SUPPLEMENTAL MATERIAL

Data S1.

SUPPLEMENTAL METHODS

Mice expressing human Nox5 in smooth muscle cells.

The generation of Nox5 transgenic mice was approved by the Animal Ethics Committee of the Ottawa Hospital Research Institute, University of Ottawa and carried out according to the recommendations of the Canadian Council for Animal Care. *NOX5β* cDNA was PCR amplified from the pDONRNox5β plasmid (GeneCopoeia, Rockville, USA) using high fidelity Phusion Polymerase (New England Biolabs, Herts, UK) and modified primers with 5' NotI and 3'SalI recognition sequences. Purified PCR NOX5β gene-coding region was ligated into the Tet-responsive promoter Pbi-1 (Clontech, Mountain View, USA). A 3.7kb TetO/NOX5β fragment was excised using EcoRV/XmnI digestion and the resulting band gel-purified and provided to the University of Ottawa Core Transgenic Facility for pronuclear injection into fertilized FVB/N oocytes. Subsequent pBI-NOX5β founders on a pure FVB/N background were identified by PCR genotyping. To generate VSMC specific knock-out animals, pBI-Nox5 animals were crossed with the SM22-tTA-FVB/N mouse strain to produce Nox5+/SM22+ and control transgenic mice (SM22+, Nox5+). Animal experiments on wild-type (WT), SM22+, Nox5+ and Nox5+/SM22+ were performed in accordance with the United Kingdom Animals Scientific Procedures Act 1986 and ARRIVE Guidelines (1) and approved by the Home Office under Project Licence No 7009021.

In additional studies, adult (20 weeks) WT, SM22+, Nox5+ and Nox5+/SM22+ mice were infused with Ang II (600 ng/Kg/day) for 4 weeks by osmotic minipumps (model 2004, Alzet, Cupertino, CA) implanted under isoflurane (3% induction; 1.5% maintenance) anaesthesia. At the end of treatment, animals were euthanized by exsanguination following cardiac puncture with immediate dissection of tissues that were rinsed, snap-frozen in liquid nitrogen and stored at − 80 °C or fixed in formalin for preparation of histological analysis.

Measurement of blood pressure

Systolic blood pressure (SBP) was assessed by tail-cuff plethysmography (2,3). Mice were trained to the apparatus (Visitech Systems model BP-2000) for two consecutive weeks until stable readings were obtained. Once baseline SBP measurements were obtained, osmotic mini-pumps with Ang II were implanted and SBP was assessed weekly for the duration of the experimental protocols.

Plasma Biochemistry

Blood was collected immediately prior to sacrifice by cardiac puncture in heparinized tubes. After collection, plasma was separated by centrifugation (2000 RPM, 10 min) (Heraeus Megafuge 16R; ThermoScientific), snap frozen, aliquoted and stored at -80^{o}C. Plasma biochemistry (Ca^{2+}, phosphate (PO_4^-), sodium (Na^+), potassium (K^+), chloride (Cl^-), magnesium (Mg^{2+}) were determined by an automated analyzer (Roche/Hitachi cobas c systems - cobas c 311 Autoanalyser).

Measurement of plasma lipid peroxidation products

Plasma lipid peroxidation was determined by quantifying malondialdehyde (MDA) (a naturally occurring product of lipid peroxidation) using the thiobarbituric acid-reactive

substances (TBARS) assay kit (Cambridge Biosciences, UK). TBARS were calculated by plotting the obtained absorbance against an MDA concentration standard curve.

Cardiac histology and fibrosis staining

After collection, hearts were rinsed in cold PBS and fixed in 4% buffered (in PBS) formalin for 48 hours. Fixed tissues were stored in 70% ethanol and then processed. Tissue processing was performed by dehydration, diafanization and Paraplast embedding. Heart samples were cut into 5mm sections and placed on silane–coated slides. For histochemical analysis, the sections were deparaffinized, rehydrated, and washed with H_2O. To assess collagen content, heart sections were stained with picrosirius red (0.1% w/v). Total collagen content (%) was measured in the whole tissue under polarised light using an Olympus BH-2 microscope (Olympus, Japan).

To assess cardiomyocyte area, H&E staining of heart sections was performed. Images of cardiac tissue were taken using an EVOS (Life Technologies) microscope at 100x magnification, and 5 fields of each section of heart were captured. Finally, images were used to measure the area of 10 cells per field (50 cells measured per animal). Data were quantified by digital image analysis software (ImageJ) with the observer blinded to group identity.

Myography to assess vascular functional, structural and mechanical properties.

Vascular function was assessed in resistance arteries by wire myography as we previously described (2,3). Briefly, second-order branches of mesenteric artery without perivascular fat were isolated (2 mm in length) from WT, SM22+, Nox5+ and Nox5+/SM22+ mice and mounted on a wire myograph (DMT myograph; ADInstruments Ltd., Oxford, U.K.). Vessel segments were equilibrated in Krebs Henseleit-modified physiological salt solution (in mmol/L: 120 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.18 KH_2PO_4, 1.18 $MgSO_4$, 2.5 $CaCl_2$, 0.026 EDTA, and 5.5 glucose) at 37°C, continuously bubbled with 95% O_2 and 5% CO_2, pH 7.4. At the beginning of each experiment, arteries were contracted with 62.5 mmol/L KCl to test for functional integrity. Endothelium-dependent relaxation was assessed in all vessels by concentration-responses to ACh (1 nmol/L-100 µmol/L) where vessels were precontracted with U46619 (a thromboxane A2 analogue) at a concentration to achieve approximately 80% of maximal response (30 nmol/L). Thereafter endothelium-independent relaxation was assessed by concentration-responses to sodium nitroprusside (SNP, 1 nmol/L-100 µmol/L) and contractile responses mediated by U46619 (0.1 nmol/L-1 µmol/L) were evaluated in endothelium-intact arteries. At the end of relaxation and contraction curves, ET-1 induced contraction was evaluated, where arteries were exposed to a single concentration of ET-1 (10 nmol/L). Maximal contraction to KCl was also evaluated in all vessels, before and after addition of pharmacological inhibitors. In some experiments, arteries were pre-incubated with N-acetylcysteine (ROS scavenger; 10 µmol/L), GKT137831 (Nox1/Nox4 inhibitor; 10 µmol/L), diltiazem (Ca^{2+} channels blocker, 10 µmol/L), calmidazolium (calmodulin inhibitor, 1 µmol/L) and dantrolene (ryanodine receptor (Ryr) blocker that inhibits ER Ca^{2+} release, 10 µmol/L).

Vascular structure and mechanical properties were assessed in resistance arteries prepared as pressurised systems on a pressure myograph as we previously described (3). For the assessment of structural and mechanical properties, second order branches of the mesenteric artery (2 to 3 mm in length) were slipped onto 2 glass microcannulae, one of which was positioned until vessel walls were parallel, in a

pressure myograph (DMT myograph; ADInstruments Ltd., Oxford, U.K.). Vascular structure and mechanics were assessed under Ca^{2+}-free conditions to eliminate the effects of myogenic tone. Vessels were perfused for 30 min with Ca^{2+} free Krebs solution containing 10 mmol/L EGTA. Measurement of media thickness and lumen diameter were taken at stepwise increments of luminal pressure (10 to 120 mmHg). Vascular structural and mechanical parameters were calculated as previously described (3).

Mouse vascular smooth muscle cell culture

VSMCs from adult male WT and Nox5+SM22+ mice were studied. Mice (20 weeks old) were euthanized by overdose of an anaesthetic gas (isofluorane) followed by neck dislocation. Mesenteric arteries were cleaned from fat/connective tissue and VSMCs extracted and culture as we described previously (4) above for human VSMCs. At subconfluence, cells were rendered quiescent with 0.5% FBS medium for 16h. Low-passage cells (passages 4–7) were studied.

Experimental protocols

Mouse VSMCs were stimulated with U46619 (0.1 nmol/L, 5 and 15 mins) for ROS assessment by lucigenin chemiluminescence. In some experiments, cells were incubated with calmidazolium (1 µmol/L), verapamil (Ca^{2+} channel blocker, 10 µmol/L) or dantrolene (10 µmol/L).

Immunofluorescence

Nox5 immunofluorescence was performed in aortas isolated from WT, SM22+, Nox5+ and Nox5+SM22+ mice. Briefly, paraffin-sections (4 µm) were deparaffinised in xylene (3 x 7 min) and rehydrated in descending series of ethanol solutions (100%, 100%, 95%, 95%, 70% and 50%) for 5 mins each, washed in dH2O for 5 min, followed by a final rinse in 1xTBS for 5 mins. Antigen retrieval was performed by boiling the slides in 1 mM EDTA (pH 8.0) for 15 mins. After cooling at room temperature for 30 mins, the slides were washed three times with 1xTBS for 5 mins. Sections were blocked in 10% goat serum in 1xTBS for 1 hr at RT in a humidified chamber. Sections were incubated with a rabbit polyclonal anti-human Nox5 antibody (1:75, gift from David J. Lambeth, Emory University, USA) overnight at 4^{o}C in a humidified chamber. For negative controls, rabbit IgG matched isotype controls were used (Cell Signalling, DA1E, Cat. Number: 3900). The following day, sections were washed three times with 1xTBS for 10 min and incubated with Alexa-fluor-488-conjugated goat anti-rabbit secondary antibody (1:300; Life technologies, Molecular Probes) for 45 min at room temperature (protected from light exposure). Slides were washed three times in 1xTBS for 10 mins. Primary and secondary antibodies were diluted in 5% goat serum in 1xTBS. Fluorescence imaging was recorded using an Axiovert 200M microscope with a laser scanning module LSM 510 (Carl Zeiss AG, Heidelberg, Germany). DAPI was excited at 405 nm; Alexa-fluor 488 at 488 nm and Lectin-conjugated with Rhodamine at 543 nm. Images were recorded using the 'Physiology Evaluation' software package (Zeiss) and image processing was performed using Image J software.

Measurement of nitrotyrosine levels

Nitrotyrosine, a measure of peroxynitrite ($ONOO^{-}$) formation, was assessed in aorta tissue from WT and Nox5+/SM22+ mice using an ELISA kit (#ab113848, Abcam, Cambridge, UK), according to manufacturer's instructions. The plate was read in the kinetic mode for 15 min at 3 min intervals at absorbance of 600 nm using a microplate reader and results were normalized to protein concentration.

Lucigenin-enhanced chemiluminescence assay

Vascular ROS generation was measured by a luminescence assay with lucigenin as the electron acceptor and NADPH as the substrate. VSMCs from mesenteric arteries were homogenized in assay buffer (in mmol/L: 50 KH_2PO_4, 1 EGTA, and 150 sucrose, pH 7.4) with a glass-to-glass homogenizer. The assay was performed with 100 µL of sample, 1.25 µL of lucigenin (5 µmol/L), 25 µL of NADPH (0.1 mmol/L) and assay buffer to a total volume of 250 µL. Luminescence was measured for 30 cycles of 18 seconds each by a luminometer (Lumistar Galaxy, BMG Lab technologies, Germany) (2). Basal readings were obtained prior to the addition of NADPH to the assay. The reaction was started by the addition of the substrate. Basal and buffer blank values were subtracted from the NADPH-derived luminescence. Superoxide anion production was expressed as relative luminescence units (RLU)/µg protein.

Quantification of Superoxide Production by HPLC in cardiac tissue

Superoxide levels in hearts from WT, Nox5+/SM22+, SM22+ and Nox5+ were measured by high-performance liquid chromatography (HPLC) (5). This protocol measures 2-hydroxyethidium (2-OHE), which is a highly specific product of the reaction between superoxide and dihydroethidium (DHE). Heart tissue was homogenised in cold phosphate buffered saline. Protein levels were determined and 50 µg of sample was used in all reactions in a final volume of 70 µL. Two sets of samples were prepared, where one set was treated with tiron (10 mM, 10 min, Sigma Chemical Co, St. Louis, MO, USA). After treatment of one set with tiron, all samples were incubated with DHE (25 mM, Thermo Fisher Scientific Inc, Wilmington, DE, USA) for 30 min avoiding exposure to light. Methanol (100 µL) was then added to the samples and they were vortexed. Ice cold HCl (0.1N - 100 µL) was then added and the samples were centrifuged at 13000 RPM for 5 min at 4oC. The supernatant was collected and transferred to glass amber vials. HPLC was carried out with two mobile phases (A: 0.1 % trifluoroacetic acid and B: 0.085 % trifluoroacetic acid in acetonitrile. Sigma Chemical Co , St. Louis, MO, USA). The non degradated DHE was eluted at 5.8 min, the specific product produced by oxidation of DHE by superoxide, 2-hydroxyethidium, was eluted at 15 min and, finally, the unspecific product of the degradation of DHE, ethidium, was eluted at 16.5 min. The specificity of the signal is confirmed by comparison of 2-OHE production in the presence of tiron (superoxide scavenger). Superoxide levels were determined from the difference between the signal intensity with and without tiron.

Real-time PCR

Total RNA was isolated using the Trizol reagent (Life Technologies) according to the manufacturer's instructions and diluted in nuclease-free H_2O (Ambion/Life Technologies, Paisley, UK). cDNA was generated from total RNA using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Warrington, UK). Real-time polymerase chain reaction was performed with the Applied Biosystems 7900HT Fast Real-Time PCR system, using Power SyBr Green Master Mix (Applied Biosystems) and specific mouse primers, as follows: Nox2 (FW: CGCCCTTTGCCTCCATTCTC; RW: CCTTTCCTGCATCTGGGTCTCC); Nox4 (FW: CCAGAATGAGGATCCCAGAA; RW: AGCAGCAGCAGCATGTAGAA). Relative gene expression was calculated by the $2^{-\Delta\Delta}$ cycle threshold method (6). Data are shown as the fold change in expression of the target gene relative to the internal control gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Western blotting

Total protein was extracted from human and mouse VSMCs and from mesenteric arteries from WT, SM22+, Nox5+ and Nox5+/SM22+ mice. Samples were homogenized in 50 mmol/L Tris-HCl (pH 7.4) lysis buffer containing 1% Nonited P-40, 0.5% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% SDS, 2 mmol/l sodium orthovanadate (Na_3VO_4), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL pepstatin A, 1 µg/mL leupeptin and 1 µg/mL aprotinin. Total protein extracts were cleared by centrifugation at 10,000 rpm for 10 min and the pellet was discarded. VSMCs were lysed in 50 mmol/L Tris-HCl (pH 7.4) lysis buffer containing 1% Nonited P-40, 0.5% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% SDS, 2 mmol/l sodium orthovanadate (Na_3VO_4), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL pepstatin A, 1 µg/mL leupeptin and 1 µg/mL aprotinin. Total protein extract were sonicated and cleared by centrifugation at 10,000 rpm for 10 min and the pellet was discarded. Protein concentrations were determined using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Proteins from homogenates (30 µg) were separated by electrophoresis on a polyacrylamide gel, and transferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with 0.01 % Tween for 1 hour at room temperature. Membranes were then incubated with specific antibodies overnight at 4°C. Antibodies were as follows: MYPT1 [Thr696] (Santa Cruz, #SC25618); p-MLC20 [Thr19/Ser 19] (Cell Signalling, #3674S); Nox1 (Sigma, #SAB4200097); Nox2 (Abcam, #ab31092); Nox4 (Abcam, #ab133303) and Nox5 (Sigma, #SAB4503153). Antibodies to β-actin or GAPDH (Sigma) were used as internal housekeeping controls. After incubation (1h) with secondary fluorescence-coupled antibodies (Licor), signals were visualized by an infrared laser scanner (Odyssey Clx, LICOR). Protein expression levels were normalized to loading controls and expressed as absolute values or percentage (%) of the control.

Measurement of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in VSMCs.

$[Ca^{2+}]_i$ was measured in VSMCs from WT and Nox5+/SM22+ mice using the fluorescent Ca^{2+} indicator, Cal-520 acetoxymethyl ester (Cal-520/AM; Abcam; 10 µmol/L). Cells, grown in 6-well plates were incubated with 10 µmol/L Cal-520 AM in 0.5% FBS at 37°C for 75 minutes followed by 30 minutes at room temperature. Following incubation, the dye solution was replaced with HEPES physiological saline solution (130 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L CaCl, 1 mmol/L MgCl , 20 mmol/L HEPES, and 10 mmol/L D-glucose, pH 7.4) for 30 minutes prior to imaging. $[Ca^{2+}]_i$ was measured in the absence and presence of ionomycin (1 µmol/L). Fluorescence measurement of Cal-520 AM-Ca^{2+} signals were performed using an inverted epifluorescence microscope (Axio Observer Z1 Live-Cell imaging system, Zeiss, Cambridge, UK) with excitatory wavelengths of 490 nm and emission at 535 nm. Images were acquired and analysed using Zen Pro (Zeiss, Cambridge, UK).

Nox5-expressing insect model - *Rhodnius prolixus*.

Rhodnius prolixus (R. prolixus) were studied as an insect animal model that expresses Nox5 endogenously (7). The *R. prolixus* colony was maintained at 28 °C and 70–80% relative humidity at the Instituto de Bioquímica Médica Leopoldo de Meis, UFRJ. Insects were fed with rabbit blood and adult females were studied in this study. All animal care relative to rabbits and experimental protocols were conducted in accordance with the guidelines of the Committee for Evaluation of Animal Use for Research (Federal University of Rio de Janeiro, CAUAP-UFRJ) and the NIH Guide for

the Care and Use of Laboratory Animals (ISBN 0-309-05377-3). The protocols were approved by the ethics committee of CAUAP-UFRJ (#IBQM155/13).

siRNA downregulation of Nox5 in *R. prolixus*

A 457-bp fragment from the Nox5 gene was amplified from reverse-transcribed RNAs extracted from *R. prolixus* midguts using the primer pairs NOX5Ds1. The amplification products were subjected to nested PCR with an additional pair of primers (Nox5Ds2) that included the T7 promoter sequence in each fragment. The primers were: Nox5Ds1, forward, 5_-CAGACTGTCGGCAATGAAAA-3_ and reverse, 5_-GTTTTGGCGGTATCAACCAG-3_; NOX5Ds2, forward, 5_-TAATACGACTCACTATAGGGCAGACTGTCGGCAATGAAAA-3_ and reverse, 5_-TAATACGACTCACTATAGGGGTTTTGGCGGTATCAACCAG-3. The nested PCRs generated 497-bp fragment of Nox5. These fragments were used as a template to synthesize double-stranded RNA (dsRNA) specific for Nox5 (dsNox5) using the MEGAscript RNAi kit (Ambion, Austin, TX) according to the manufacturer's instructions. An unrelated dsRNA (dsMal) specific for the *Escherichia coli* MalE gene (Gene ID: 948538) was used as a control for the off-target effects of dsRNA. The Mal fragment was amplified from the Litmus 28i-mal plasmid (New England Biolabs) with a single primer (T7, 5_-TAATACGACTCACTATAGGG-3_) specific for the T7 promoter sequence that is on both sides of the MalE sequence. For gene silencing experiments, adults *R prolixus* were injected in the hemocoel with 1 µL sterile distilled water containing 1 mg/mL dsRNA using a 10 µL Hamilton syringe. Six days after dsRNA injection, the insects were fed with rabbit blood.

RNA Extraction, PCR and qPCR- *R. prolixus*

Total RNA was extracted from anterior midguts using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was treated with RNase free DNase I (Fermentas International Inc., Burlington, Canada) and cDNA was synthesized using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA from anterior midguts were PCR-amplified using the PCR master mix (Fermentas International Inc.) and the same primers were used for qPCR (described below). The fragments were separated by agarose gel electrophoresis (2% w/v), and their sizes were compared with GeneRulerTM 100 bp Plus DNA ladder fragments (Fermentas International Inc.). qPCR was performed on a StepOnePlus real-time PCR system (Applied Biosystems) using the Power SYBR Green PCR master mix (Applied Biosystems). The comparative *Ct* method (Livak et al, 2001) was used to compare gene expression levels. The *R. prolixus EF-1 S* rRNA gene was used as an endogenous control. The primer pairs used for the amplification of Nox5 and EF-1 cDNA fragments for both conventional and real-time PCR, named Nox5Rt and EF1Rt, respectively, were *Nox5Rt*, forward 5'-GCATGGTGGCGTTTAAGAAT-3' and reverse 5'-AACGGAGCTTTTTGAAGCAA-3'; *EF1Rt*, forward, 5'-GATTCCACTGAACCGCCTTA-3' and reverse, 5'-GCCGGGTTATATCCGATTTT-3'.

VAS2870 injection in *R. Prolixus*

VAS2870 is a Nox1/2/4 inhibitor (8). *R. prolixus* were injected in the hemocoel with 5 µL of 0.1 mM VAS2870 (Enzo Life Sciences) dissolved in 1% DMSO/saline 6 days before feeding. All insects were maintained at 28 ◦C in a humid chamber then were fed with rabbit blood. The controls were injected with the same concentration of vehicle (DMSO/*Rhodnius* physiological saline) (130 mM NaCl, 8.6 mM KCl, 8.3 mM $MgCl_2$, 10.2 mM $NaHCO_3$, 4.3 mM Na_2HPO_4, 34 mM Glucose, 2 mM $CaCl_2$).

Images and video acquisition of gut contractions in Nox5-silenced *R. prolixus*

Nox5-siRNA-injected and VAS2870-treated insects were studied 7 days after a blood meal. Peristaltic contractions of the anterior midguts were recorded for 2 minutes by video (Olympus MVX10 macroview fluorescence microscope equipped with a Olympus DP-72 color CCD camera without filters and an external LED white light source). Videos were deframed using VirtualDub (http://virtualdub.org) and images analyzed in ImageJ (Schindelin et al, 2015). Image sequences were imported and an area including the anterior midgut and ovaries were selected. Using the Multimeasure option, we recorded contractions and their magnitude as the peak response of each contraction.

Statistical analysis

Data are presented as mean±standard error of the mean (SEM). Statistical comparisons were made with 1-way ANOVA followed by Dunnet's post-hoc or 2-tailed Student's t test when appropriate. $P<0.05$ was considered statistically significant.

Table S1. Plasma biochemistry (mmol/L) of WT, Nox5+SM22+, SM22+ and Nox5+ mice before and after treatment with Ang II. * p<0.05 vs non-treated group.

	Ca^{2+}	Na^{+}	K^{+}	Mg^{2+}	Cl^{-}	PO_4
WT	2.2±0.1	157.6±2.3	6.4±0.3	0.76±0.03	118.2±2.2	1.62±0.1
WT+Ang II	2.0±0.1	150.2±1.7	5.77±0.2	0.76±0.03	107.4±2.2	2.97±0.3 *
Nox5+SM22+	2.2±0.06	154.8±1.5	5.8±0.4	0.74±0.02	114.9±1.1	1.72±0.06
Nox5+SM22+ + Ang II	1.89±0.2	153.9±2.5	6.72±0.5	0.74±0.02	112.6±4.6	3.52±0.8 *
SM22+	2.3±0.02	155.1±1.2	6.0±0.2	0.83±0.02	112.6±1.3	1.8±0.06
SM22+ + Ang II	1.71±0.4	158.6±2.5	5.48±0.2	0.83±0.02	119.6±5.7	4.34±1.1 *
Nox5+	2.01±0.2	156.6±2.4	5.9±0.4	0.8±0.06	115.7±3.3	1.73±0.13
Nox5+ + Ang II	2.29±0.1	147.7±1.7	5.9±0.5	0.80±0.05	105.3±4.6	2.77±0.7

Ca^{2+} - calcium, Na^{+} - sodium, K^{+} - potassium, Mg^{2+} - magnesium, Cl^{-} - chloride, PO_4 – phosphate, WT – wild-type, Ang II – angiotensin II.

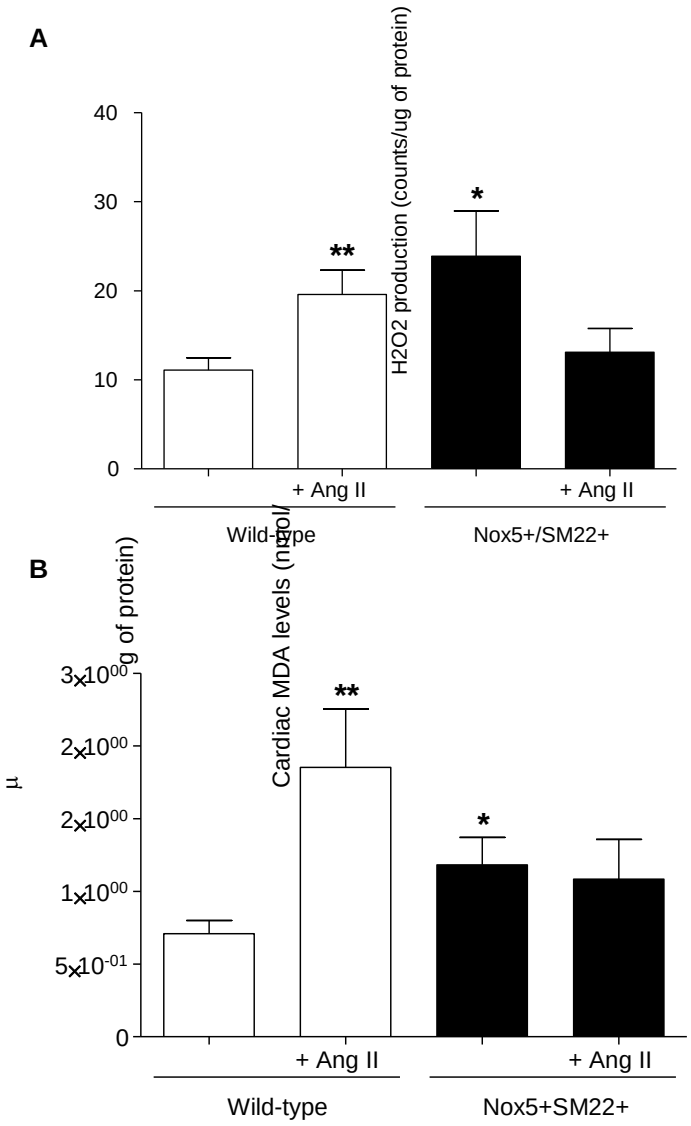

Figure S1. Cardiac H_2O_2 and MDA levels in Nox5+SM22+ mice. (A) H_2O_2 levels in hearts from WT (open bars) and Nox5+SM22+ (closed bars) mice, before and after Ang II-treatment, measured by Amplex Red. (B) MDA levels, a marker of lipid peroxidation, in hearts from WT (open bars) and Nox5+SM22+ (closed bars) mice, before and after Ang II-treatment, measured by ELISA. Results are mean±SEM of 5-8 mice/group. *p<0.05 vs. WT group; **p<0.05 vs. untreated group. Abbreviations: H_2O_2 – hydrogen peroxide**,** MDA – malondialdehyde, WT – wild-type, Ang II – angiotensin II.

Sup Figure 2

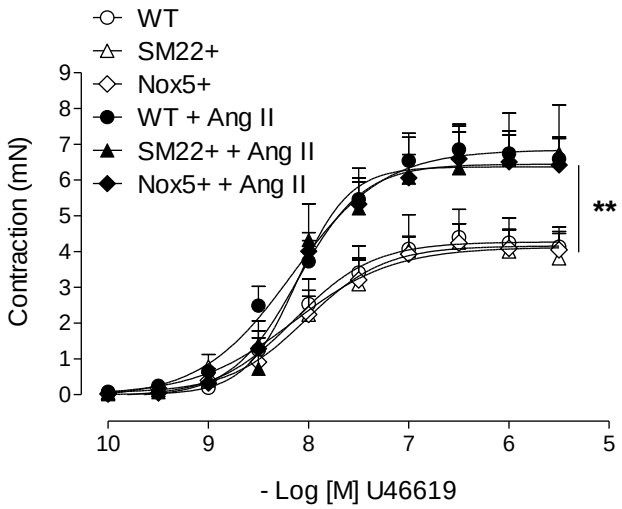

Figure S2. Vascular contraction in mesenteric arteries from control mice (Wild-type, SM22+ and Nox5+ mice). (A) Vascular contractility to U46619 assessed by wire myography in mesenteric arteries from WT (open circle), SM22+ (open triangle), Nox5+ (open rhombus) before and after Ang II treatment (closed symbols). Results are mean±SEM of 5-8 mice/group. **p<0.05 vs. untreated WT, SM22+ or Nox5+ (open symbols). Abbreviations: WT – wild-type, Ang II –angiotensin II.

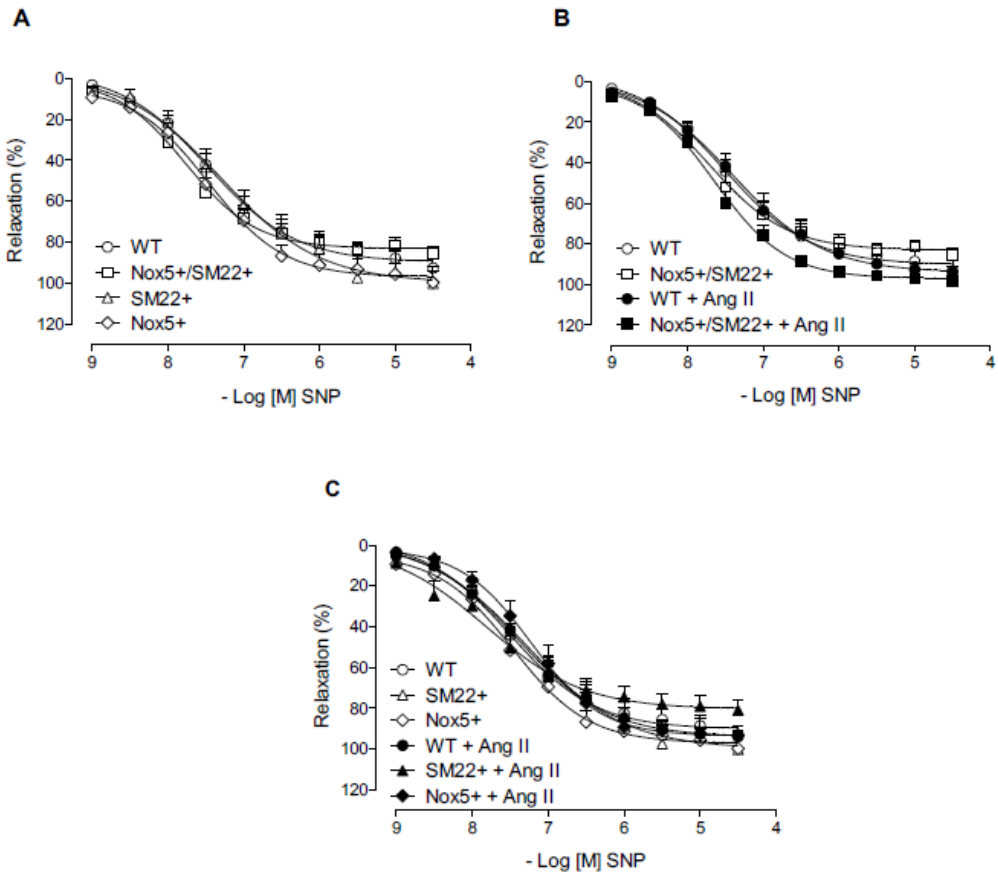

Figure S3. Endothelium-independent relaxation in mesenteric arteries from Nox5+SM22+ and control mice. (A) Vascular relaxation to SNP (endothelium-independent) in arteries from WT (open circle), SM22+ (open triangle), Nox5+ (open rhombus) and Nox5+SM22+ (open square). (B) Vascular relaxation to SNP in arteries from WT (open circle), WT + Ang II (closed circle), Nox5+SM22+ (open square) and Nox5+SM22+ + Ang II (closed square) mice. (C) Vascular relaxation to SNP assessed by wire myography in mesenteric arteries from WT (open circle), SM22+ (open triangle), Nox5+ (open rhombus) before and after Ang II treatment (closed symbols). Results are mean±SEM of 5-8 mice/group. **p<0.05 vs. untreated WT, SM22+ or Nox5+ (open symbols). Abbreviations: SNP – sodium nitroprusside, WT – wild-type, Ang II –angiotensin II.

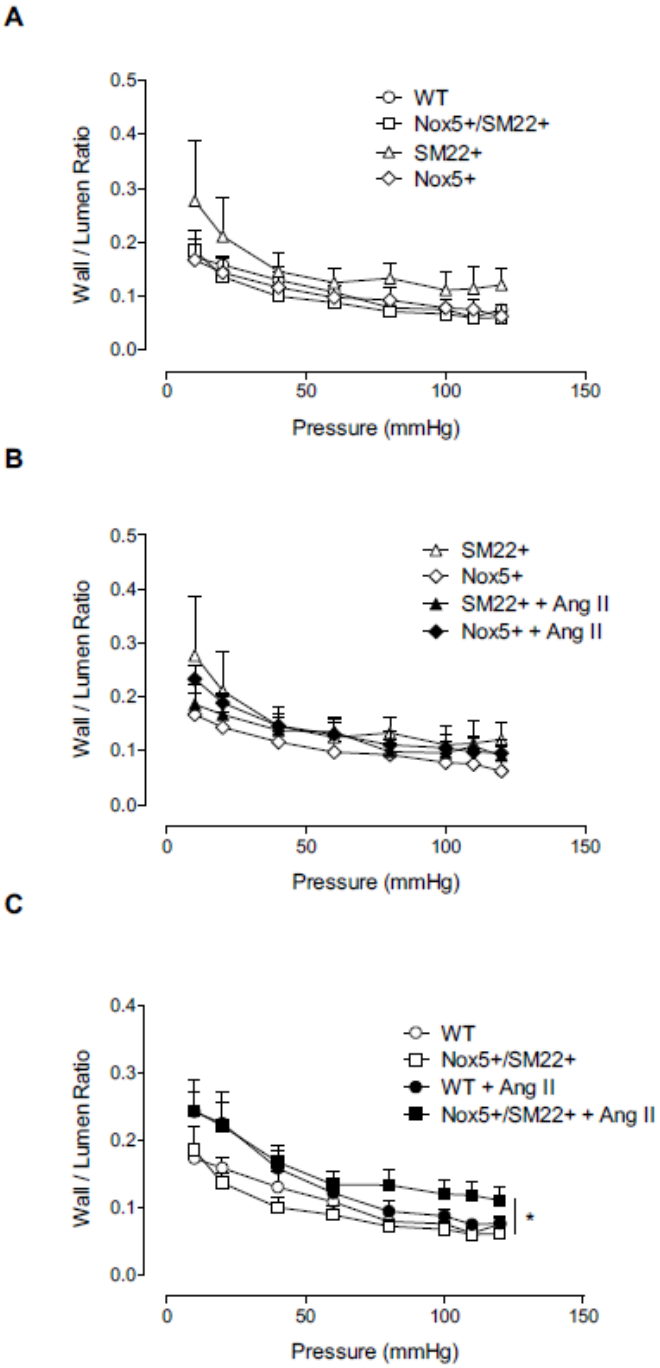

Figure S4. Wall to lumen ratio in resistance arteries Nox5+SM22+ mice. (A) Vascular wall to lumen ratio assessed by pressure myography in mesenteric arteries from WT (open circle), SM22+ (open triangle), Nox5+ (open rhombus) and Nox5+SM22+ (open square). (B) Vascular wall to lumen ratio assessed by pressure myography in mesenteric arteries from SM22+ (open triangle) and Nox5+ (open rhombus), before and after Ang II treatment (closed symbols). (C) Vascular wall to lumen ratio assessed by pressure myography in mesenteric arteries from WT (open circle) and Nox5+SM22+ (open square), before and after Ang II treatment (closed symbols). Results are mean±SEM of 5-8 mice/group. *p<0.05 vs. untreated Nox5+SM22+. Abbreviations: WT – wild-type, Ang II –angiotensin II.

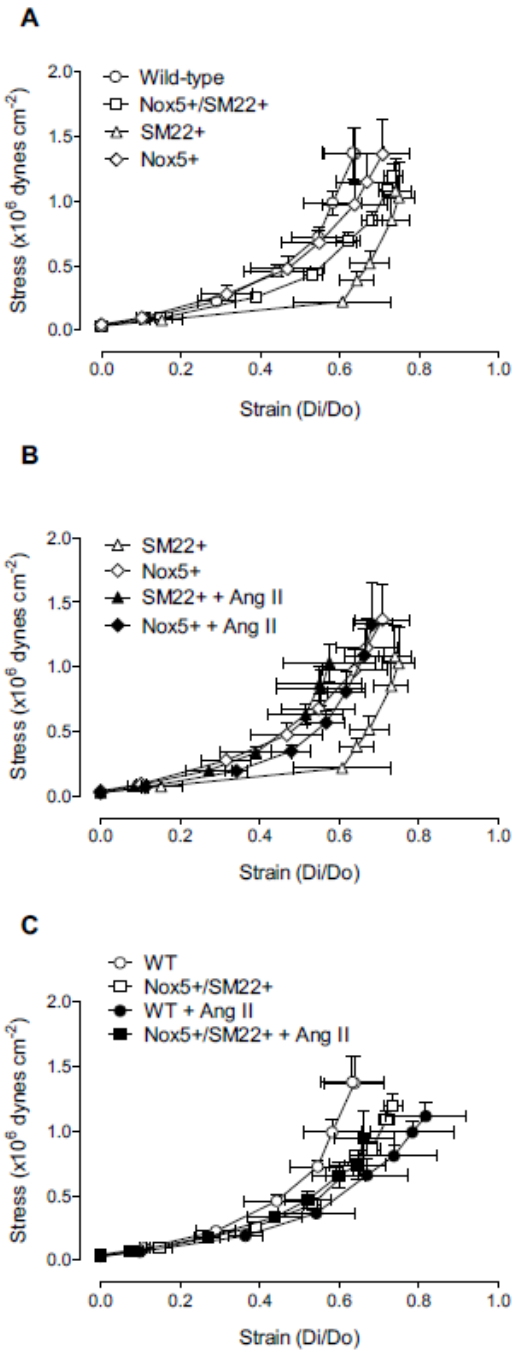

Figure S5. Vascular stiffness in Nox5+/SM22+ mice. (A) Vascular stiffness (strain vs stress curves) assessed by pressure myography in mesenteric arteries from WT (open circle), SM22+ (open triangle), Nox5+ (open rhombus) and Nox5+SM22+ (open square). (B) Vascular stiffness (strain vs stress curves) assessed by pressure myography in mesenteric arteries from SM22+ (open triangle) and Nox5+ (open rhombus), before and after Ang II treatment (closed symbols). (C) Vascular stiffness (strain vs stress curves) assessed by pressure myography in mesenteric arteries from WT (open circle) and Nox5+SM22+ (open square), before and after Ang II treatment (closed symbols). Results are mean±SEM of 5-8 mice/group. Abbreviations: WT – wild-type, Ang II –angiotensin II.

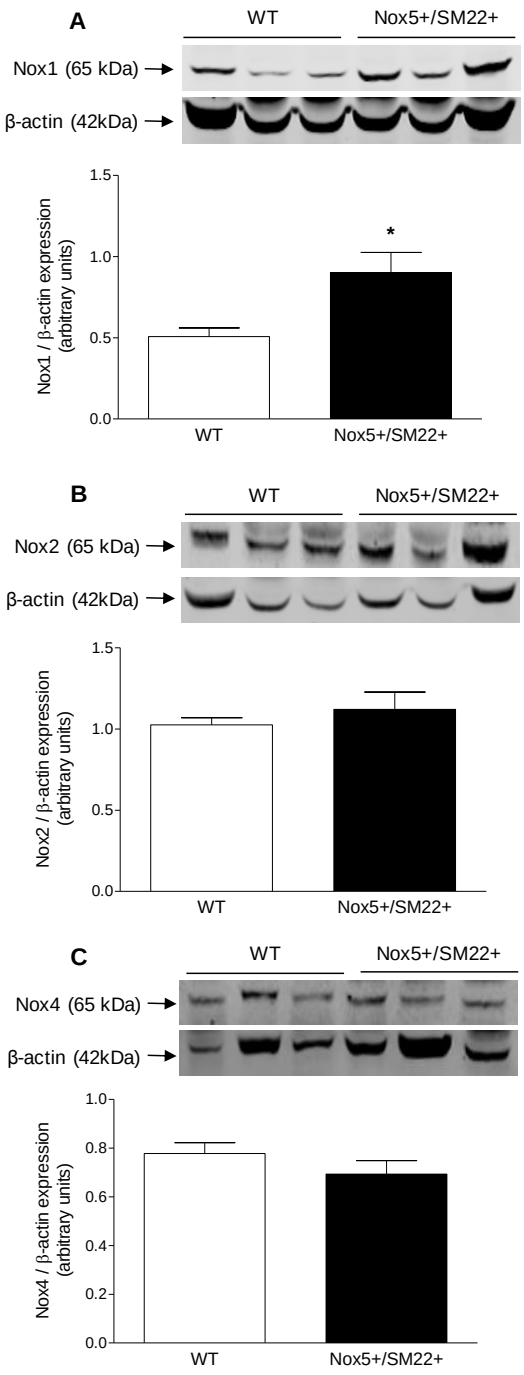

Figure S6. Nox1, Nox2 and Nox4 regulation in arteries from Nox5+SM22+ mice. (A) Nox1 protein expression in mesenteric arteries from WT and Nox5+SM22+ mice assessed by immunoblotting. (B) Nox2 and (C) Nox4 protein expression in mesenteric arteries from WT and Nox5+SM22+ mice assessed by immunoblotting. Results are mean±SEM of 3 experiments. *$p<0.05$ vs. WT. Abbreviations: WT – wild-type, Ang II –angiotensin II.

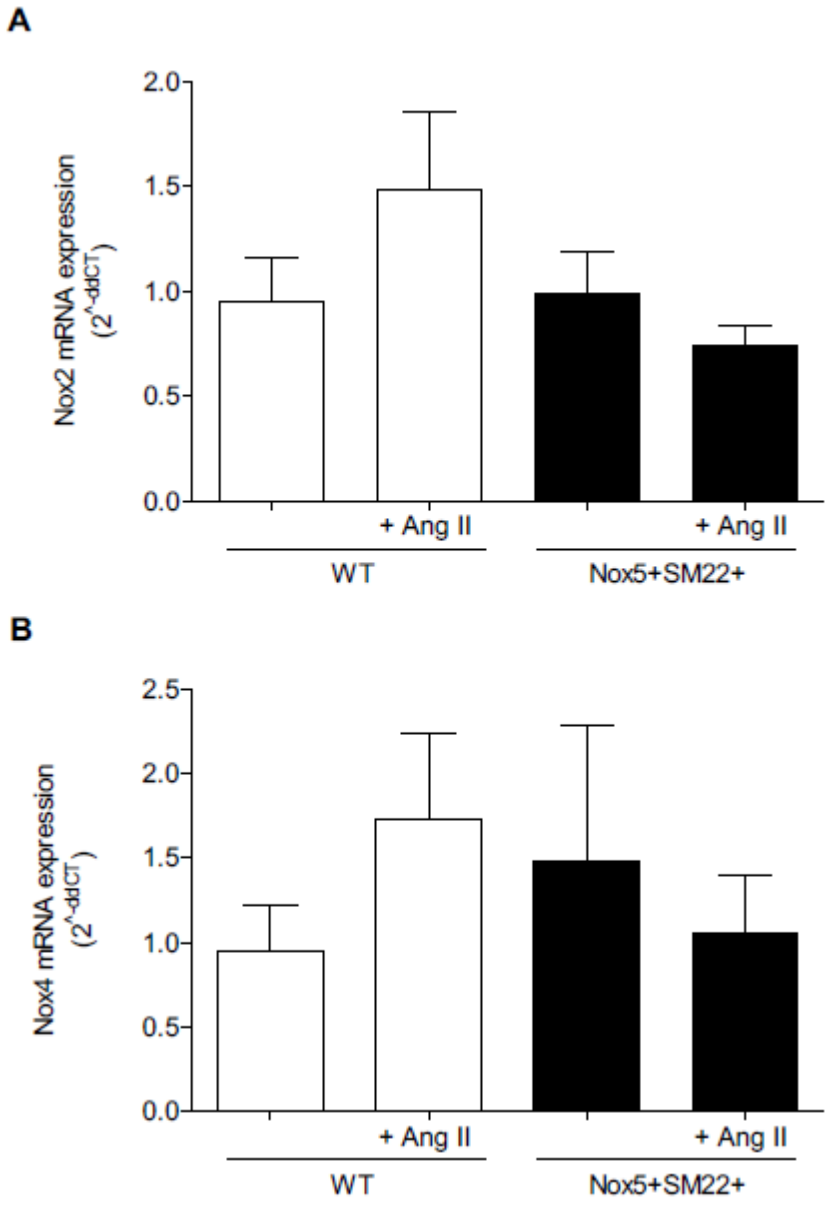

Figure S7. Cardiac mRNA levels of Nox isoforms in Nox5+SM22+ and control mice. Gene levels of Nox2 (A) and Nox4 (B) in Nox5+SM22+ (closed bars) and control groups (open bars), before and after Ang II treatment. Results are mean±SEM of 5-8 mice/group. Abbreviations: WT – wild-type, Ang II –angiotensin II.

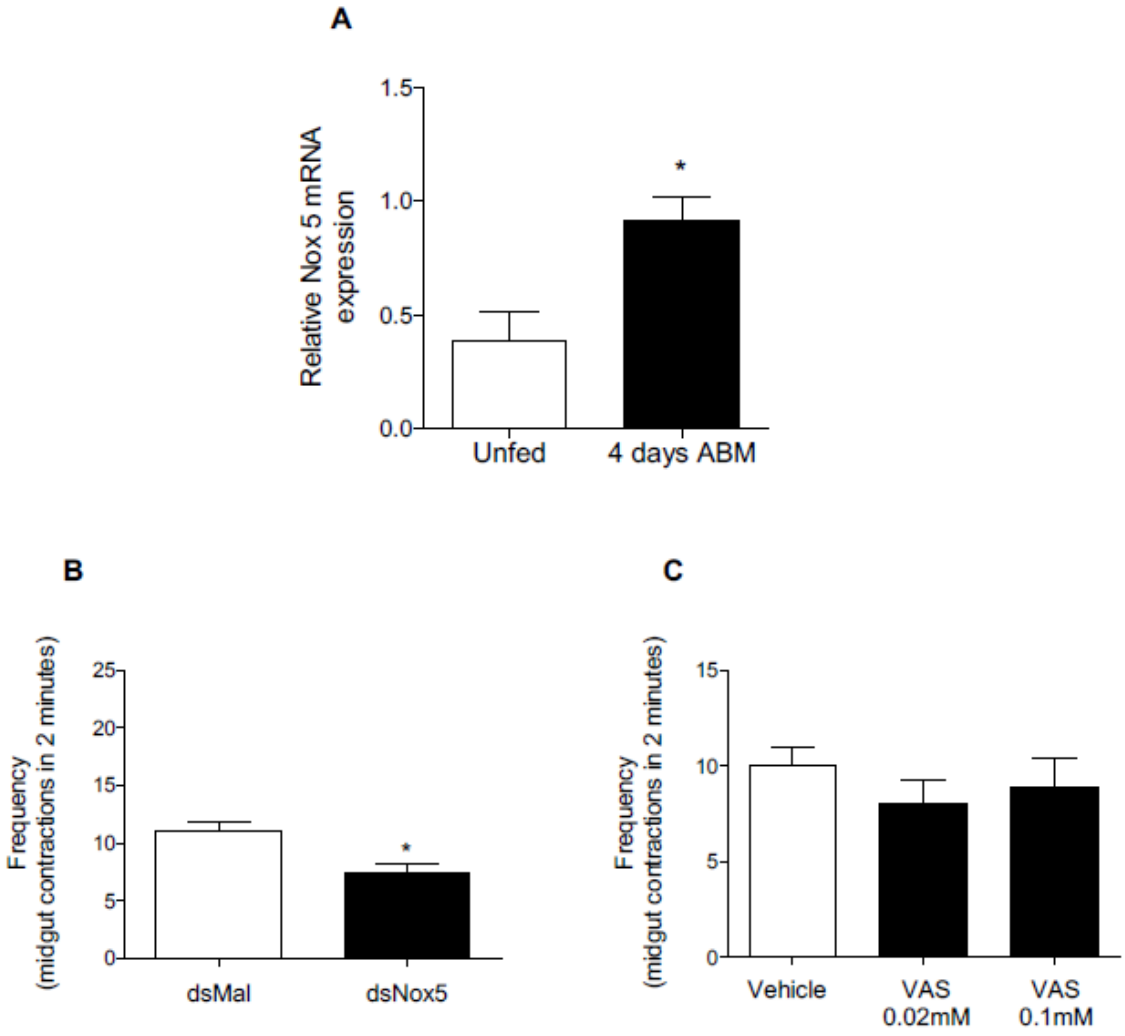

Figure S8. Nox5 is important in midgut contraction of *Rhodnius Prolixus*. (A) Nox5 gene expression in female *R. Prolixus* midgets before and after a bloody meal assessed by video fluorescence microscopy. Post-feeding midgut contraction (peristalsis) in female R. Prolixus after Nox5 downregulation (B) and Nox1/Nox4 inhibition (VAS2870 - VAS) (C). Results are mean±SEM of 5 experiments. *p<0.05 vs. unfed insects (A) or dsMal (B).

Supplemental References:

1. Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG, Group NCRRGW. Animal research: Reporting in vivo experiments: The arrive guidelines. *Br J Pharmacol*. 2010;160:1577-1579.

2. Callera GE, Antunes TT, He Y, Montezano AC, Yogi A, Savoia C, Touyz RM. C-src inhibition improves cardiovascular function but not remodeling or fibrosis in angiotensin ii-induced hypertension. *Hypertension*. 2016;68:1179-1190.

3. Schiffrin EL, Park JB, Intengan HD, Touyz RM. Correction of arterial structure and endothelial dysfunction in human essential hypertension by the angiotensin receptor antagonist losartan. *Circulation*. 2000;101:1653-1659.

4. Montezano AC, Lopes RA, Neves KB, Rios F, Touyz RM. Isolation and culture of vascular smooth muscle cells from small and large vessels. *Methods Mol Biol*. 2017;1527:349-354.

5. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. *Methods*. 2001;25:402-408.

6. Zhao H., Joseph J., Fales H. M., Sokoloski E. A., Levine R. L., Vasquez-Vivar J., Kalyanaraman B. Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence. *Proc. Natl. Acad. Sci. U.S.A.* 2005;102, 5727–5732.

7. Gandara ACP, Torres A, Bahia AC, Oliveira PL, Schama R. Evolutionary origin and function of nox4-art, an arthropod specific nadph oxidase. *BMC Evol Biol*. 2017;17:92-97.

8. Altenhöfer S, Radermacher KA, Kleikers PW, Wingler K, Schmidt HH. Evolution of NADPH Oxidase inhibitors: Selectivity and mechanisms for target engagement. *Antioxid Redox Signal.* 2015;23:406-427.