SUPPLEMENTAL MATERIAL

Supplemental Methods

Cell Culture

Mouse aortic VSMC were isolated from 4-month old male C57BL/6J mice as previously described.[1] Cells were grown in DMEM containing 10% fetal bovine serum in a humidified incubator with 5% CO_2. Experiments were performed on multiple primary cultures derived from several mice. All experiments were conducted using VSMC between passage numbers 3 and 12 that were growth-arrested at 80% confluence for 72 hours with medium containing 0.1% fetal bovine serum.

Reverse transcriptase-Polymerase Chain Reaction (RT-PCR) and Sequencing of NOXA1

Total RNA was isolated from mouse aortic VSMC using RNeasy Micro Kit (Qiagene). The first-strand cDNA synthesis was carried out using SuperScript II reverse transcriptase (Invitrogen) as per manufacturer's instructions. PCR was performed with primers generated from the mouse NoxA1 sequence (5′-TTCCAGCTGCAGAGGTTCCAG-3′ as forward primer and 5′-AAGTCCAAATCCTCCGGTCT-3′ as reverse primer) to amplify a 923-bp fragment (247-1169 bp) of the NoxA1 gene. The PCR was run with the following cycle profile: 94°C for 1 min; 35 × 94°C for 30 seconds, 57°C for 30 seconds, 68°C for 1.5 minutes, and 68°C 10 min. Positive and negative controls for PCR were pcDNA3-NOXA1 plasmid (generously provided by Dr. Banfi, University of Iowa) and total RNA, respectively. The PCR product was then cloned into Topo-TA cloning vector (Invitrogen) and sequenced using M13 (forward and reverse) primers. Similarly, we performed RT-PCR to determine the expression of p67phox in mouse VSMC. PCR was performed with primers generated from the mouse p67phox sequence (5′-CTACCTGGAGCCAGTTGAGC-3′ as forward primer and 5′-

GGGCAGCCTCATAACTGAAG-3′ as reverse primer) to amplify a 636-bp fragment (876-1411 bp) of the p67phox gene.

Retroviral Vector Construction and Infection of Mouse Aortic VSMC

Retroviruses encoding hairpin shRNA of NoxA1 were constructed using pSUPER.retro.puro vector (OligoEngine). A double-stranded hairpin oligonucleotide designed to target the mouse NoxA1 cDNA nucleotides 376-394 (5′-TACAACATGGCATCAGCAC-3′) of NoxA1 gene was cloned into the BglII/HindIII site of pSUPER.retro.puro vector to generate NoxA1 shRNA. A control shRNA was constructed which encoded a 19-bp scrambled sequence (5′-GCGCGCTTTGTAGGATTCG-3′) with no significant homology to any mouse gene. Correct insertion of the oligonucleotides was confirmed by sequencing. All retroviruses were prepared in [Phi]Nx-eco cells (Orbigen) by calcium phosphate transfection. Puromycin resistant clones (selected with 2 μg/mL puromycin for 10 days) were expanded prior to their use in experiments.

Adenovirus Construction and Infection of Mouse Aortic VSMC

Hemagglutinin (HA) tag was added to the C-terminus of pcDNA3-NoxA1 by PCR recombination. The PCR product, HA-NoxA1 was subcloned into pAdTrackCMV shuttle vector, and the resulting plasmid was recombined with the pAdEasy1 vector in BJ5183 *Escherichia coli.* The recombinant adenoviral vector DNA was transfected into human embryonic kidney 293 cells with LipofectAMINE (Invitrogen), then the viruses were serially amplified in HEK 293 cells, purified on a CsCl density gradient by ultracentrifugation, and tittered as described.[2] The control adenovirus contained empty pAdTrackCMV vector. Myc tag was added to the N-terminus of human p47phox cDNA and adenoviruses containing Myc-p47phox were constructed as described above. Adenoviral infection of nearly confluent mouse aortic VSMC was performed

at a multiplicity of infection of 100 in DMEM containing 2% FBS for 48 hours. The cells were then quiesced in DMEM containing 0.1% FBS for 72 hours and treated with thrombin as indicated.

Measurement of Reactive Oxygen Species

Intracellular hydrogen peroxide levels were measured using 2',7'-dichlorofluorescein diacetate (H_2DCF-DA; Invitrogen), which is oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) by H_2O_2.[3] The medium of quiesced VSMC grown in 24-well glass-bottomed plates was replaced by Hank's balanced salt solution containing 10 μM H_2DCF-DA for 30 minutes prior to treatment with or without thrombin. Fluorescence was measured by using a WALLAC 1420 Multilabel Counter with excitation and emission wavelengths set at 485 nm and 535 nm, respectively. H_2O_2 levels were normalized to mg protein content.

Superoxide levels in mouse aortic VSMC were measured by staining with dihydroethidium (DHE; Invitrogen).[4] Cells were quiesced for 72 hours and treated with thrombin (2U/mL) for 30 min, washed with Hank's balanced salt solution (HBSS), and incubated with 10 μmol/L of DHE at 37°C for 15 minutes. DHE fluorescence was obtained at 590 nm in several visual fields using Leica DMIRB fluorescent microscope. Superoxide anion generation was also measured using chemiluminescence enhancer L012 [8-amino-5-chloro-7-phenylpyridol[3,4-d]pyridazine-1,4(2H,3H)dione].[5] Quiesced mouse aortic VSMC were harvested from 10-cm culture dishes using accutase/PBS (PAA, Pasching, Austria). 2.0×10^5 intact cells were resuspended in 300 μl HBSS containing 100 μM L012 and incubated for 15 min and treated with thrombin (1 U/mL) or vehicle for 10 min. Superoxide production was determined using WALLAC 1420 Multilabel counter and the mean light signal subtracted from the background was taken as a measure for O_2^- production.

Frozen sections of carotid artery specimens were stained with 10 μmol/L dihydroethidium for 10 min at 37°C. Confocal microscopy imaging was performed using Olympus FV500 LSM at excitation and emission set at 485 and 585 nm, respectively. Images were quantified using NIH ImageJ software.

Immunoprecipitation and Western Blot Analysis

Preparation of cell extracts, immunoprecipitation and Western blotting were preformed as described.[6] Rabbit anti-NoxA1 200 antibody was generated as described.[7] The other antibodies used were as follows: monoclonal anti-β actin, monoclonal anti-β-tubulin (Sigma), polyclonal p38 MAP kinase, polyclonal anti-phosphospecific p38 (Thr180/Tyr182), polyclonal anti-Akt1, polyclonal anti-phosphospecific Akt1 (Ser 473), polyclonal anti-phosphospecific JAK2 (Tyr1007 and Tyr1008) (Cell Signaling Technology), and monoclonal anti-cMyc (Santa Cruz Biotechnology).

[^{3}H]Thymidine Incorporation Assay

Mouse aortic VSMC, infected with pSUPER.retro. puro viruses containing either NoxA1 or scrambled shRNA, were grown to ~70% confluence in a 24-well plate, and quiesced by incubating in DMEM containing 0.1% fetal bovine serum for 72 hours. Quiesced VSMC were treated with or without 1 unit/mL thrombin for 16 hours after pretreatment with diphenyleneiodonium, an NADPH oxidase inhibitor, for 1 hour. Cells were labeled with [*methyl*-^{3}H]thymidine for 3 hours, and its incorporation into DNA was determined by liquid scintillation counting.[6]

In Vitro Migration Assay

Migration of mouse aortic VSMC was determined in a scratch wound assay. Mouse aortic VSMC were grown in 10-cm dishes until 90% confluent and then infected with either Ad-HA-NoxA1 or Ad-GFP virus for 24 hours. When the cells were confluent, the cell monolayers were scratched with a sterile rubber policeman to create cell-free zones. The cells were washed and treated with or without thrombin 1 U/mL for 16 hours. Cells were briefly stained with Hemotoxilin and visualized on an Olympus inverted microscope connected to a Color Camera.

Real-time RT-PCR to Measure NoxA1, p67phox and Nox1 Expression

Total RNA was extracted from cells using RNeasy Micro Kit (Qiagen). Reverse transcription was performed using TaqMan Reverse Transcription Reagents Kit (Applied Biosystems). Real-time PCR using TaqMan Gene Expression Assays for mouse NoxA1 (Mm00549175_m1), p67phox (Mm00726636_s1) and Nox1 (Mm00549170_m1) was carried out on ABI PRISM 7900 HT Sequence Detection System according to manufacturer's protocol. Relative expression levels were determined by normalization to 18S ribosomal RNA expression using REST2005 (Relative Expression Software Tool).[8]

Carotid Artery Injury and NoxA1 Overexpression

Ten week old wild-type mice were anesthetized with an intraperitoneal injection of pentobarbital (45 mg/kg). The right external and common carotid arteries were surgically exposed through midline neck incision, and isolated. Angioplasty guide wire (tip diameter 0.36 mm) (Guidant Corporation) was introduced into the arterial lumen through an arteriotomy made in external carotid artery just distal to the common carotid artery bifurcation and passed five times to denude the endothelium. Artery was flushed with PBS and a 1 cm segment was isolated with vascular ligatures. A 50 µl PBS or PBS containing AdNoxA1 or AdGFP vector ($1x10^9$ pfu) was injected through catheter and incubated in the common carotid artery for 30 min. After removal of this

solution, the external carotid artery was ligated and blood flow to the internal carotid artery was restored. The arteriotomy site was ligated by tying the previously placed suture and skin incision was closed with 7-0 sutures. The contralateral common carotid artery served as an uninjured control vessel. Animals were sacrificed and specimens collected 3 and 15 days after injury.

Histology and Morphometry

The vasculature was cleared by transcardial perfusion with 20 mL of PBS, followed by 20 mL of freshly prepared 4% paraformaldehyde (PFA) in PBS. Carotid artery specimens were postfixed for 24 hours in 4% PFA. Tissue samples containing a segment of common carotid artery were then blocked and embedded in paraffin. Eight adjacent 5 μm sections were cut every 250 μm, extending through the length of the tissue block. Serial sections from each artery were stained with combined Masson's trichrome elastic stain. Morphometric analysis was performed using NIH ImageJ 1.37 software. Luminal, intimal and medial areas were determined in a blinded manner. Intima/media ratio was calculated and averaged for each specimen.

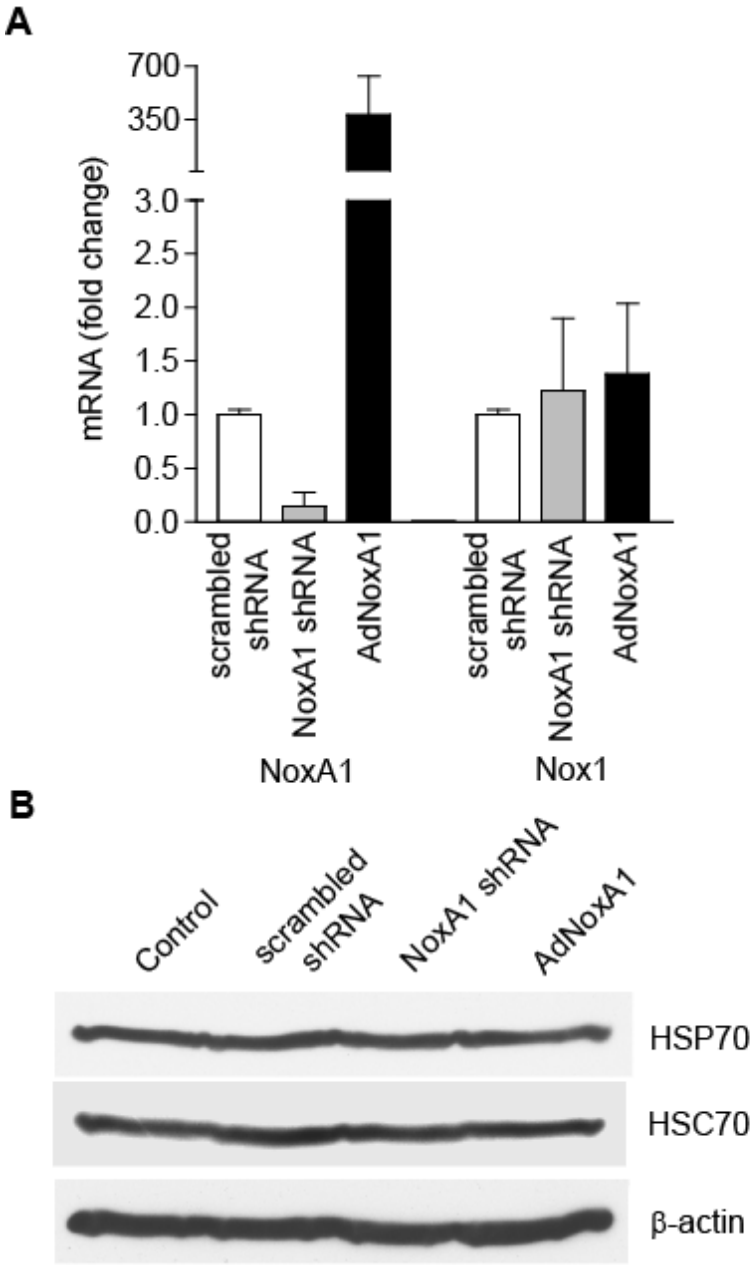
Fresh frozen sections of carotid artery specimens were prepared by clearing vasculature with PBS and freezing in OCT compound (Ted Pella). Six adjacent frozen 10 μm sections were cut every 250 μm. Whole aortas, dissected from animals perfused with PBS, were divided into arch, thoracic and abdominal parts and frozen in OCT compound. Six to eight 15 μm serial sections were cut every 500 μm. Sections from each aorta were stained with oil red O. Human carotid artery sections were stained with oil red O and hematoxylin-eosin.

Immunohistochemistry

Serial sections of carotid artery and aorta were stained using rabbit anti-NoxA1 antibody (antibody199). Immunostaining was performed using Vectastain Elite ABC Kit and ImmPACT

DAB substrate (Vector Laboratories) according to manufacturer recommendations. The sections were counterstained with Vector Methyl Green and permanently mounted with VectaMount Mounting Medium (Vector Laboratories). Human carotid artery sections were stained using rabbit anti-NoxA1 (antibody 199), rabbit anti-p67phox (Millipore) or mouse anti-smooth muscle actin (Dakocytomation) antibodies.

Suppl Figure I



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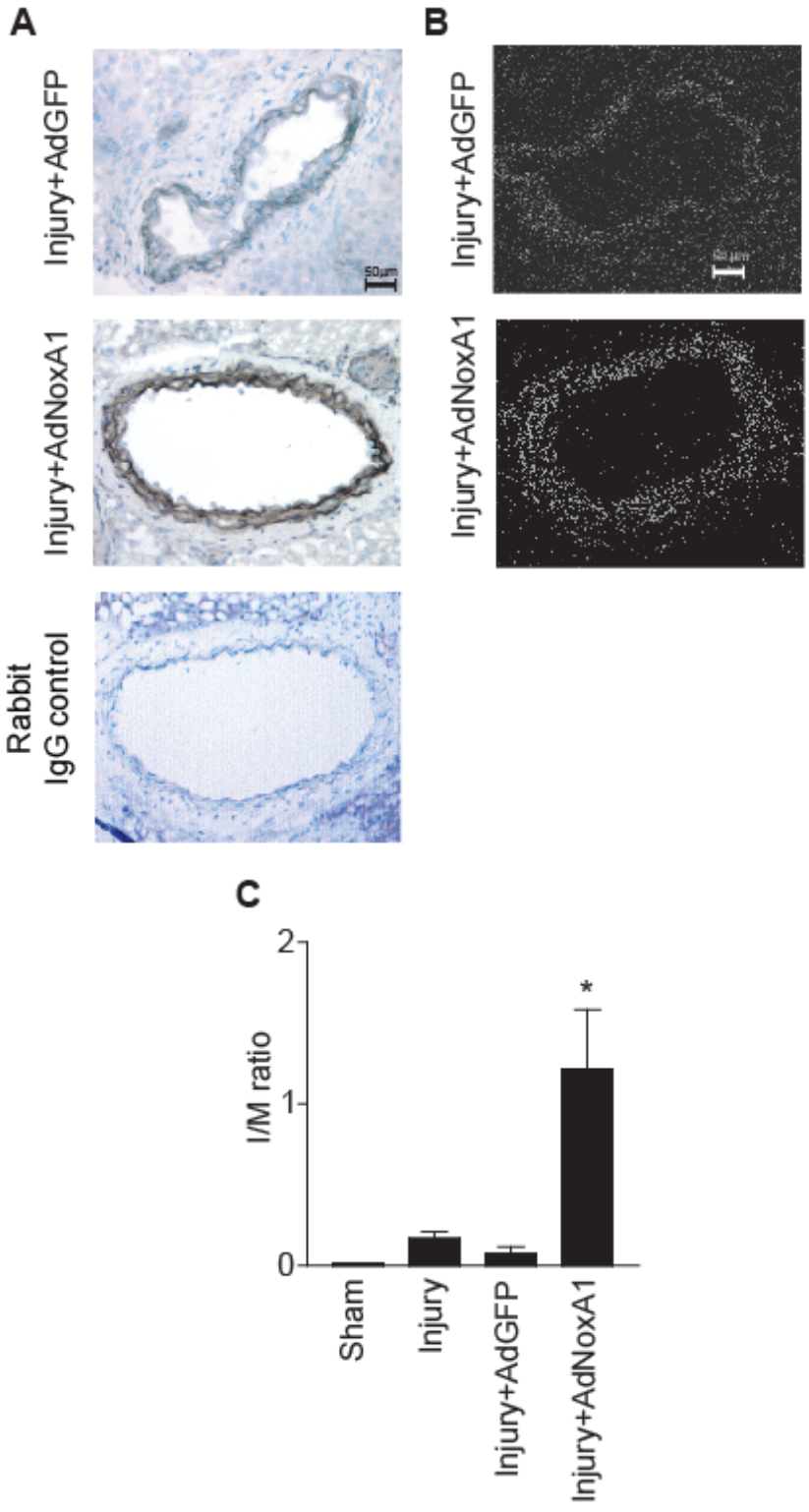
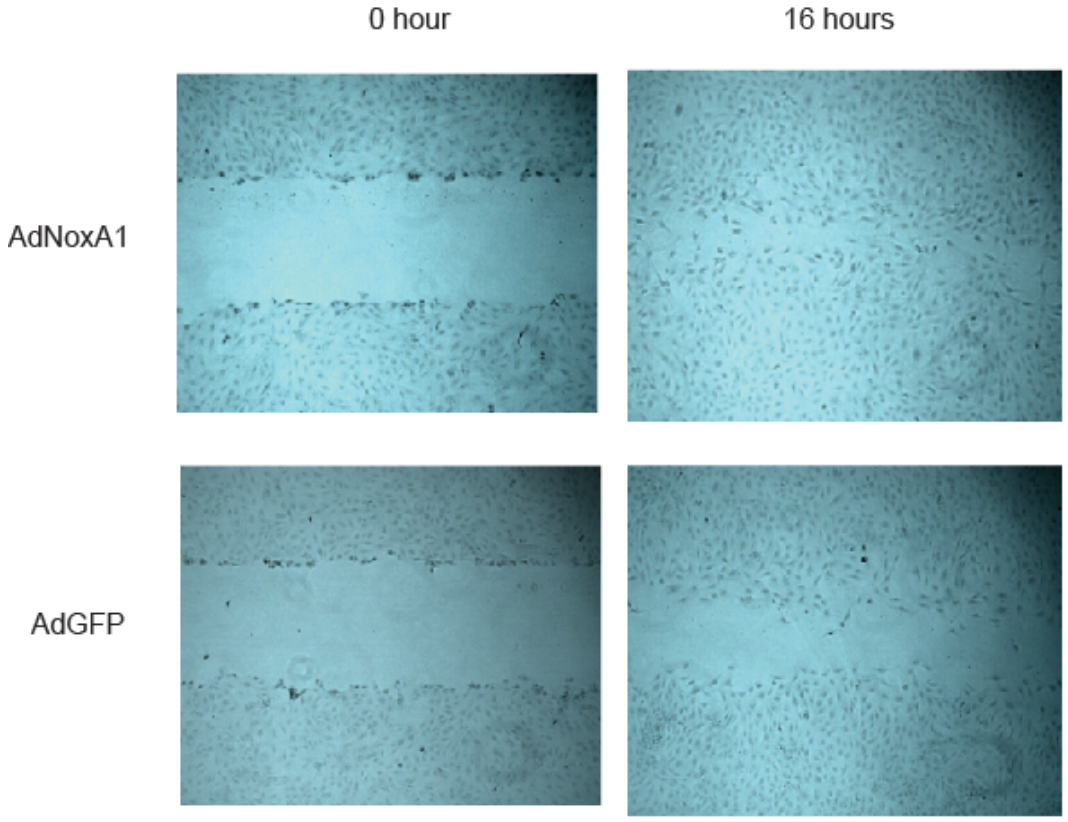


Suppl Figure III



SUPPLEMENTAL FIGURE LEGENDS

Figure I. Overexpression or knockdown of NoxA1 had no significant effect on the expression of Nox1 or cause cellular stress response. A, Real-time RT-PCR analysis of expression of NoxA1 and Nox1 genes in mouse VSMC expressing scrambled shRNA, NoxA1 shRNA or HA-NoxA1 (mean $\pm$ SEM, n=3). B, Stress response was assessed by Western blot analysis of cell lysates for HSC70 and HSP70 expression in control VSMC and VSMC expressing scrambled shRNA, NoxA1 shRNA or HA-NoxA1.

Figure II. Increased NoxA1 expression, enhanced superoxide production and neointima formation in AdNoxA1 infected mouse carotid arteries. A, Representative sections of carotid arteries, 3 days after injury and infection with adenovirus encoding NoxA1, were stained for immunoreactive NoxA1 or non-immune rabbit IgG. B, Representative LSM images of injured

carotid arteries stained with DHE. C, Morphometric analysis of intima/media ratio of carotid artery sections 15 days after injury and adenoviral infection (mean ± SEM, n=7, *P<0.05 vs injured and AdGFP-infected arteries).

Figure III. Overexpression of NoxA1 enhances VSMC migration. Mouse aortic VSMC were infected with adenoviruses encoding either NoxA1 or GFP and images were acquired at 0 and 16 hours in in vitro scratch assays. The data shown are the representative of three independent experiments with similar results.

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