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Distinct Regulatory Effects of Myeloid Cell and Endothelial Cell Nox2 on Blood Pressure

Running Title: *Sag et al.; Nox2 Dependent Regulation of Blood Pressure*

Can Martin Sag, MD[1,2]*; Moritz Schnelle, MD, PhD[1,3]*; Juqian Zhang, MD[1]; Colin E. Murdoch, PhD[1]; Sabine Kossmann, PhD[4]; Andrea Protti, PhD [1]; Celio X.C. Santos, PhD[1]; Greta Sawyer, PhD[1]; Xiaohong Zhang, MD[1]; Heloise Mongue-Din, PhD[1]; Daniel A. Richards, PhD[1]; Alison C. Brewer, PhD[1]; Oleksandra Prysyazhna, PhD[1]; Lars S. Maier, MD[2]; Philip Wenzel, MD[4]; Philip J. Eaton, PhD[1]; Ajay M. Shah MD, FMedSci.[1]

[1]King's College London British Heart Foundation Centre of Excellence, Cardiovascular Division, London, United Kingdom; [2]Klinik und Poliklinik für Innere Medizin II, Universitätsklinikum Regensburg, Regensburg, Germany; [3]Department of Cardiology and Pneumology, Medical Center Goettingen, Goettingen, Germany; [4]Center for Cardiology and Center for Thrombosis and Hemostasis, University Medical Center Mainz, Mainz, Germany

*Equal Contribution

Address for Correspondence:
Ajay M. Shah, MD, FMedSci
James Black Centre
King's College London BHF Centre of Excellence
125 Coldharbour Lane
London SE5 9NU, UK
Tel: 44-2078485189
Fax: 44-2078485193
Email: ajay.shah@kcl.ac.uk

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Abstract

Background—Hypertension due to increased renin angiotensin system (RAS) activation is associated with elevated reactive oxygen species (ROS) production. Previous studies implicate NADPH oxidase (Nox) proteins as important ROS sources during RAS activation, with different Nox isoforms being potentially involved. Among these, Nox2 is expressed in multiple cell types including endothelial cells, fibroblasts, immune cells and microglia. Blood pressure (BP) is regulated at central nervous system, renal and vascular levels but the cell-specific role of Nox2 in BP regulation is unknown.

Methods—We generated a novel mouse model with a Floxed Nox2 gene and used Tie2-Cre, LysM Cre or Cdh5-CreERT2 driver lines to develop cell-specific models of Nox2 perturbation to investigate its role in BP regulation.

Results—Unexpectedly, Nox2 deletion in myeloid but not endothelial cells resulted in a significant reduction in basal BP. Tie2-CreNox2 knockout (KO) mice (in which Nox2 was deficient in both endothelial cells and myeloid cells) and LysM Cre Nox2KO mice (in which Nox2 was deficient in myeloid cells) both had significantly lower BP than littermate controls whereas basal BP was unaltered in Cdh5-CreERT2 Nox2 KO mice (in which Nox2 is deficient only in endothelial cells). The lower BP was attributable to an increased NO bioavailability which dynamically dilated resistance vessels *in vivo* under basal conditions, without change in renal function. Myeloid-specific Nox2 deletion had no effect on angiotensin II-induced hypertension which, however, was blunted in Tie2-CreNox2KO mice along with preservation of endothelium-dependent relaxation during angiotensin II stimulation.

Conclusions—We identify a hitherto unrecognized modulation of basal BP by myeloid cell Nox2 whereas endothelial cell Nox2 regulates angiotensin II-induced hypertension. These results identify distinct cell-specific roles for Nox2 in BP regulation.

Key-Words: NAD(P)H oxidase; blood pressure; angiotensin II; genetically altered mice

Clinical Perspective

What is new?

- NADPH oxidases generate superoxide and are implicated in the pathogenesis of hypertension.
- The NADPH oxidase Nox2 isoform is expressed in several cell types (such as endothelial cells and inflammatory cells) but exactly how it impacts on blood pressure (BP) is unclear.
- This study uses novel gene-modified mouse models to show that Nox2 in myeloid cells modulates basal BP whereas endothelial cell Nox2 is involved in angiotensin II-dependent hypertension.
- This is the first demonstration that myeloid cell Nox2 regulates basal BP.

What are the clinical implications?

- The finding that Nox2 in different cell types has distinct effects on BP suggests that different disease conditions may alter BP through effects of Nox2 in distinct cell types.
- It is conceivable that the effects of myeloid cells on basal BP may be enhanced in inflammatory settings where these cells are more activated.
- On the other hand, endothelial cell Nox2 activation may be more relevant to renin-angiotensin system-dependent hypertension.
- The current results are relevant to the design of novel therapeutic approaches for hypertension by targeting NADPH oxidases.

Introduction

The renin angiotensin system (RAS) plays a central role in blood pressure (BP) regulation and its chronic activation contributes to hypertension. Both animal and human studies have implicated increased reactive oxygen species (ROS) production in the pathophysiology of angiotensin II (AngII)-dependent hypertension.[1,2] ROS have complex cell-, source- and context-specific roles, ranging from physio-pathologic redox signaling to inactivation of nitric oxide (NO) and detrimental oxidation of cellular biomolecules.[3,4] In experimental models, ROS scavengers can attenuate the hypertensive response to AngII[1] but randomized clinical trials of general antioxidant approaches have failed to demonstrate reduction in cardiovascular morbidity and mortality;[5] such global inhibition approaches may affect beneficial redox signaling as well as detrimental oxidative stress. A better understanding of the roles of ROS in BP regulation and hypertension is therefore necessary in order to develop novel and more refined therapeutic approaches.

NADPH oxidase (Nox) family proteins are major sources of ROS in the cardiovascular system and are important in redox signaling.[6,7] They contain a Nox catalytic subunit that mediates ROS generation through electron transfer from NADPH to molecular oxygen. Five different oxidases, each based on a distinct Nox catalytic subunit (Nox1-5), have been identified. These have tissue-specific distribution and differing modes of activation, based on their varying requirements for accessory subunits. Different Nox isoforms may have distinct roles even in the same cell type, thought to be related to their coupling to different intracellular signaling pathways and/or production of different ROS (superoxide versus hydrogen peroxide).[6] This is relevant from a therapeutic perspective because isoform-selective Nox inhibitors are currently being developed.[8] Previous work suggested an involvement of Nox1 in the genesis of AngII-

dependent hypertension. Mice globally deficient in $p47^{phox}$, a subunit required for both Nox1 and Nox2 function, display a reduced hypertensive response to AngII[9] as do global Nox1 knockout mice,[10] whereas vascular smooth muscle-targeted Nox1 transgenic mice develop exaggerated AngII-induced hypertension.[11] The role of Nox2 is less clear. This isoform is expressed in endothelial cells, fibroblasts, cardiomyocytes, inflammatory cells and microglia,[6] sites that are of interest given that BP is regulated at central nervous system (CNS), renal, vascular and cardiac levels. Nox2 is involved in the genesis of endothelial dysfunction in diverse models.[6,7] Interestingly, ROS production in the subfornical organ in the brain is implicated in the vasopressor effects of AngII[12] and the knockout of $p22^{phox}$ - a subunit required for Nox1, Nox2 and Nox4 function - at this site blunted AngII-induced hypertension.[13] $p47^{phox}$ knockout mice have reduced renal afferent arteriolar constrictor responses to AngII[14] and the hypertensive effect of AngII has been shown to involve infiltration of the vasculature by $p47^{phox}$-containing T lymphocytes[15] and Nox2 competent monocytic cells.[16] While these studies suggest an involvement of Nox proteins in AngII-dependent hypertension, they do not directly establish the role of Nox2, in particular its potential cell-specific role. In this study, we have used a novel mouse model with a Floxed Nox2 gene to study the role of endothelial and myeloid cell Nox2 in BP regulation. Unexpectedly, we found that myeloid cell Nox2 has an essential role in the basal regulation of BP whereas activation of endothelial Nox2 contributes to AngII-dependent hypertension.

Methods

Generation of mice with a Floxed Nox2 allele and cell-specific knockouts

Animal studies complied with the UK Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 and institutional guidelines. The generation of $Nox2^{fl/fl}$ mice was commissioned from Genoway (France). The targeting construct was electroporated into 129sv embryonic stem cells. Recombinant clones were identified by PCR and Southern blotting. After successful targeting, the neomycin cassette was excised using flanking Flippase Recognition Target sites. Clones were injected into C57BL/6 blastocysts. Heterozygous Floxed mice obtained from germline chimeras were back-crossed >10 generations with C57BL/6 mice. For generation of cell-specific knockout (KO) and littermate controls, $Nox2^{fl/fl}$ females were crossed with male Tie2-Cre,[17] LysM-Cre[18] or Cdh5-CreERT2[19] transgenic mice. Inducible deletion of Nox2 in the Cdh5-CreERT2 model was achieved by Tamoxifen treatment (40 mg/kg i.p. for 3 consecutive days). Adult male mice aged 8-16 weeks with cell-specific Nox2 deletion were compared with Cre-negative Flox littermates.

PCR

Confirmation of cell-specific Nox2 deletion by PCR was based on the amplification of a 225 bp product that is only formed after excision of exons 1 and 2 of the cybb gene (forward: GGAATTGAGTTGTAAGAATCAAATGAC, reverse: ATGATGTGTCCCAAATGTGC). Primer GGGGCTGAATGTCTTCCTCT was included in the reaction to detect the 467 bp wildtype Nox2 sequence.

Real-time RT-PCR with SYBR Green was used to quantify mRNA expression levels. Delta delta Ct values were calculated using GAPDH as denominator. Primer sequences were

(forward, reverse): GAPDH: ATGACAACTTTGTCAAGCTCATTT,

GGTCCACCACCCTGTTGCT;

Nox2: ACTCCTTGGGTCAGCACTGG, GTTCCTGTCCAGTTGTCTTCG

$p22^{phox}$: GCCCTCCACTTCCTGTT, GCAGATAGATCACACTGGCAAT;

$p40^{phox}$: CTGCTTTTCTGACTACCCACAG, AAGCTGCTCAAAGTCGCTCT;

$p47^{phox}$: GGACACCTTCATTCGCCATA, CTGCCACTTAACCAGGAACAT;

$p67^{phox}$: TTGAACCTGTCACACAGCAAT, CCAGCACACACACAAACCTT;

superoxide dismutase-1 (SOD1): GGACCTCATTTTAATCCTCACTCTAAG,

GGTCTCCAACATGCCTCTCTTC;

SOD2: CACACATTAACGCGCAGATCA, GGTGGCGTTGAGATTGTTCA;

SOD3: ACACCTTAGTTAACCCAGAAATCTTTTC, GGGATGGATCTAGAGCATTAAGGA

Catalase: GCTGAGAAGCCTAAGAACGCAAT, CCCTTCGCAGCCATGTG.

Immunoblotting

Snap-frozen aortic tissue was homogenized for immunoblotting. Primary antibodies were: Nox2 (1:1000; BD Biosciences, UK), $p22^{phox}$ (1:1000; Santa Cruz, USA), β-actin (1:4000; Sigma, UK), Nox4 (1:1000),[20] eNOS (1:1000; BD Biosciences, UK). Actin (Sigma) was used as a loading control. Protein bands were visualized using enhanced chemiluminescence and quantified by densitometry.

Blood pressure measurement

BP telemeters (model TA11PA-C10, Data Sciences International, Netherlands) were implanted subcutaneously under isoflurane anesthesia, with a 1-week recovery period prior to measurements.[21] Analyses were performed using Dataquest ART analysis software. AngII (1.1 mg/kg/day) was administered via subcutaneous osmotic minipumps (Model 1002, Alzet,

Cupertino, CA), implanted under 2% isoflurane.[21] In some experiments, Nω-Nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich, UK; 100 mg/kg/day) was administered in the drinking water.

Magnetic resonance imaging (MRI)

MRI was performed in prone mice on a 7T horizontal scanner (Agilent Technologies, Varian Inc., Palo Alto, CA) under isoflurane anesthesia.[22] Body temperature was maintained at 37ºC and heart rates above 400 bpm. Temporally resolved dynamic short-axis images of the carotid arteries were acquired with a cine-FLASH sequence using ECG and respiratory gating. Endothelium-dependent relaxation *in vivo* was assessed using acetylcholine (18.8 mg/kg i.p.).[22] Pixels encompassing the blood pool were clustered based on signal intensity and the vessel wall borders. Images were analyzed in end-systolic and end-diastolic phases using ClinicalVolumes segmentation software (King's College London, UK; www.clinicalvolumes.com).

Other *in vivo* procedures

Echocardiography was performed using a Vevo 2100 System with a 40-MHz linear probe (Visualsonics, Canada), under 1.5% isoflurane anesthesia.[23] Renal function was assessed in response to an acute saline challenge (40 ml/kg 0.9% w/vol. saline, i.p. injection). Animals were placed in an individual metabolic chamber (Tecniplast 3600M021, UK) for 4 h, without access to food or water, and urine was collected at hourly intervals.[24] Metabolites were analyzed on an Advia 2400 Chemistry System (Siemens AG, Germany).

***Ex vivo* vascular function**

Isometric tension was quantified in descending thoracic aortic rings suspended in a Krebs buffer solution containing indomethacin (3 μmol/L) at 37°C, pH 7.4.[21] Endothelium-dependent relaxation was assessed from the cumulative dose-response to acetylcholine (ACh) of rings pre-

constricted to 70% of the maximal contraction to phenylephrine. In some experiments, rings were incubated with AngII (0.1 μmol/L, 4 h) in the presence or absence of the superoxide scavenger MnTMPyP (10 μmol/L) prior to addition of other vasoactive agents. Basal NO bioavailability was assessed from the response to a single dose of N-methyl-L-arginine (L-NMMA, 100 μmol/L) in rings pre-constricted to 30% of the maximal phenylephrine response.

Vascular segments from mesenteric (second order) arteries were studied in a tension myograph (Danish Myo Technology, Denmark) in Krebs buffer at 37 °C.[25] Endothelium-dependent relaxation was assessed from the cumulative dose-response to ACh in rings pre-constricted with U46619 (0.1 μmol/L; Sigma).

ROS assays

HPLC-based detection of dihydroxyethidium (DHE) oxidation products was performed as described previously.[26] Aortic segments were incubated with or without AngII (0.1 μmol/L, 3.5 h, 37 °C) before addition of DHE (100 μmol/L) for 30 min at 37°C in the dark. Tissue was harvested in acetonitrile, sonicated and centrifuged. Supernatants were dried under vacuum and pellets stored at -80°C. For analysis, samples were re-suspended in 120 μl PBS/DTPA and injected into the HPLC system. The superoxide specific 2-hydroxyethidium (EOH) signal was normalized to tissue weight.

ROS production in bone marrow and blood mononuclear cells was assessed using flow cytometry of cells loaded with DHE (10 μmol/L, 10 mins at 37 °C), after stimulation with phorbol 12-myristate 13-acetate (PMA, 100 ng/ml) to activate the Nox2 oxidase complex.[27] Bone marrow cells were harvested as described previously.[28] Monocytes were isolated by Ficoll gradient centrifugation and CD11b Microbeads (Miltenyi Biotech, Germany).

Vascular morphology

In vivo perfusion fixation with 4% PFA under pressure followed by paraffin embedding was used.[21] Vascular media thickness and intima-media area were quantified in 6 µm sections stained with hematoxylin and eosin, using Volocity software (Volocity v5.0, Perkin Elmer, American Fork, UT).

Coronary microvascular endothelial cells (CMEC) were isolated from mouse hearts as described previously.[29]

Flow cytometry (FACS)

Quantitative analyses of leukocyte number and phenotype were performed by FACS on aortic tissue digests, using a FACS CantoII® instrument (BD Biosciences, Oxford, UK). Briefly, animals under terminal anesthesia were perfused with saline through the LV to eliminate circulating blood. The blood free descending and abdominal aorta was digested in a mixture of collagenase IV, DNase and hyaluronidase at 37°C for 30 min, followed by trituration and filtration through a 70 µm nylon mesh. Cell suspensions were washed and blocked with anti-CD16/CD32 antibodies prior to staining. Monocytes ($CD45^+CD11b^+Ly6G^-$), macrophages ($CD45^+CD11b^+F4/80^+$), neutrophils ($CD45^+CD11b^+Ly6G^+$), T-cells ($CD45^+TCR\beta^+$) and B-cells ($CD45^+CD19^+$) were identified. Zombie-Aqua dye (Biolegend) was used to identify dead cells prior to fixation with 1% PFA. Fluorescence-minus-one (FMO) stained samples were used as negative controls. Data analysis was performed with FlowJo software (Tree Star Inc., Ashland, OR).

NO measurement by Electron Paramagnetic Resonance (EPR)

Aortic NO formation was measured as described previously[30] by EPR-based spin trapping with iron-diethyldithiocarbamate ($Fe(DETC)_2$) colloid using a Miniscope MS400 table-top X-band

spectrometer (Magnettech, Berlin, Germany). In brief, aortae were either stimulated with calcium ionophore for measurement of eNOS-derived NO levels or with LPS to detect iNOS-derived NO formation. For LPS stimulation, freshly prepared aortae were cut into 3-mm rings and incubated with 10 µg/ml LPS in RPMI 1640 medium +10% FCS + 1%penicillin/ streptomycin for 21 h at 37°C, 5% CO_2. Afterwards rings were transferred in a 24-well plate filled with 1 ml Krebs-HEPES-solution (pH 7.35, containing NaCl 99.01 mM, KCl 4.69 mM, $CaCl_2$ 2.50 mM, $MgSO_4$ 1.20 mM, $NaHCO_3$ 25.0 mM, K_2HPO_4 1.03 mM, Na-HEPES 20.0 mM, D-glucose 11.1 mM). For activation of eNOS, 10 µM calcium ionophore (A12187, Sigma) was added to freshly prepared aortic rings in 1 ml Krebs-HEPES-solution two minutes before $Fe(DETC)_2$ spin trap addition. 1 ml colloid $Fe(DETC)_2$ was then added to each well (0.4 mM in PBS Ca^{2+}/Mg^{2+}) and incubated at 37°C, 10% CO_2 for 1 h. After incubation aortic rings were snapfrozen in a 1 mL syringe and recordings were performed at 77°K, using a Dewar flask. Instrument settings were: B0-field=3300 G, sweep=110 G, sweep time=30 s, steps 4096, number pass 10, modulation=7000 mG, MW alten = 10 milliwatts, gain=9 E2. Levels of NO are expressed as intensity of signal (A.U.) per weight of wet sample.

Statistics

All data are expressed as mean±SEM. Comparisons were made by Student's t-tests, 2-way ANOVA or 2-way repeated measures ANOVA followed by Newman-Keuls post hoc tests as appropriate. Concentration-response curves were fitted with a sigmoid dose response curve with fixed Hill slope (also known as four-parameter logistic equation). Curves were compared by non-linear regression analysis followed by the extra sum-of-squares F test. Data were analyzed on GraphPad Prism v6 or SigmaStat v3.5. $P<0.05$ was considered significant.

Results

Tie2-Cre targeted deletion of Nox2 in mice *in vivo* reduces basal BP

We first generated a mouse model with a "Floxed" Nox2 allele on a C57Bl6 background such that Cre-mediated recombination deletes a 3kb fragment of Nox2 including the transcription initiation site and the first two exons (Fig. 1A,B). Homozygous Floxed mice ($Nox2^{fl/fl}$) were crossed with Tie-2 Cre mice to achieve endothelial-targeted deletion of Nox2 (Tie2-Nox2KO), although bearing in mind that myelo-monocytic cells may also be targeted with this promoter.[31] Tie2-Nox2KO mice were born at the expected Mendelian ratio and had no gross abnormalities up to 6 months of age. Body and major organ weights were similar between Tie2-Nox2KO and Flox littermates (e.g. body weight 26.8±0.3g vs. 26.4±0.1g at 10 weeks age; n≥10/group). The aorta of Tie2-Nox2KO mice showed significant reduction in Nox2 mRNA levels but no changes in the mRNA levels of $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, SOD1-3 or catalase (Fig. 1C). Nox2 mRNA levels were also substantially reduced in coronary microvascular endothelial cells (CMEC) from Tie2-Nox2KO mice compared to control (Suppl. Fig. 1A). Nox2 protein levels were significantly decreased in Tie2-Nox2KO mouse aorta compared to control Flox mice, but there were no differences in $p22^{phox}$, Nox4 or endothelial NO synthase (eNOS) protein levels (Fig. 1D). A Tie2-driven deletion of Nox2 was therefore not accompanied by significant changes in antioxidant genes, Nox4 or eNOS.

Assessment of ambulatory BP by telemetry revealed that Tie2-Nox2KO mice had a significantly lower basal systolic and mean BP than control Flox mice by approximately 10 mmHg (Fig. 2A,B). There were no differences between groups in heart rate, activity levels or cardiac structure and function assessed by echocardiography (Fig. 2B and Suppl. Fig. 1B).

Lower basal BP in Tie2-Nox2 KO is not accounted for by altered renal function, vascular remodeling or endothelium-dependent vasodilation

There were no differences between Tie2-Nox2KO and control mice in urinary volume, electrolytes or osmolarity in response to an acute saline challenge (Fig. 3A). To look for vascular remodeling as a basis for the lower BP, we quantified intima-media thickness and area in aortic sections but this was also similar between groups (Fig. 3B). Endothelium-dependent relaxation assessed from the vasodilator response to acetylcholine in isolated aortic rings was no different between Tie2-Nox2KO and control mice (Fig. 3C). The vascular smooth muscle response to the NO donor sodium nitroprusside and the constrictor response to phenylephrine were similar in both groups. Acetylcholine-induced vasodilation in mesenteric arteries was also similar between groups (Fig. 3D).

Vascular and BP responses of Tie2-Nox2KO mice during AngII stimulation

After exposure of aortic rings to AngII (0.1 μmol/L, 4 h), the phenyephrine-induced vasoconstriction was similar in both groups, indicating comparable baselines. Acetylcholine-induced vasodilation was significantly greater in Tie2-Nox2KO than control (Fig. 4A, upper right panel). Aortic superoxide levels were similar in Tie2-Nox2KO and control aorta at baseline but were significantly higher in the control group after AngII treatment (Fig. 4A, upper left panel). In line with this, the superoxide scavenger MnTMPyP (10 μmol/L) improved endothelium-dependent relaxation in the control group but had no significant effect in Tie2-Nox2KO (Fig. 4A, lower panels). We also found that acute NOS inhibition with L-NMMA (100 μmol/L) induced greater vasoconstriction in AngII-treated Tie2-Nox2KO aortic rings than AngII-treated control rings (Suppl. Fig. 2A), suggestive of a greater amount of bioactive NO in the former setting. *In vivo*, the hypertensive response observed in control mice with a 2-week

infusion of AngII at 1.1 mg/kg/d was significantly blunted in Tie2-Nox2KO animals, without difference in the heart rate response (Fig. 4B). Thus, the blunted hypertensive response to AngII observed in Tie2-Nox2KO mice may be attributable to a lower AngII-induced increase in endothelial superoxide, a consequent higher level of NO bioavailability and a greater extent of endothelium-dependent vasorelaxation.

Tie2-Nox2KO mice have increased basal NO bioavailability *in vivo*

Although basal *ex vivo* endothelium-dependent vascular function was similar between Tie2-Nox2KO and control, it is possible that this might be different *in vivo* and that an increase in NO-bioavailability due to reduction in Nox2-derived superoxide may account for the lower basal BP in Tie2-Nox2KO. We tested this idea by assessing the ambulatory BP response to the NOS inhibitor, L-NAME (100 mg/kg/d orally for 2 days). The hypertensive response to L-NAME was found to be significantly greater in Tie2-Nox2KO than control mice, such that BP levels were similar in the two groups after L-NAME treatment (Fig. 5A). This finding suggests that *in vivo* NO bioavailability at baseline may be higher in Tie2-Nox2KO than control mice. To assess if L-NAME-mediated changes in BP involved the vasculature, we used MRI-based measurement of resistance artery calibre *in vivo* (by imaging the carotid artery). The carotid artery luminal area in Tie2-Nox2KO was significantly higher than in matched control mice at baseline (0.46±0.03 mm^2 vs. 0.38±0.02 mm^2, n=10 each, $P<0.05$, Fig. 5B,D), consistent with basal vasodilatation. Acetylcholine induced a similar extent of vasodilatation in both groups of mice (Fig. 5E), consistent with the results in *ex vivo* vessels. After L-NAME treatment, however, Tie2-Nox2KO carotid arteries constricted to a greater extent than in control mice such that luminal vessel diameters were now similar in both groups (Fig. 5C,F). These results are consistent with the

notion that a higher *in vivo* NO bioavailability may increase basal resistance vessel calibre in Tie2-Nox2KO mice.

Contrasting roles of endothelial versus myeloid immune cell Nox2 in BP regulation

The data presented so far suggest that the lower basal BP in Tie2-Nox2KO animals involves an NO-dependent mechanism but cannot be accounted for by altered endothelial function. Since Tie2 is expressed not only in endothelial cells but also in myelo-monocytic cells,[31] we considered the possibility that the results observed in Tie2-Nox2KO mice might be related to Nox2 deletion in myeloid cells. Indeed, Tie2-Nox2KO mice showed clear evidence of Cre-mediated recombination in bone marrow-derived cells (Fig. 6A). Furthermore, Tie2-Nox2KO bone marrow cells and circulating mononuclear cells displayed functionally deficient Nox2-derived ROS production (Fig. 6B).

To investigate whether Nox2 in myeloid cells contributes to the effects on basal BP, we next generated a myeloid-specific Nox2 KO model using a LysM-Cre mouse line which targets myelo-monocytic cells[17] (Suppl. Fig. 2B). LysM-Cre-Nox2 KO mice showed no obvious gross phenotype and had comparable amounts of myeloid inflammatory cells in the vessel wall (Suppl. Table 1). However, ambulatory BP monitoring by telemetry revealed a significant reduction in basal systolic BP as compared to control Flox mice, similar to that observed in Tie2-Nox2KO animals, with no alteration in heart rate (Fig. 6C). To assess if this reduction in basal BP was related to increased *in vivo* NO bioavailability, LysM-Cre-Nox2 KO mice and controls were treated with L-NAME. Indeed, we found that after L-NAME treatment, which had hypertensive effects in both groups of mice, the systolic BP was similar in LysM-Cre-Nox2 KO and controls (Fig. 6D). To more directly assess basal vascular NO levels, we performed EPR with NO-$Fe(DETC)_2$ spin-trapping (Fig 6E). Aortic NO levels were significantly higher in LysM-Cre-

Nox2KO mice than control, consistent with the higher NO bioavailability *in vivo* (Fig. 6D). Interestingly, while basal aortic NO levels were increased in LysM-Cre-Nox2KO, iNOS-derived NO formation as assessed after lipopolysaccharide stimulation was lower than in controls (Suppl. Fig. 3A). We also studied the response of LysM-Cre-Nox2 KO mice to AngII infusion. In contrast to the differences in basal BP, the hypertensive response to AngII was similar in LysM-Cre-Nox2 KO and control mice (Suppl. Fig. 3B), indicating that myeloid cell Nox2 does not appear to modulate AngII-dependent hypertension.

To elucidate the specific role of Nox2 in the endothelium, we then generated an inducible endothelial Nox2KO model using a Cdh5-CreERT2 driver line.[19] In this model, endothelial-specific Nox2 deletion was achieved in adult mice by tamoxifen treatment (Suppl. Fig. 2C). Tamoxifen treatment had no effect on cardiac function (Suppl. Fig. 2D). In contrast to LysM-Cre-Nox2 KO mice, Cdh5-CreERT2-Nox2 KO animals showed no difference in basal BP compared to matched controls as assessed by ambulatory telemetry (Fig. 7A). The hypertensive response to L-NAME was also similar in both groups (Fig. 7B), suggesting a comparable NO bioavailability under basal conditions in this model. The quantification of basal aortic NO levels by EPR confirmed that these were similar between groups (Fig. 7C). AngII-induced hypertension, however, was significantly attenuated in Cdh5-CreERT2-Nox2KO mice (Suppl. Fig. 3C) pointing to endothelial Nox2 as a crucial player in AngII-mediated hypertension.

Discussion

This study provides significant new insights into the role of Nox2 in the regulation of BP. With the use of several novel cell-specific Nox2 knockout models, we were able to dissect out and distinguish between the effects of myelo-monocytic cell Nox2 on basal BP and those of

endothelial cell Nox2 on AngII-induced hypertension. We accordingly identify cell-specific and context-specific roles for Nox2 in BP regulation which indicates that the roles of ROS are highly complex, not only among different ROS sources but also for the same source in different cell types.

An important and unexpected initial finding was that Tie2-Nox2KO mice, which we generated as a model of endothelial-specific Nox2 deletion, exhibited significantly lower basal BP compared to matched controls. While some previous studies have reported a lower BP in global Nox2KO mice,[32,33] it was not immediately obvious why the deletion of endothelial Nox2 should affect basal BP. For example, previous work from our group[21] and others[34] showed that endothelial-specific overexpression of Nox2 had no effect on basal BP. Compensatory changes in other enzymes such as the antioxidants SOD1-3 and catalase, eNOS or Nox4 could conceivably be involved (and Nox4 was previously linked to lower BP[26]), but we found no differences in the levels of these enzymes between Tie2-Nox2KO and control mice. The investigation of *ex vivo* endothelium-dependent function in Tie2-Nox2KO mouse aorta and mesenteric artery did not reveal any enhancement of relaxation, in line with the finding that basal vascular O_2^- production was unaltered. We also found no changes in cardiac or renal function nor any evidence of structural vascular remodeling to explain the lower BP. Nevertheless, the lower BP in Tie2-Nox2KO mice was related to an enhanced *in vivo* NO bioavailability as indicated by the normalization of the BP difference upon treatment with L-NAME. Furthermore, *in vivo* assessment of resistance vessel function by MRI revealed that Tie2-Nox2KO mice had a pronounced vasodilatation under basal conditions that could be reversed by L-NAME treatment but showed no evidence of altered endothelium-dependent relaxation. Taken together, these findings led us to consider the possibility that non-endothelial Nox2-containing cells may be

involved in the effect on basal BP. In this regard, it is known that Tie2 is expressed not only in endothelial cells but also in certain hematopoietic cells, notably monocytic cells.[31,35] Indeed, we confirmed that the Tie2-Cre approach not only targeted endothelial cells but also induced functional Nox2 deletion in myelo-monocytic cells.

The generation of novel LysM-Cre-Nox2 KO and Cdh5-CreERT2-Nox2 KO mice then allowed us to establish that the reduction in basal BP was in fact related to deletion of Nox2 from myelo-monocytic cells rather than endothelial cells, with an associated increase in *in vivo* NO bioavailability. Using EPR with NO-Fe(DETC)$_2$ spin-trapping, we found that the aortae of LysM-Cre-Nox2 KO mice had increased NO levels, pointing to the vasculature as the likely site of action of myelo-monocytic cells with disrupted Nox2. Conversely, Cdh5-CreERT2-Nox2 KO aortae had unaltered vascular NO levels under basal conditions. It should be noted that the magnitude of difference in NO levels observed in aorta cannot necessarily be extrapolated to the resistance vasculature, which is the more important vascular site for BP regulation. The effect of myelo-monocytic cells in the vasculature is most likely to reflect the inactivation of endothelial-derived NO by myeloid Nox2-derived superoxide, before the NO can affect vascular smooth muscle cell relaxation. When myelo-monocytic Nox2 is disrupted, the levels of bioactive NO would accordingly rise. Consistent with this idea, we could demonstrate the presence of myelo-monocytic cells in the aortae of both LysM-Cre-Nox2 KO and control mice but with no difference in the number of cells between groups. Myelo-monocytic cells could in principle produce NO from inducible NOS (iNOS) but we found that iNOS-derived NO (after LPS stimulation) was decreased in LysM-Cre-Nox2 KO mice - consistent with previous reports that Nox-dependent signaling can increase iNOS expression[36] – and making it unlikely that myeloid cell iNOS plays a role in the observed changes in basal BP. The reason why only the deletion of

myelo-monocytic Nox2 but not the deletion of endothelial Nox2 affects basal BP is most likely because the abundance of Nox2 is much lower in endothelial cells than myeloid cells.[37] We did note, however, that the reduction in basal BP was slightly higher in the Tie2-Nox2KO mice (where both myeloid and endothelial cells Nox2 is deleted) than in LysM-Cre-Nox2 KO. This could indicate that the effects of myelo-monocytic Nox2 KO may be enhanced by the concomitant knockout of endothelial cell Nox2. The current results are to our knowledge the first indication that myelo-monocytic cell Nox2 modulates basal BP.

AngII is a potent activator of both Nox1 and Nox2 and an impact of Nox enzymes on AngII-induced hypertension has been documented in many previous studies, as discussed in the Introduction. However, the specific roles of different Nox isoforms and different cell types has remained unclear. Quite strong evidence supports a role for vascular smooth muscle Nox1 in AngII-induced hypertension, involving changes in vascular remodeling.[10,11] The cell-specific role of Nox2 is unclear - a pertinent question because Nox2 is expressed in endothelial cells, cardiomyocytes, fibroblasts, certain vascular smooth muscle cells and inflammatory/immune cells.[6] Nox2 is involved in the genesis of endothelial dysfunction in diverse models,[6,7] and previous studies from our lab[21] and others[32] found that endothelium-targeted overexpression of Nox2 enhanced AngII-induced hypertension. However, the role of endogenous endothelial Nox2 was not established in those studies. The current results in Cdh5-CreERT-Nox2KO mice and Tie2-Nox2KO mice clearly establish that endogenous endothelial Nox2 augments AngII-induced hypertension, at least in the relatively short-term (2 weeks). Interestingly, AngII-induced hypertension was comparable in LysM-Cre-Nox2 KO and respective control mice, indicating that constitutive deficiency of Nox2 in myelomonocytic cells is apparently not important in this setting. On the other hand, previous data suggest that T cell Nox2[15] as well as Nox2 in the

subfornical organ,[12,13] and possibly Nox2 in renal afferent arterioles,[14] also modulate AngII-induced hypertension. Furthermore, it was also shown that mice can be protected from arterial hypertension when LysM-positive cells are depleted prior to beginning of AngII-infusion, and that BP can be restored by adoptive transfer of Nox2-competent monocytes into these mice.[16] Taken together, these data indicate complex roles during AngII-induced hypertension for Nox2 in multiple cell types, some of which involve altered NO bioavailability and others which may involve Nox2-dependent redox signaling events. The endothelium-dependent effects of Nox2 defined in the current study appear to involve an increased inactivation of NO by ROS, which affects endothelium-dependent relaxation as suggested by our *ex vivo* studies, but could potentially also affect other NO-dependent functions.

The major new finding of this study is the potential for myelo-monocytic cells to affect basal BP in a reversible manner. While the absolute change in basal BP is modest, it is of a similar magnitude to changes that would be considered clinically or prognostically significant, for example in hypertension. From a pathophysiological perspective, it is interesting to speculate whether the effects of these cells on BP might be enhanced in disease settings where monocytes are activated, e.g. inflammatory conditions. In this regard, patients with chronic granulomatous disease (CGD), who have functionally deficient Nox2 activity, might be of particular interest. More broadly, the current results suggest the potential for different disease conditions to alter BP through Nox2-dependent effects in distinct cell types, i.e. myeloid cells or endothelial cells. In terms of RAAS-dependent hypertension, Nox2 inhibition would be anticipated to be beneficial and combined Nox1/Nox2 inhibitors could be of particular value given the effects of both isoforms to increase BP. In summary, this study identifies distinct effects of myeloid cell Nox2

and endothelial cell Nox2 on basal and AngII-dependent BP respectively, and suggests that Nox2 may be a master regulator of BP.

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Disclosures

None

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Figure Legends

Figure 1. Generation of Tie2-Nox2KO mice.

A. Targeting strategy for generation of $Nox2^{flox}$ mice (Flox). The endogenous Nox2 locus is shown at the top and the targeting vector at the bottom. LoxP sites are represented by blue triangles and FRT sites by double red triangles. After successful targeting, the Neomycin (Neo) cassette was excised using the FRT sites. Cre-mediated recombination deletes a 3kb fragment of Nox2 including the transcriptional initiation site and the first two exons. **B.** Southern blot analysis of genomic DNA from selected ES cell clones screened for 5'-homologous (upper panel) and 3'-homologous recombination (lower panel). The clones 1A3 and 7C11 were used for blastocyst injection to generate $Nox2^{flox}$ mice. **C.** mRNA levels of Nox2, accessory subunits and antioxidant genes in Tie2-Nox2KO and Flox control aortae. **D.** Protein levels of Nox2, $p22^{phox}$, Nox4 and eNOS in Tie2-Nox2KO and control aortae. Representative immunoblots are shown above and mean data below. #, $P<0.05$ vs. Flox.

Figure 2. Tie2-Nox2KO mice have reduced basal blood pressure.

A. Representative telemetric BP traces in Tie2-Nox2KO mice and Flox controls. **B.** Mean data for BP, heart rate and activity level. #, $P<0.05$ vs. Flox.

Figure 3. Renal function, vascular remodeling and *in vitro* vascular function in Tie2-Nox2KO mice.

A. Renal function assessed by response to an acute saline challenge. Changes in urine osmolarity, volume, and sodium and potassium concentrations are shown. RM-TW-ANOVA =

repeated measures two-way ANOVA; N.S. = not significant between groups. **B.** Representative histological sections of aorta from Tie2-Nox2 KO and Flox mice (x40 magnification), and mean intima-media area and thickness. **C.** Concentration-response curves for response of aortic rings to the NO donor sodium nitroprusside (SNP), phenylephrine (PE) and acetylcholine (ACh). **D.** Concentration-response curves for response of mesenteric arteries to ACh.

Figure 4. Tie2-Nox2KO mice demonstrate blunted *in vitro* and *in vivo* responses to AngII.

A. *Upper left panel*: Superoxide levels in Tie2-Nox2KO and Flox aorta at baseline and after AngII stimulation, assessed by HPLC of the specific dihydroethidium oxidation product, 2-hydroxyethidium (EOH). $^{\#}$, $P<0.05$ vs. Flox; *, $P<0.05$ vs. control conditions (TW-ANOVA). *Upper right panel*: Effect of AngII pre-treatment on vasodilation of aortic rings to ACh. *Lower panels*: Effect of MnTMPyP on ACh responses in AngII-treated rings from Flox mice (left) and Tie2-Nox2KO mice (right). **B.** Effect of chronic AngII infusion (1.1 mg/kg/day) on telemetric BP and heart rate in Tie2-Nox2KO and Flox mice. RM-TW-ANOVA = repeated measures two-way ANOVA; $^{\#}$ indicates significant difference between genotypes; * indicates significant interaction.

Figure 5. Tie2-Nox2KO mice have increased basal NO bioavailability *in vivo.*

A. BP response to L-NAME in Tie2-Nox2KO and Flox mice, assessed by telemetry. $^{\#}$ indicates significant difference between genotypes, * indicates significant interaction as tested by RM-TW-ANOVA. **B, C.** Representative MRI images of the carotid artery in Tie2-Nox2KO and Flox mice at baseline (B) and after L-NAME treatment (C). Scale bar, 1 mm. **D-F.** Mean data for

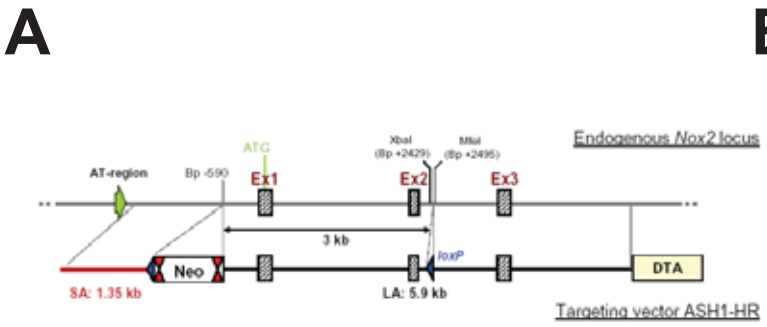
diastolic area of the carotid artery at baseline (D), following ACh treatment (E), and after L-NAME treatment (F). #, $P<0.05$ vs. Flox; *, $P<0.05$ vs. control conditions (TW-ANOVA).

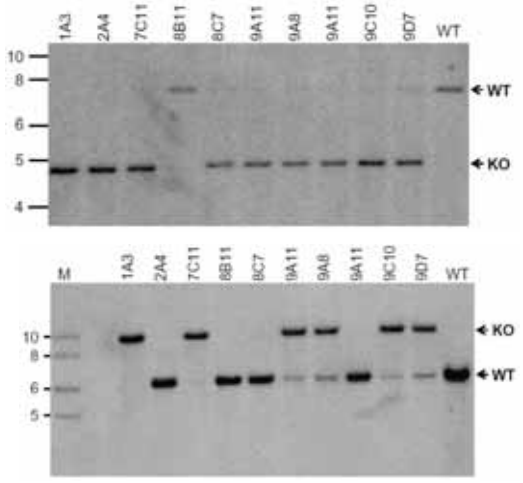
Figure 6. LysM-Cre-Nox2KO mice have reduced basal BP and increased vascular NO bioavailability.

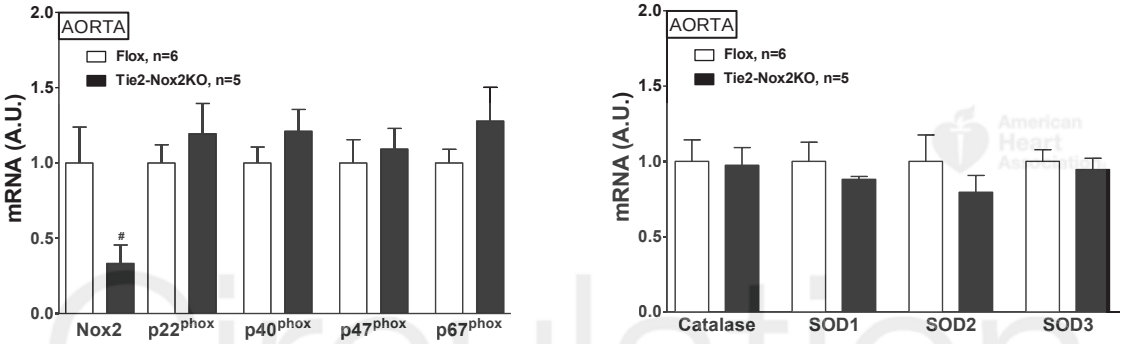
A. Cre-mediated recombination in bone marrow-derived cells from Tie2-Nox2KO mice. **B.** Functionally deficient ROS production in phorbol ester (PMA)-stimulated bone marrow cells and circulating mononuclear cells from Tie2-Nox2KO mice, assessed by flow cytometry in cells loaded with dihydroethidium (DHE). **C.** Reduced basal BP (telemetry) in LysM-Cre-Nox2KO mice. #, $P<0.05$ vs. Flox. **D.** In vivo response to L-NAME in LysM-Cre-Nox2KO mice and controls. # indicates significance vs. Flox as tested by RM-TW-ANOVA. **E**. Increased aortic NO levels in LysM-Cre-Nox2KO under basal conditions, as assessed by EPR. Representative EPR spectra are shown to the right. #, $P<0.05$ vs. Flox.

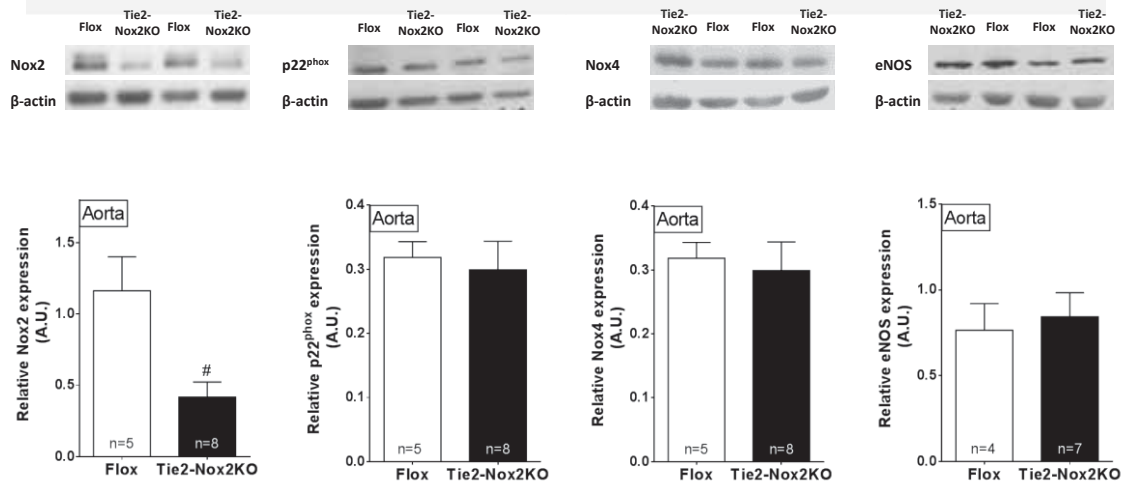
Figure. 7. Cdh5-CreERT2-Nox2KO mice have unaltered basal BP.

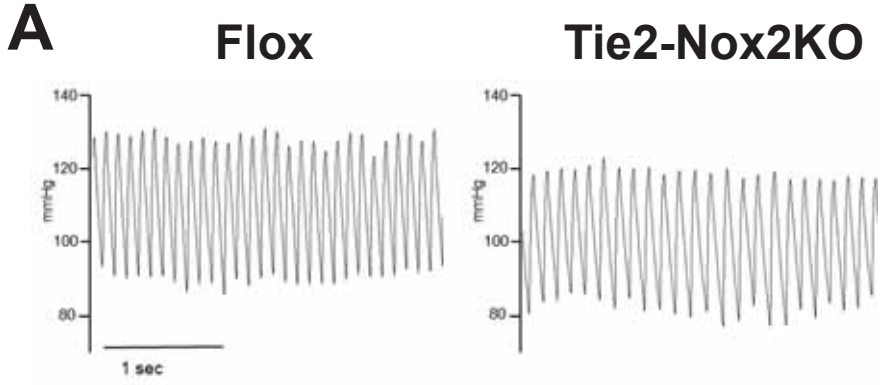
A. Telemetric BP and heart rate at baseline. **B.** BP responses to L-NAME in Cdh5-CreERT2-Nox2KO and Flox mice. L-NAME increased BP in both groups but there was no significant difference between groups by RM-TW-ANOVA. **C**. Comparable aortic NO levels in Cdh5-CreERT2-Nox2KO and controls under basal conditions, as assessed by EPR. Representative EPR spectra are shown to the right.

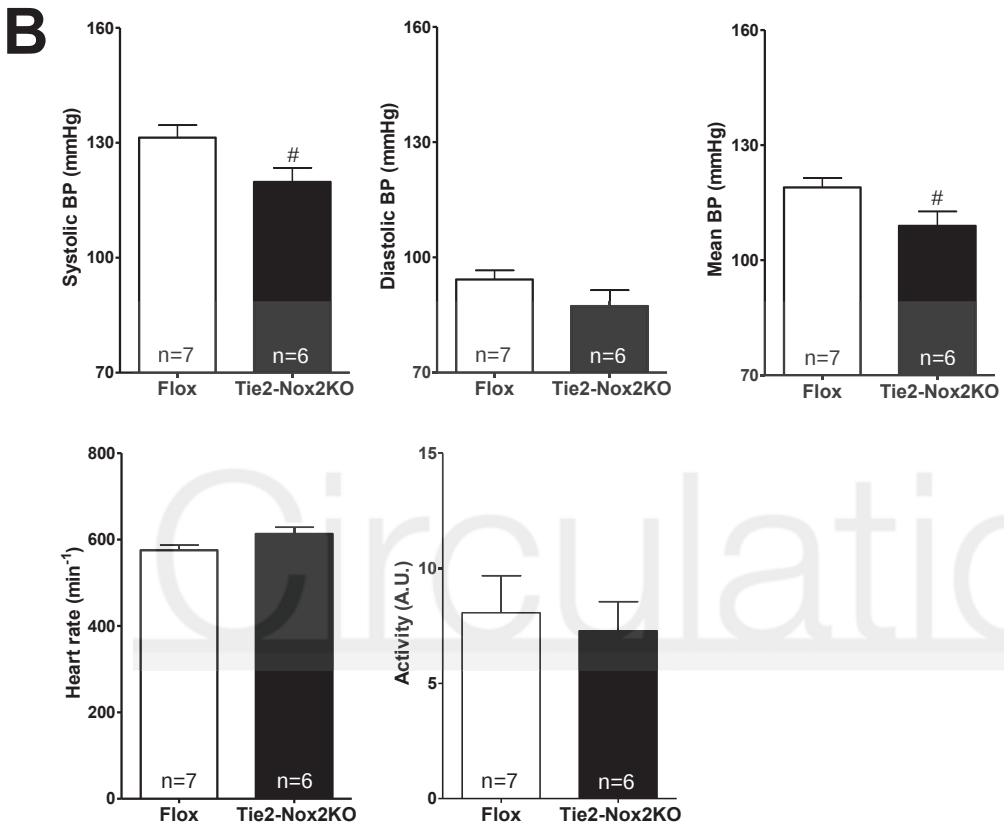


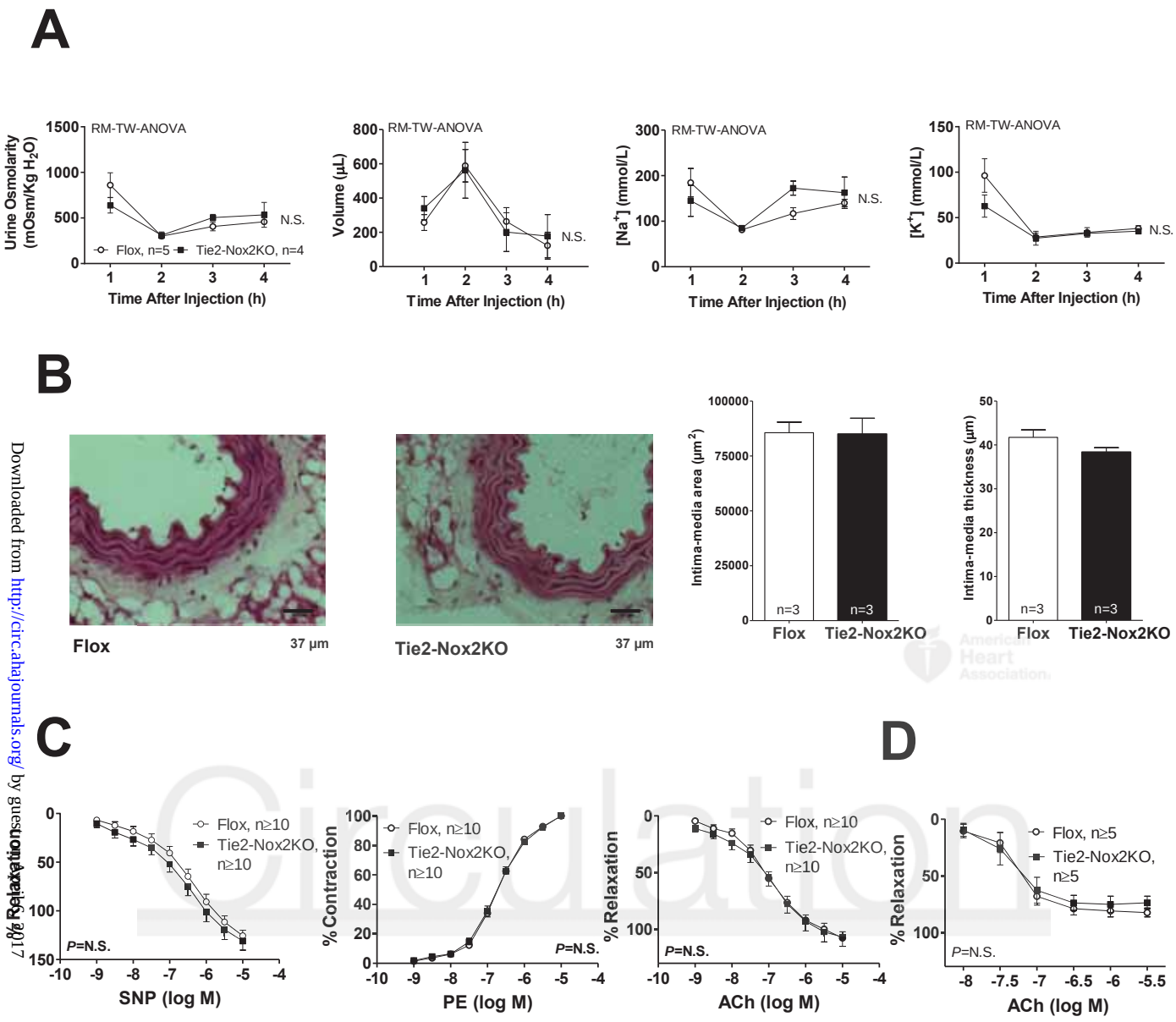


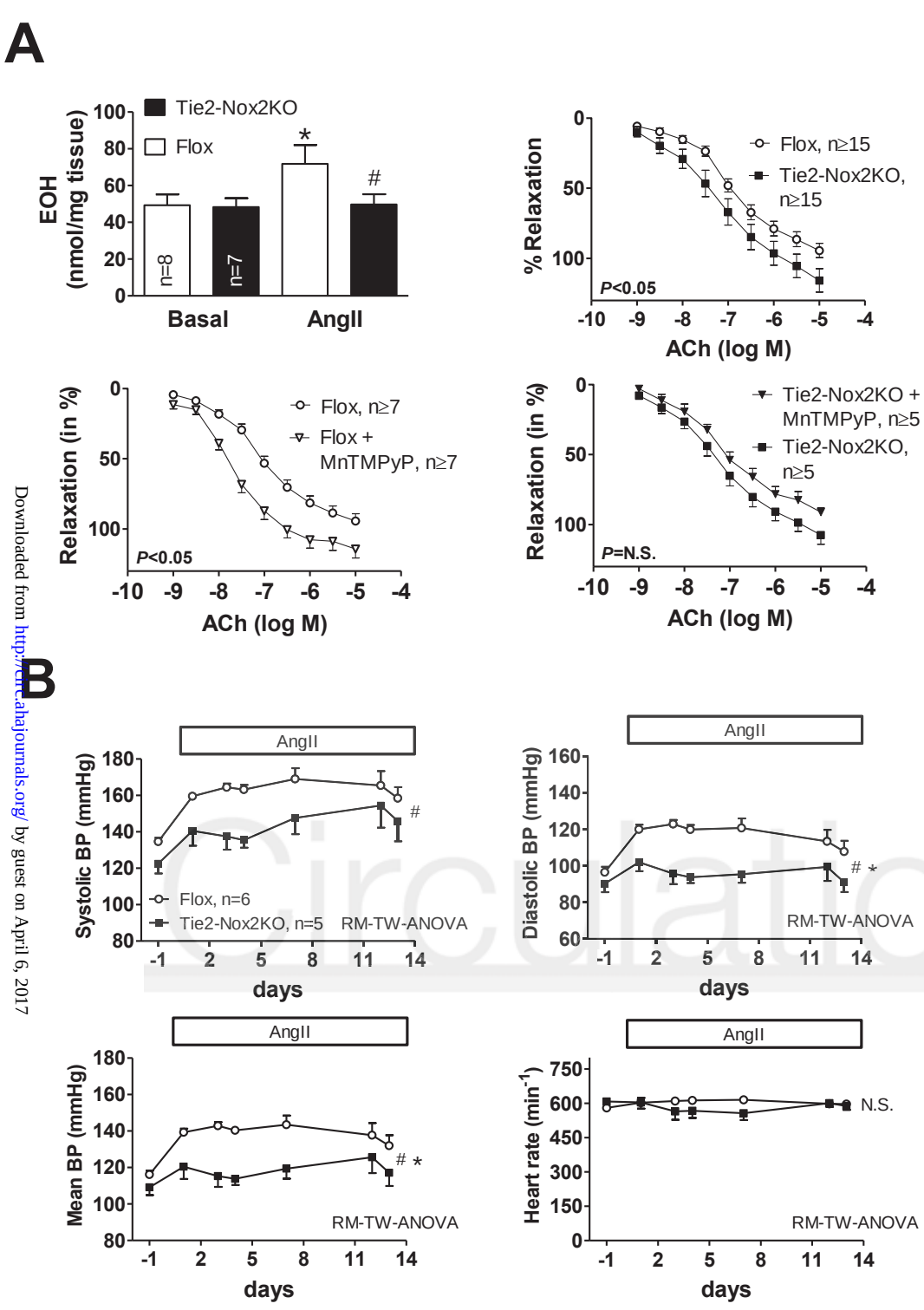


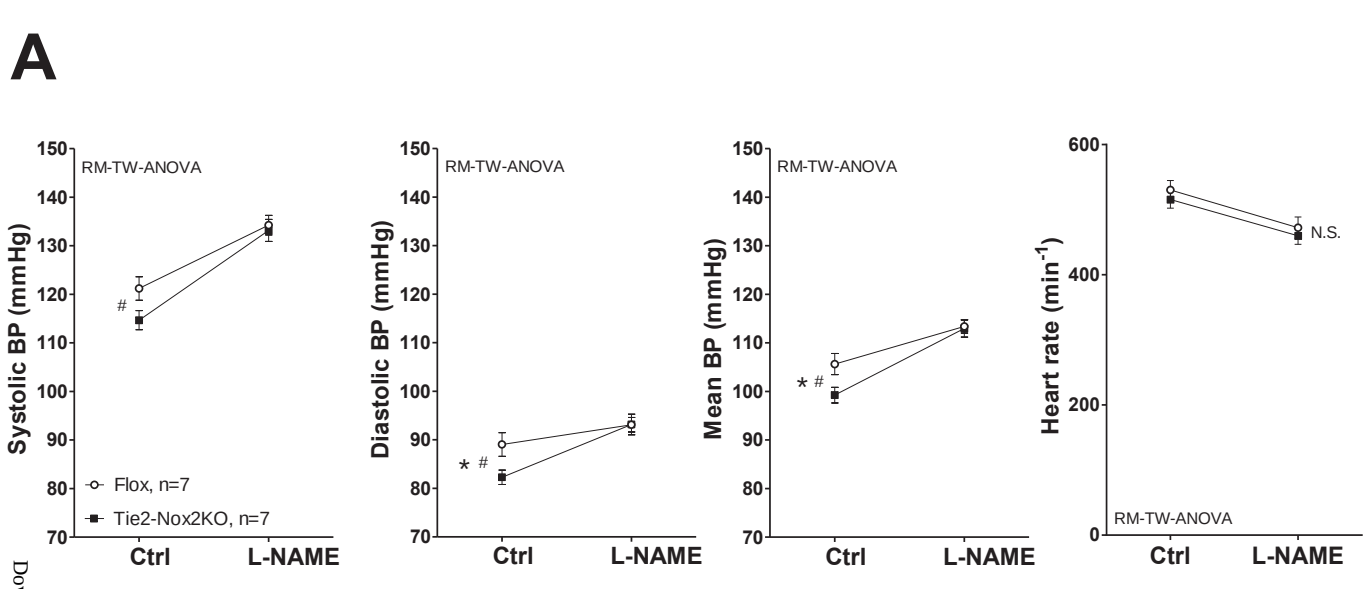


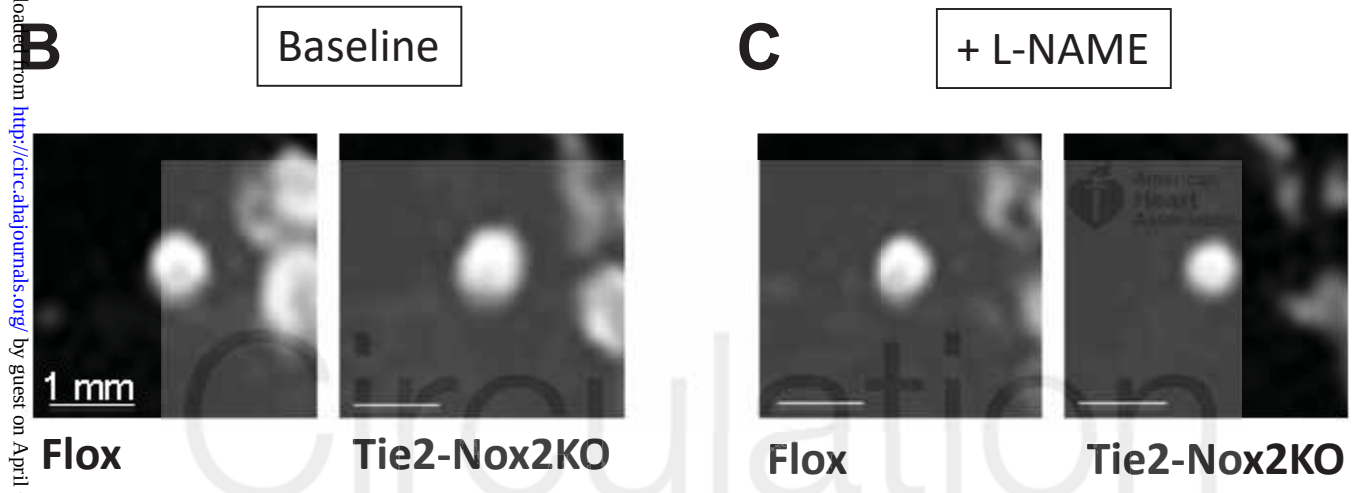


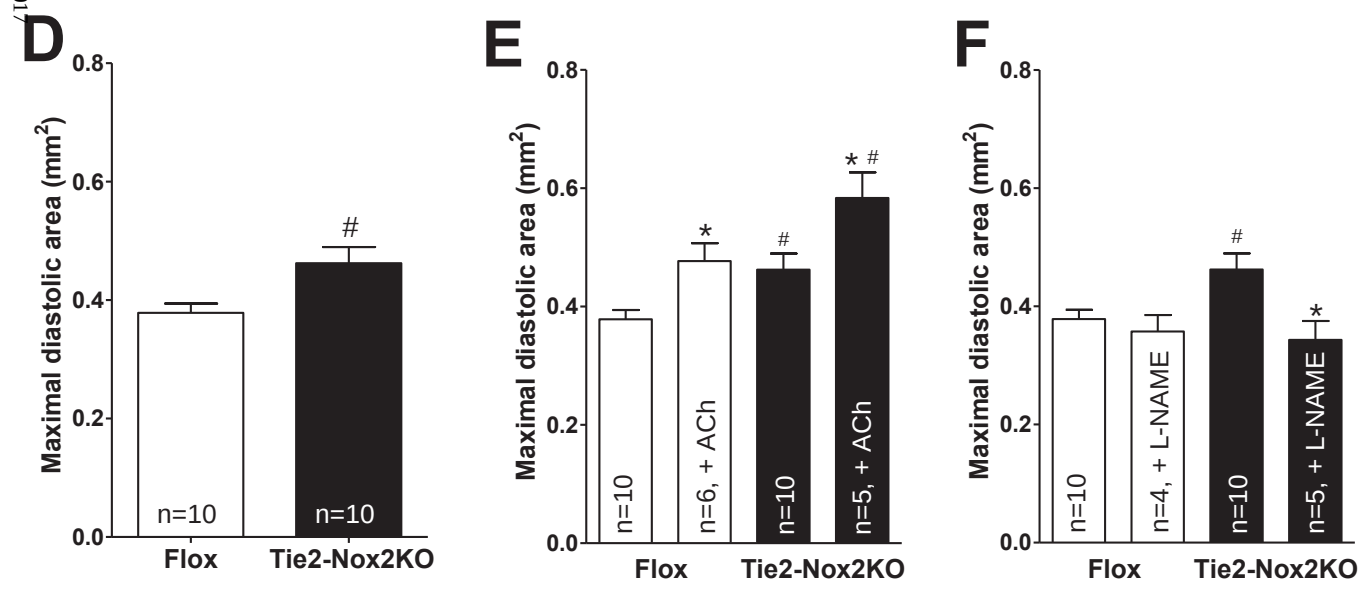


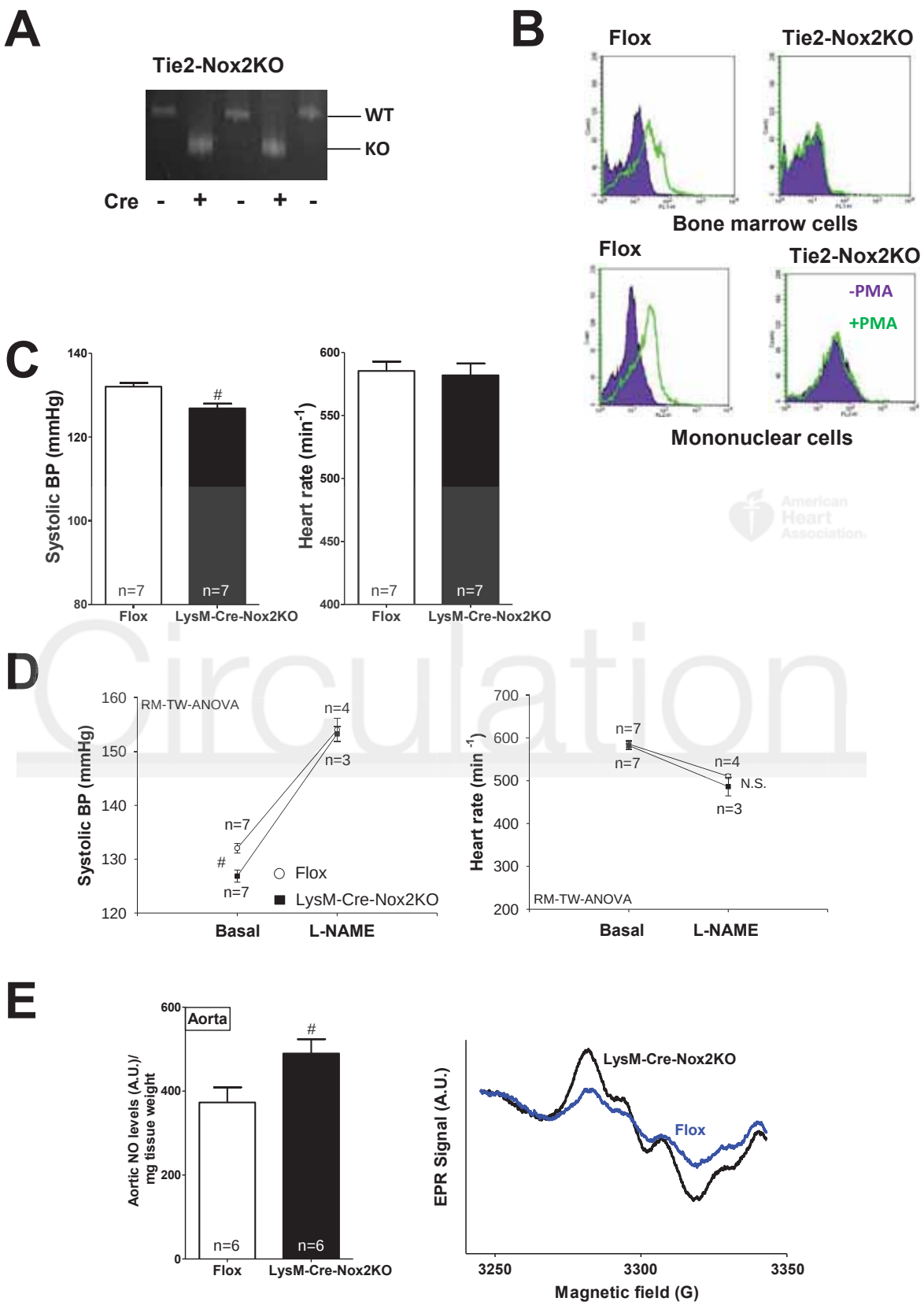


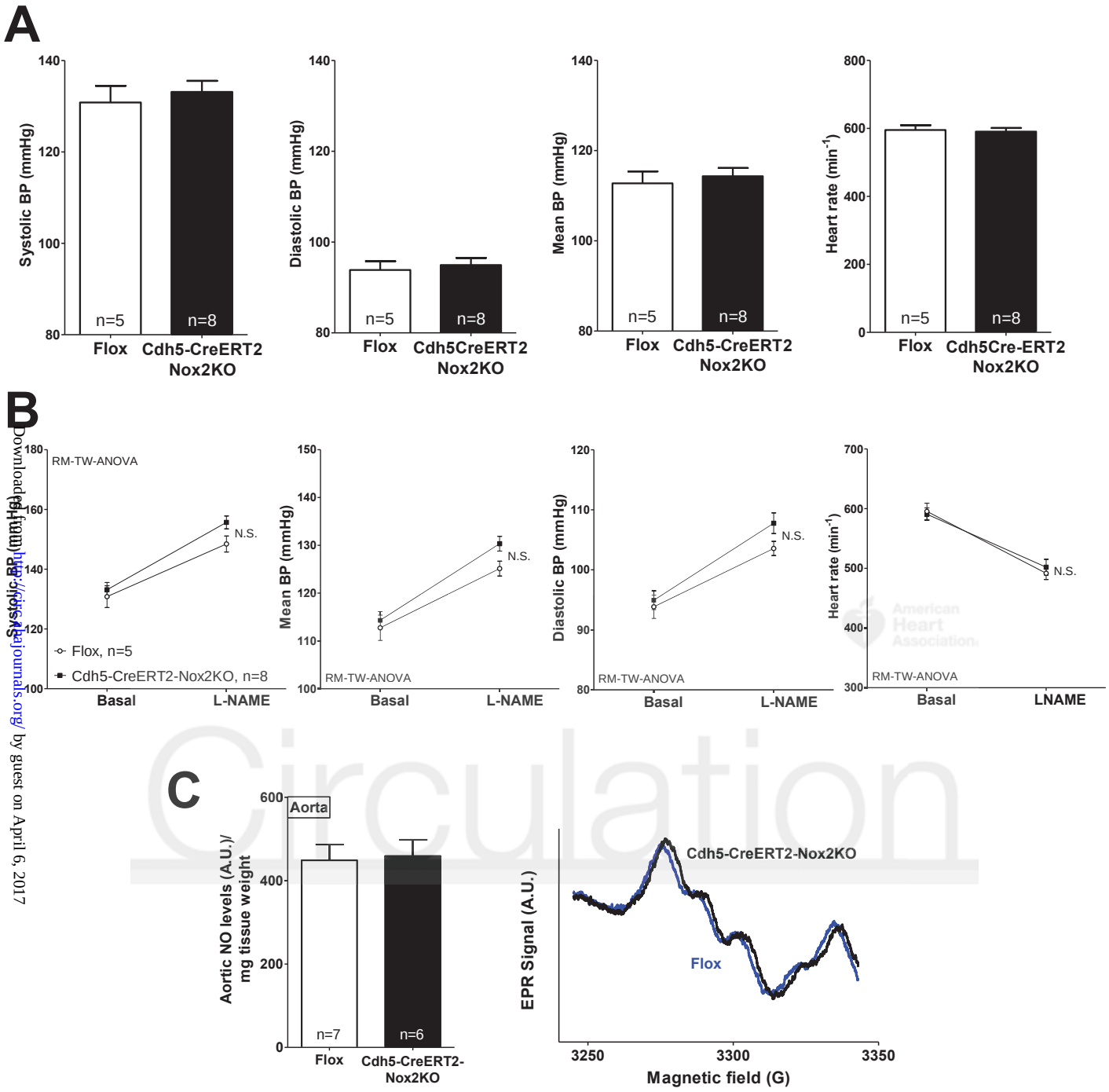












Distinct Regulatory Effects of Myeloid Cell and Endothelial Cell Nox2 on Blood Pressure

Can Martin Sag, Moritz Schnelle, Juqian Zhang, Colin E. Murdoch, Sabine Kossmann, Andrea Protti, Celio X. C. Santos, Greta J. Sawyer, Xiaohong Zhang, Heloise Mongue-Din, Daniel A. Richards, Alison C. Brewer, Oleksandra Prysyazhna, Lars S. Maier, Philip Wenzel, Philip J. Eaton and Ajay M. Shah

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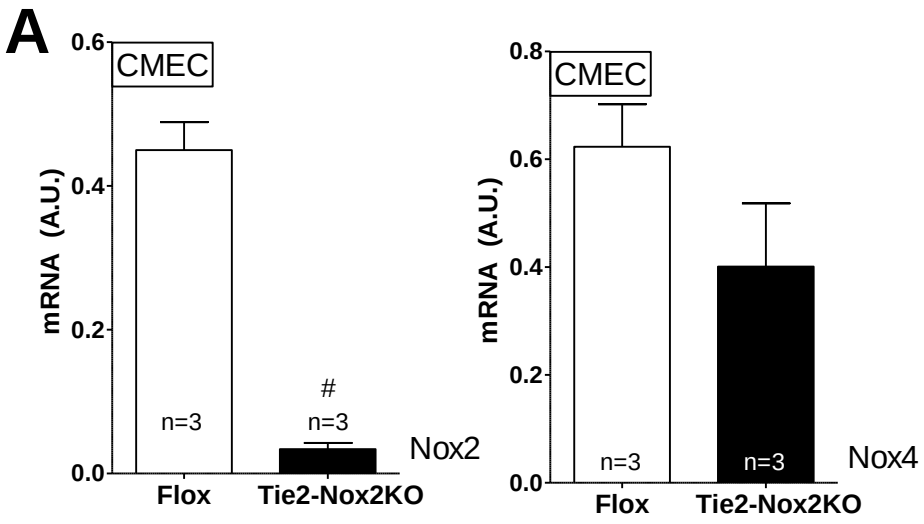
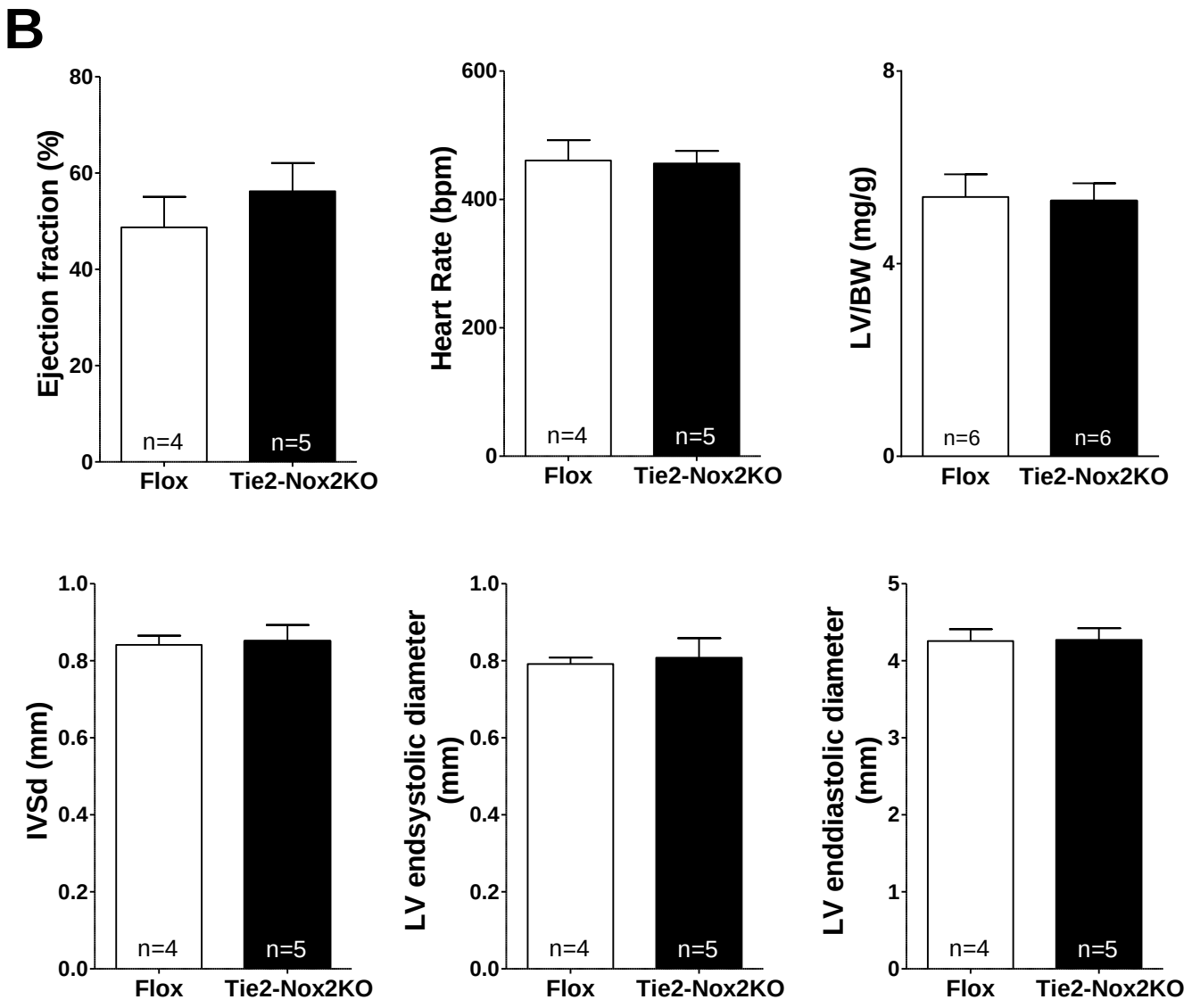
SUPPLEMENTAL MATERIAL

Supplementary Table 1. Aortic myelo-monotic and lymphatic cells in Flox control and LysM-Cre-Nox2KO mice.

	Flox control		**LysM-Cre Nox2KO**		
Cell type	**Number of cells / 10 mg aortic tissue**	**n**	**Number of cells 10 / mg aortic tissue**	**n**	***P***
$CD45^+CD11b^+ Ly6G^-$ (Monocytes)	100 ± 60	4	120 ± 20	5	N.S.
$CD45^+CD11b^+ F4/80^+$ (Macrophages)	3.5 ± 2.2	4	8.1 ± 1.3	5	N.S.
$CD45^+CD11b^+ Ly6G^+$ (Neutrophils)	540 ± 230	5	580 ± 80	5	N.S.
$CD45^+TCR\beta^+$ (T-Lymphocytes)	790 ± 340	5	810 ± 110	5	N.S.
$CD45^+CD19^+$ (B-Lymphocytes)	640 ± 310	5	640 ± 90	5	N.S.

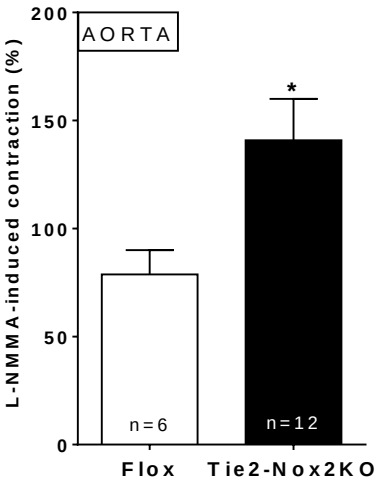
Data are mean±SEM.


A
CMEC
mRNA (A.U.)
0.6
0.4
0.2
0.0
n=3
#
n=3
Nox2
Flox
Tie2-Nox2KO
CMEC
mRNA (A.U.)
0.8
0.6
0.4
0.2
0.0
n=3
n=3
Nox4
Flox
Tie2-Nox2KO


B
Ejection fraction (%)
80
60
40
20
0
n=4
n=5
Flox
Tie2-Nox2KO
Heart Rate (bpm)
600
400
200
0
n=4
n=5
Flox
Tie2-Nox2KO
LV/BW (mg/g)
8
4
0
n=6
n=6
Flox
Tie2-Nox2KO
IVSd (mm)
1.0
0.8
0.6
0.4
0.2
0.0
n=4
n=5
Flox
Tie2-Nox2KO
LV endsystolic diameter (mm)
1.0
0.8
0.6
0.4
0.2
0.0
n=4
n=5
Flox
Tie2-Nox2KO
LV enddiastolic diameter (mm)
5
4
3
2
1
0
n=4
n=5
Flox
Tie2-Nox2KO

Suppl. Fig. 1

A



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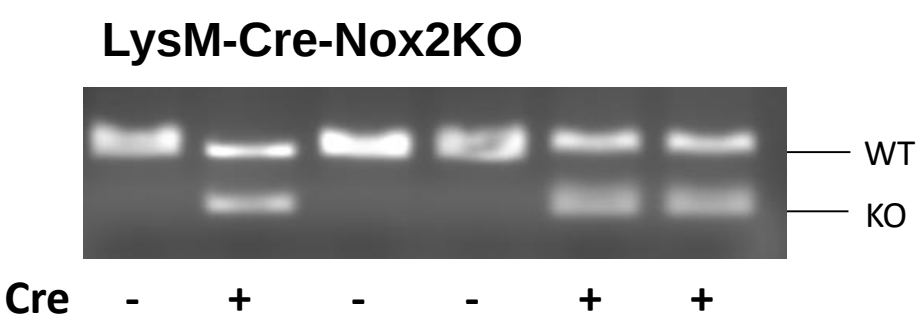
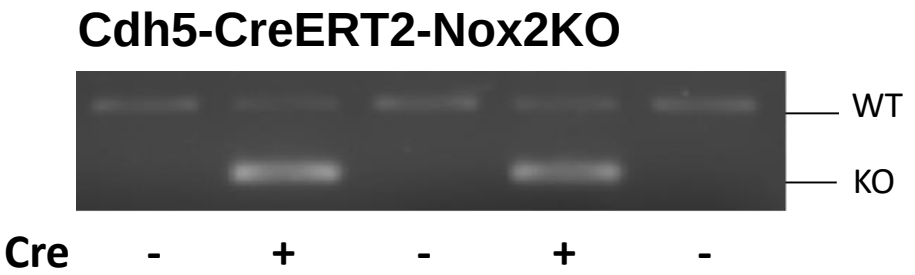
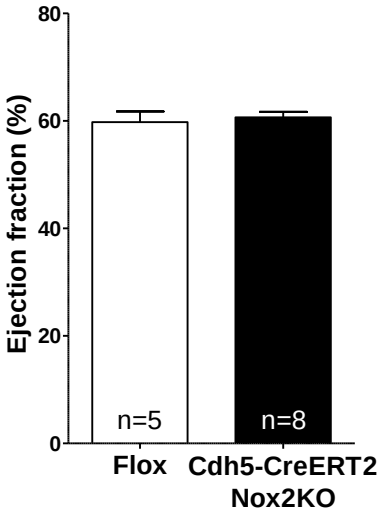


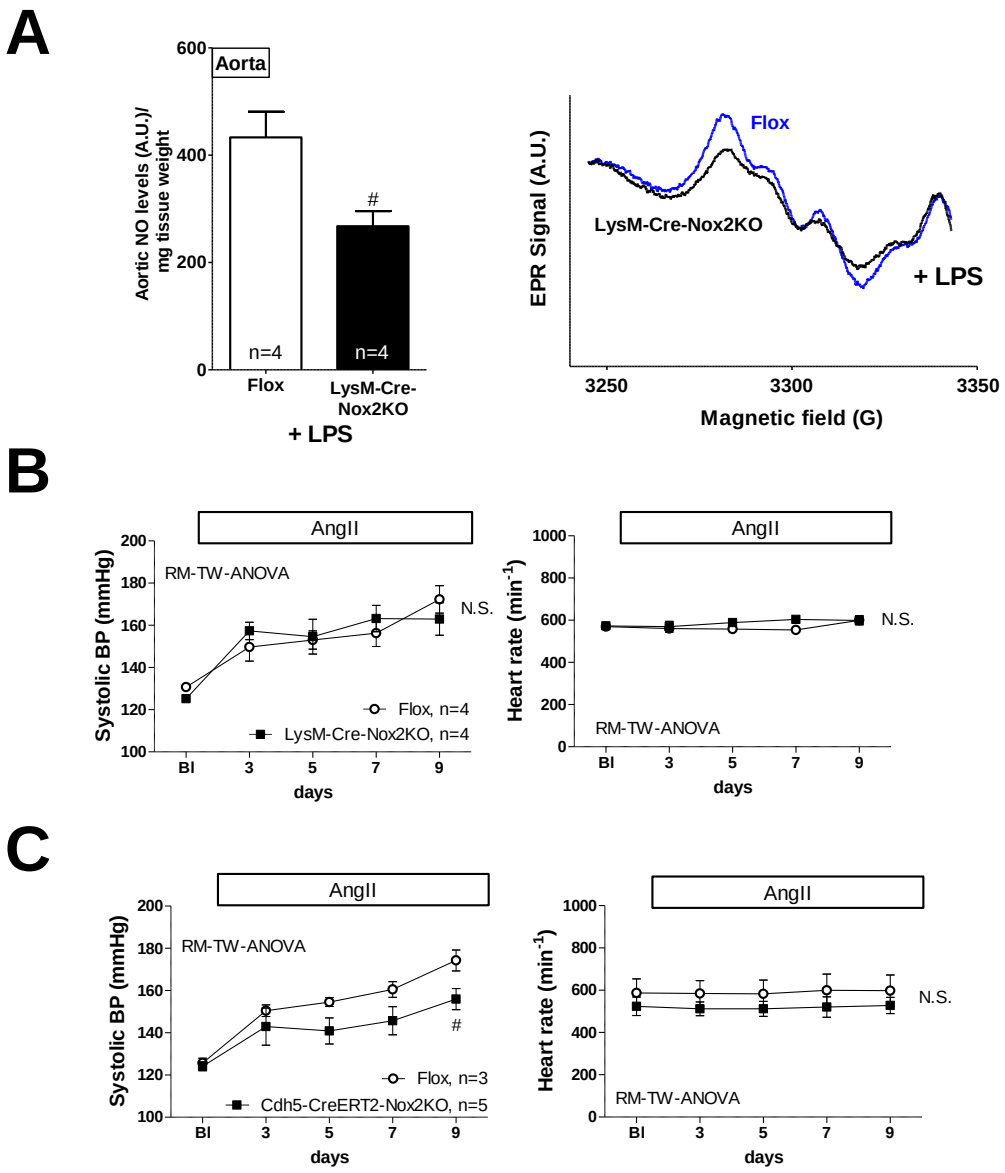
C



D



Suppl. Fig. 2


A
Aorta
Aortic NO levels (A.U.)/ mg tissue weight
600
400
200
0
#
n=4
n=4
Flox
LysM-Cre-Nox2KO
+ LPS
Flox
LysM-Cre-Nox2KO
+ LPS
EPR Signal (A.U.)
3250
3300
3350
Magnetic field (G)
B
AngII
RM-TW-ANOVA
Systolic BP (mmHg)
200
180
160
140
120
100
N.S.
Flox, n=4
LysM-Cre-Nox2KO, n=4
Bl
3
5
7
9
days
AngII
Heart rate (min-1)
1000
800
600
400
200
0
N.S.
RM-TW-ANOVA
Bl
3
5
7
9
days
C
AngII
RM-TW-ANOVA
Systolic BP (mmHg)
200
180
160
140
120
100
#
Flox, n=3
Cdh5-CreERT2-Nox2KO, n=5
Bl
3
5
7
9
days
AngII
Heart rate (min-1)
1000
800
600
400
200
0
N.S.
RM-TW-ANOVA
Bl
3
5
7
9
days

Suppl. Fig. 3

Supplementary Figure 1. Coronary microvascular endothelial cell mRNA levels and cardiac echocardiography.

A. mRNA levels of Nox2 and Nox4 in coronary microvascular endothelial cells (CMEC). $^{\#}$, $P<0.05$ vs. Flox. **B.** Echocardiographic parameters of cardiac structure and function, and left ventricle/body weight ratio (LV/BW). IVSD, interventricular septal diameter.

Supplementary Figure 2. Generation of LysM-Cre-Nox2KO and Cdh5-CreERT2-Nox2KO mice.

A. Magnitude of L-NMMA-induced constriction in AngII-treated aortic rings from Tie2-Nox2KO mice and Flox controls. *, $P<0.05$ vs. Flox. **B.** Cre-mediated recombination in Cre^{+} bone marrow cells from LysM-Cre-Nox2KO mice. **C.** Cre-mediated recombination in Cre^{+} lung tissue from Cdh5-CreERT2-Nox2KO mice. **D.** Left ventricular ejection fraction in Cdh5-CreERT2-Nox2KO cf. Flox mice following Tamoxifen treatment.

Supplementary Figure 3. Hypertensive response to AngII in LysM-Cre-Nox2KO and Cdh5-CreERT2-Nox2KO mice.

A. Reduced aortic iNOS-derived NO formation in LysM-Cre-Nox2KO after LPS stimulation. $^{\#}$, $P<0.05$ vs. Flox. Representative EPR spectra shown to the right. **B.** *In vivo* response to AngII infusion in LysM-Cre-Nox2KO mice and Flox control. **C.** *In vivo* response to AngII infusion in Cdh5-CreERT2-Nox2KO mice and respective control. RM-TW-ANOVA = repeated measures two-way ANOVA. $^{\#}$, $P<0.05$ vs. Flox control.