam, no sequence homology with Hb α X-FITC or Hb α; 1/100 in blocking solution) was perfused into the lumen for 10 minutes and the TD arteries were removed from the cannula and placed in an individual well of a 96 well-plate filled with blocking solution and primary antibody and incubated overnight at 4° C. The next day, the TD arteries were re-cannulated to wash out excess primary antibody. After washing, Rhodamine Red-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, 1/50 in blocking solution) was perfused luminally and abluminally. The TD artery was placed in a well of a 96 well-plate containing the secondary antibody in blocking solution for 30 minutes at room temperature. The TD arteries were then re-cannulated and washed both lumenally and ablumenally with calcium-free Krebs-HEPES to wash out excess secondary antibody. Lastly, the lumen was perfused with AlexaFluor633-conjugated sodium hydrazide (Molecular Probes, 0.2 μM in calcium-free Krebs-HEPES) to mark elastin for 20 minutes and the excess dye was further washed for 10 minutes. This technique was based upon previously published methods for protein visualization within the holes of the IEL[2, 3]. At the end of the experiment, the TD artery was removed from the cannula at one end while the other end was still secured. The TD artery was then cut longitudinally from the unattached end and placed on a glass slide with the luminal side facing down and the excess saline solution removed. A single drop of DAPI mounting medium (ProLong Gold, Invitrogen) was placed next to the vessel and a coverslip was positioned on the vessel. The mounting medium was allowed to diffuse between the slide and the coverslip for 10 minutes while a weight was placed on the coverslip to ensure flattening of the artery for microscopy. The coverslip was sealed with nail polish and viewed on an Olympus Fluoview 1000 laser scanning confocal microscope. For quantification purposes, the peptide and Hb α were each determined to be present in the holes of the IEL if fluorescence was >50% maximal intensity, and only within or on the physical boundaries of the hole as determined by the AlexaFluor633 sodium hydrazide.

Proximity ligation assay and quantitation on thoracodorsal arteries: Isolated TD arteries were perfused with Scr X or Hb α X peptide (5 μmol/L) for 20 minutes and immediately immersed in 4% paraformaldehyde, paraffin-embedded and sectioned as previously described [4]. Next, sections were de-paraffinized, blocked and incubated with mouse anti-eNOS (BD Biosciences; 1:500), rabbit anti-Hb α (Abcam; 1:500) and mouse anti-caveolin-1 (BD Biosciences; 1:500) primary antibodies overnight at 4°C. The following day, secondary antibodies conjugated with oligonucleotide PLA probes were added, ligated and rolling circle amplification with fluorescent oligonucleotides identified positive interaction sites as previously described[1]. All images were visualized and captured using an Olympus Fluoview 1000 laser scanning confocal microscope. For proximity ligation assay quantification, positive interactions indicated by red punctates on the endothelium were counted and divided by the circumference of the lumen using Metamorph software.

Western blot analysis of coronary endothelial cell lysates: Endothelial cells were harvested in lysis buffer, sonicated and subjected to electrophoresis using 10% Bis-Tris polyacrylamide gels (Invitrogen) as previously described[5]. Proteins were transferred to nitrocellulose, incubated with phospho-eNOS S1177 (BD Biosciences) or total eNOS (Sigma) and visualized and quantitated using a Li-Cor Odyssey Imager as previously described[6].

Nitrite and NO_x measurements from media of coronary endothelial and red blood cells: Quantification of nitrite in culture medium was measured by chemiluminescence using a Sievers nitric oxide analyzer according to the manufacturer's instructions. Quantification of peaks was analyzed using Origin Pro 6.0 software as previously described [1]. For red blood cell (RBC) studies, veinous blood was withdrawn from the inferior vena cava and placed in an EDTA tube. Scr X or Hb α X (5 μmol/L) were incubated with RBCs for 30 minutes and spun on centrifuge through a 30 KDa molecular weight cutoff filter. NOx was measured on the filtrate using a Cayman Chemical assay (nitrate/nitrite).

cGMP assay on thoracodorsal arteries: Isolated TD arteries were cannulated and pressurized to 80 mmHg as previously described [5]. Arteries were perfused with 5 μmol/L Scr X or Hb α X with addition of 0.5 mmol/L of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (all conditions) or 100 μmol/L of the nitric oxide synthase inhibitor L-NG-Nitroarginine Methyl Ester (L-NAME) for 20 minutes. Arteries were then stimulated with 50 μM phenylephrine for 10 minutes and immediately immersed in lysis buffer provided in the cGMP XP assay Kit (Cell Signaling) according to the manufacturer's instructions. Briefly, a competition enzyme-linked immunoassay was used to generate a standard curve of known cGMP concentrations followed by calculating cGMP concentrations of experimental samples. The cGMP concentration in experimental samples was normalized to total protein concentration.

Pressure myography on thoracodorsal arteries: Thoracodorsal arteries were isolated, cannulated and pressurized in a Danish Myo Technology (DMT) pressure myograph as previously described[5]. Following 10 minutes of equilibration, vessels were perfused lumenally with Scr X or Hb α X peptide and incubated for an additional 20 minutes. Contractile responses were studied using cumulative concentrations (10^{-9} - 10^{-3} mol/L) of phenylephrine in the presence or absence of 100 μmol/L L-NAME. After completion of dose responses to phenylephrine, potassium chloride (40 mmol/L) was added to ensure vessels could contract equally. Following constriction, the maximum diameter was measured by incubating the vessel in calcium-free Krebscontaining ethylene glycol tetraacetic acid (EGTA, 1 mmol/L) and sodium nitroprusside (10μmol/L). Quantification of vessel diameter was performed using DMT vessel acquisition software and data are expressed as the percentage of initial inner diameter. Half maximal effective concentration (EC_{50}) and maximum drug concentration (E_{max}) were calculated at previously described[7].

Abdominal Aorta Ring Assay: Abdominal aortas were isolated, cut into 2 mm wide rings and mounted on a DMT wire myograph system with low bath volumes as previously described[8]. Briefly, rings were stretched at 1.2 × resting length in Krebs solution and allowed to equilibrate for 30 min at 37 °C prior to depolarization with 154 mm K^+. Following the K^+ contraction, rings were returned to Krebs solution and incubated with 5 μmol/L Scr X or Hb α X peptide for 20 minutes. Cumulative concentrations of phenylephrine (PE) (10^{-10} - 10^{-4} mol/L) were added to the rings and the magnitude of the tension response measured in milli-newtons.

Blood pressure analysis: Blood pressure was measured in conscious C57Bl/6 or $eNOS^{-/-}$ mice under unrestrained conditions using implanted radio telemetry units. Continuous blood pressure measurements were recorded using Dataquest A.R.T. 20 software (DSI). To do so, mice were anesthetized with isoflurane and the catheters (TA11PA-C10, Data Sciences International (DSI)) were implanted in the left carotid artery. The catheter was tunneled through to the radiotransmitter, which was placed in a subcutaneous pouch along the flank. Mice were allowed to recover for seven days after surgery to regain normal circadian rhythms before arterial pressure measurements and experiments were initiated. For acute blood pressure measurements, a continuous base line reading was obtained thirty minutes prior to peptide injection. Then, a bolus injection of Scr X or Hb α X peptide, or saline was administered via an intraperitoneal injection at 5 mg/kg. One hour post-injection, blood pressure was recorded for a 30 minute duration. For time course studies, the blood pressure measurements involved continuous

recording for 5 minutes every hour throughout the day. In adult C57Bl/6 mice (N=6) Hb α X peptide (5mg/kg) was injected daily at 9 am and blood pressure monitored continuously. A separate set of age matched adult C57Bl/6 mice was injected with a scrambled version of Hb α X peptide (5mg/kg). These injections occurred 7 days in a row. Baseline per mouse was calculated by averaging 24 hours of recording prior to any injection. Change from baseline per mouse was calculated by averaging the blood pressure measurements one hour after injections for 9 hours (10 am – 7 pm) and subtracting from baseline. For induction and maintenance of hypertension, mini-osmotic pumps were implanted with angiotensin II at a dose of 1000 ng/kg/min for five days. An averaged hypertensive baseline per mouse was obtained with two-hour pre-injection measurements collected daily throughout the duration of the experiment. In all cases, the change in blood pressure was calculated by subtracting the average pre injection blood pressure from the average post-injection blood pressure.

Statistics: Statistics on individual comparisons were performed using Student's *t*-test. For multiple comparisons a one-way ANOVA was used and for dose response curves a two-way ANOVA followed by a Bonferroni's *post-hoc* test was used. All statistics were computed using GraphPad Prism 5.

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