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Supplemental Material

Methods

$OA-NO_2$ synthesis. Nitro-oleic acid ($OA-NO_2$) used in this study was synthesized via nitroselenation. Oleic acid (OA) (NuCheck Prep, >99%) was converted to a nitro-phenylselenylated intermediate in the presence of mercuric salts, and then oxidized with hydrogen peroxide (30% aqueous) to yield the nitroalkene regioisomers of $OA-NO_2$. Product purification was performed by column chromatography on silica gel, with purity determined by ^1H NMR and HPLC-electrospray mass spectrometry. $OA-NO_2$ produced by this method yields an equimolar distribution of 9- and 10-nitro-octadec-9-enoic acid regioisomers.

Wire-mediated vascular injury. All animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Approval 0702181). Mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg body weight; Aveco) and xylazine (5 mg/kg, Lloyd Laboratories) diluted 0.9% sodium chloride solution. Osmotic mini-pumps (21 day delivery, ALZET®, Durect Corporation, CA, USA) containing either vehicle (V), OA (2 mg/kg/d), or $OA-NO_2$ (2 mg/kg/d) were subcutaneously implanted in wildtype C57BL/6 male mice, 8-10 weeks of age (Jackson Laboratories, Bar Harbor, ME), immediately prior to left common femoral artery injury. $OA-NO_2$ was stabilized in mini-pumps by co-solvation in polyethylene glycol (PEG 400) containing 15% ethanol. In another cohort of C57BL/6 male mice, Sn(IV) protoporphyrin (SnPP) was administered to mice (i.p., 50 μmol/kg) one time immediately prior to mini-pump implantation and femoral wire injury and then every 3 days for 21 days.

Additionally, age-matched HO-$1^{-/-}$ mice on a C57BL/6 background (kindly provided by Dr. Anupam Agarwal) were treated with either vehicle or $OA-NO_2$ and subjected to femoral artery injury.

To achieve unilateral femoral artery injury, the left common femoral artery was exposed using a midline leg incision, and blunt dissected to separate from the femoral vein and nerve. The femoral artery was clamped proximally and ligated distally with an 8-0 silk suture. An additional silk suture was looped around the femoral artery just proximal to the site of arteriotomy. A 0.014-inch flexible angioplasty guidewire was introduced into the femoral artery and the proximal clamp is removed. Endothelial denudation injury of the left common femoral artery is performed using wire withdrawal injury and three passes along the common femoral artery. Before removal of the guidewire, the femoral artery is clamped proximally. The wire is then removed and the femoral artery is ligated just proximal to the arteriotomy site.

At 21 days, mice received one intraperitoneal (i.p.) injection of Nembutal® sodium solution (65 mg/kg) (Abbott Laboratories, North Chicago, IL) for anesthesia and were perfused with PBS followed by 4% paraformaldehyde prior to removal of the femoral arteries. Femoral arteries were then post-fixed in 4% paraformaldehyde overnight and dehydrated in 30% sucrose for an additional 24 hours. Vessels were then embedded in OCT compound for sectioning. To obtain mRNA from femoral arteries, a cohort of mice were killed 3 days after femoral artery injury.

Detection and quantitation of OA-NO_2 in serum. Blood was removed from mice and transferred to a Microtainer® Brand Serum Separator Tube (Becton Dickinson and Company, Franklin Lakes, NJ) and allowed to clot for 2 hours at room temperature. The serum was obtained by centrifugation at 6000 x g at room temperature for 5 minutes. Serum was combined with cold (-20 °C) acetonitrile (1:4) and centrifuged at 2500 rpm for 15 minutes at 4 °C, to obtain lipid extracts. An internal standard ([^{13}C]OA-NO_2) was added during extraction to correct for losses due to sample preparation. A hybrid triple quadrupole/linear ion trap mass spectrometer (4000 Q-Trap LC/MS/MS; Applied Biosystems/MDS Sciex) was used to quantitate OA-NO_2 levels. The mass spectrometer interfaces with an HPLC system, enabling OA-NO_2 to be resolved via unique chromatographic properties and retention time. A C18 reverse phase column was utilizing a gradient solvent system as follows: **A** (H_2O containing 0.1% formic acid) and **B** ($CNCH_3$ containing 0.1% formic acid) using the following conditions: 60% **B** (2 minutes); 60-95% **B** over 5 minutes; 95% **B** (3 minutes); 95-60% **B** over 0.1 minutes; and 60% **B** equilibration (4 minutes). Multiple reaction monitoring (MRM) scanning in the negative ion mode allows for transitions of intact and ionized species to be predicted and monitored. The MRM transitions used are based upon the precursor ion *m/z* and the most abundant product ion. Values for OA-NO_2, *m/z* 326/279, as well as [^{13}C]OA-NO_2, *m/z* 344/279 are based upon the common loss of the nitro group which occurs during collision-induced dissociation of nitrated fatty acids. Following integration of the area under the peak(s), the ratios of analyte to internal standard areas are determined, permitting OA-NO_2 to be quantified using a standard curve with Analyst 1.4 software (Applied Biosystems/MDS Sciex).

Vessel Morphometry. To measure intimal and medial cross-sectional areas of injured and non-injured femoral arteries, three sets of three serial 6 μm thick cross-sections of each artery, spaced at 0.3 mm intervals, were cut. To visualize smooth muscle actin and endothelial cells, sections were permeabilized with 0.1% TritonX in phosphate buffered solution for 15 minutes, blocked for 45 minutes with 2% bovine serum albumin and incubated for 1 hour with a primary monoclonal anti-CD 31 antibody (BD Pharmingen, Franklin Lakes, NJ) and a monoclonal anti-α-smooth-muscle actin antibody conjugated with Cy3 (Sigma, Saint-Louis, MO). Subsequently, sections were incubated for 1 hour with a Cy5-conjugated Affini Pure antibody (Jackson ImmunoResearch, Baltimore, MD). Autofluorescence was used to visualize internal and external elastic membranes. Cross-sectional images were collected using an Olympus Provis I fluorescence microscope. Intimal and medial areas were quantified by image analysis (MetaMorph software, MDS, Toronto, Canada).

Immunofluorescence. Cross-sections (6 μm thick) of injured femoral arteries were blocked in 2% bovine serum albumin and incubated for 1 hour with a primary antibody to HO-1 (Stressgen, Victoria, Canada). Bound primary antibody was detected using a secondary Cy5-conjugated anti-rabbit antibody (Jackson ImmunoResearch, Baltimore, MD). Images were obtained using a Zeiss confocal microscope. To detect Ki67 positive cells femoral arteries were incubated with a primary antibody to Ki67 (Abcam, Cambridge, MA) followed by a secondary Alexa Fluor® 546 (Invitrogen, Carlsbad, CA) antibody. Nuclei were stained using Hoechst stain (10 mg/ml, Sigma-Aldrich, Inc, St Louis, MO).

Cells and cell culture. Rat aortic smooth muscle cells (RASMC) were isolated via explant and cultured in DMEM containing 10% FBS in 5% CO_2 at 37 °C. All experiments were performed using RASMC between passage 3 and 8. For cell proliferation studies, RASMC were plated onto 96 well plates at a density of 10,000 cells/cm^2 and allowed to attach overnight. For some experiments, RASMC were transfected with 50 μM siRNA against HO-1 or non-targeting control siRNA (Dharmacon Lafayette, CO) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). Transfections were performed using a reverse transfection protocol according to manufacturer´s instructions.

Cells were serum starved for 24 hours and then stimulated to proliferate with DMEM containing 2% serum and treated with 2.5 μM OA or 1 or 2.5 μM OA-NO_2. After 24 hours cell proliferation was assessed using the Cyquant NF proliferation assay as described by the manufacturer (Invitrogen, Carlsbad, CA).

For migration studies, RASMC were seeded into 6 well plates and grown to near confluence. RASMC were serum starved overnight and then scratched with a sterile pipette tip to produce a cell-free zone bordered by straight wound edges. Cells were stimulated to migrate with 20 ng/ml PDGF and concomitant addition of either 250 nM OA, or 25, 50, 100 or 250 nM OA-NO_2, with or without 50 μM SnPP (Frontier Scientific, Logan, UT). For some experiments RASMC were transfected with non-targeting or 50 μM HO-1 siRNA as described above. At baseline as well as after 18h pictures were obtained with an IX-71 Olympus microscope and analyzed using Adobe Photoshop CS3 Extended software.

Real-time quantitative PCR. Total RNA from RASMC and femoral artery tissue was isolated with TRIzol® and/or further purified using the RNeasy Mini, RNA isolation kit (Qiagen, Valencia, CA). Complimentary DNA was obtained from 100 ng RNA using iScript reagents (Bio-Rad Laboratories, Hercules, CA) or SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quantitative mRNA expression was assessed using real-time PCR with TaqMan Fast Universal PCR Master Mix or Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA). Specific gene expression assays used are as follows: HO-1 (Rn00561387-m1), actin (4352340E) or primer sequences for mouse HO-1: forward, 5'-TCA GTC CCA AAC GTC GCG GT-3'; reverse, 5'-GCT GTG CAG GTG TTG AGC C-3', HO-2: forward, 5'-ACT ACT CAG CCA CAA TGT CT-3'; reverse, 5'-GTG AAT CCG ATC CAC ATA CT-3' and mouse GAPDH: forward, 5'TGA AGG TCG GTG TGA ACG GAT TTG GC-3'; reverse, 5'CAC CAC CTG GAG TAC CGG ATG TAC-3', where dissociation curves were observed for undesirable formation of primer-dimers. Samples were run in triplicate on the StepOne or Prism 7000 detection systems (Applied Biosystems, Foster City, CA).

Western blot analysis. Cells were washed twice with cold PBS and then lysed in buffer containing 50mM Tris, 1%NP-40, 1mM EDTA, 125mM NaCl, 20mM Deoxycholic Acid, 1mM Sodium Orthovanadate, 20mM Sodium Fluoride, 1mM Sodium Pyrophosphate, and Protease Inhibitors (Sigma cat: P8340). The lysates were slowly rotated at 4ºC for 1 hour. The samples were centrifuged at 14,000 rpm for 5 minutes and the supernatant was collected and assayed for protein concentration. Equal amounts of protein were loaded onto an SDS page gel (4% stacking, 10% resolving) and separated by electrophoresis. Protein was transfered to a nitrocellulose membrane and the membrane blocked in tris buffered saline, 0.1% tween 20

(TBST) containing 5% dry milk (w/v). HO-1 (1:5,000) and HO-2 (1:1000) were detected using Stressgen antibodies (Stressgen Biotechnologies, Ann Arbor, MI) followed by the corresponding horse radish peroxidase linked secondary antibodies. Proteins were visualized using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL).

Heme Oxygenase Enzyme activity. Heme oxygenase activity was measured by bilirubin generation in microsomal preparations from mouse liver as described previously[1]. Liver microsomes were incubated with rat liver cytosol, a source of bilirubin reductase (3 mg), hemin (20 μmol/L), glucose-6-phosphate (2 mmol/L), glucose-6-phosphate dehydrogenase (0.2 units), and NADPH (0.8 mmol/L) for 1 h at 37°C in the dark. The formed bilirubin was extracted with chloroform and the change in optical density, 464 to 530 nm, was measured (extinction coefficient, 40 $mmol/L^{-1}. cm^{-1}$ for bilirubin). Enzyme activity was expressed as nmol of bilirubin formed/mg protein/hour.

Statistical analysis. Results are expressed as mean ± SD or SEM. Statistical analysis was performed using one-way ANOVA and unpaired students t-test as appropriate. Differences between groups were assessed by Bonferroni post hoc test. A value of $p < 0.05$ was considered statistically significant. SPSS 15.0 was used for all calculations.

1. Agarwal A, Balla J, Alam J, Croatt AJ, Nath KA. Induction of heme oxygenase in toxic renal injury: a protective role in cisplatin nephrotoxicity in the rat. *Kidney Int.* 1995;48:1298-1307.