ONLINE SUPPLEMENT

Vascular Nox compartmentalization, protein hyperoxidation and ER stress response in hypertension

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Running title: Vascular Nox and ER stress in SHR

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Methods

Animals. Sixteen-week-old male normotensive Wistar Kyoto (WKY) and hypertensive rats spontaneously hypertensive rats (SHR) were euthanized and mesenteric arteries isolated for primary culture of VSMC. Mesenteric arteries from WKY and SHRSP (stroke-prone spontaneously hypertensive rats) rats were used for assessment of vascular function by myography. All animal procedures were conducted in conformity with Home Office regulations regarding experiments with animals in the United Kingdom (Scientific Procedures) Act 1986.

Isolation and culture of vascular smooth muscle cells from rat mesenteric arteries Primary VSMCs were isolated from mesenteric arteries by enzymatic digestion, as we previously described[1]. Mesenteric beds were excised from 4-8 animals and pre-digested in F12 medium containing collagenase type I (9 mg/ 3 mL/ artery), for 15 min at 37°C under rotation. Adipose and connective tissue were subsequently stripped off and the clean arteries were pooled. VSMCs were dissociated by digestion in an F12 enzymatic mix containing collagenase type I (5 mg/ 2.5 mL/ artery), elastase (0.3 mg/ 2.5 mL/ artery), soybean trypsin inhibitor (0.9 mg/ 2.5 mL/ artery), and bovine serum albumin (5 mg/ 2.5 mL/ artery), for 60-90min at 37°C under rotation. Digested arteries were then passed four times through a 21-gauge needle to disperse cells, and filtered through a 100 μm nylon filter (BD Biosciences, Oxford, UK) to remove debris. Cell suspension was centrifuged at 800 g for 3 min and re-suspended in growth medium (DMEM supplemented with 10% FBS and 5% antibiotics (100 IU/ml penicillin – 100 μg/ml streptomycin)). Medium was refreshed after 24 h. At 80-90% confluence cells were treated with 0.025% trypsin-EDTA and passaged. VSMCs were studied at passage 5-7.

Cell transfection. VSMCs from WKY and SHR (8×10^5 cells/well) were plated and cultured for 24h in growth medium (DMEM supplemented with 10% FBS and 5% antibiotics (100 IU/ml penicillin – 100 μg/ml streptomycin)). VSMCs were incubated with 50nM of Nox1 siRNA or Nox4 siRNA (Stealth RNAi™ siRNA, Thermo Fischer Scientific, Illinois, USA) complexed with Lipofectamine™ RNAiMAX (Thermo Fischer Scientific, Illinois, USA) as transfection reagent in DMEM without serum and antibiotics for 6 h. A sequence not homologous to any gene in the vertebrade transcriptome was used as control siRNA (Stealth RNAi™ siRNA Negative Control Lo GC, Thermo Fischer Scientific, Illinois, USA). After transfection, medium was replaced by growth medium (DMEM with 10% FBS and 5% antibiotics) and experiments were conducted 48h after transfection.

Cell Fractionation. Plasma membrane proteins were obtained using a plasma membrane isolation kit according to manufacturer instructions (Promokine, Heidelberg, Germany). Endoplasmic reticulum (ER) and mitochondria were obtained by differential centrifugation. Briefly, cells were trypsinized and resuspended in hypotonic buffer (10 mmol/L HEPES, 1 mmol/L EGTA, 25 mmol/L KCl) incubated for 20 min at 4°C. Cells were centrifugated and resuspended in isotonic buffer (10 mmol/L HEPES, 250 mmol/L sucrose, 1 mmol/L EGTA, 25 mmol/L KCl) and homogenized. Cell homogenate was centrifuged at 1000 x g for 10min, supernatant was transferred to a new tube and centrifuged again at 10,000 x g for 10min. For mitochondria isolation, the pellet was resuspended in PBS and incubated for 1h with magnetic beads coupled with antibody against mitochondrial outer membrane protein TOM22 (Abcam, Cambridge, UK). The magnetic beads were prepared using the Dynabeads antibody coupling kit (Thermo Scientific, Illinois, USA). Thereafter the beads were separated from the supernatant and resuspended in lysis buffer (in mmol/L: 50 $Na_4P_2O_7$, 50 NaF, 50 NaCl, 5 Na_2EDTA, 10 Hepes, 0.5% (v/v) triton X-100, pH 7.4, supplemented with 2 Na_3VO_4, 1 PMSF and 1 μg/mL of aprotinin, leupeptin and pepstatin) and prepared for western blotting.

The post-mitochondrial fraction was centrifuged at 100,000 x g for 1h. The obtained pellet comprising ER was resuspended in lysis buffer and prepared for Western blotting. Nuclear fractions were obtained using a nuclear fraction kit (Active Motif, La Hulpe, Belgium).

ROS measurements. ROS levels were measured in VSMCs that had been pre-treated (30 mins) with NoxA1ds (Nox1 inhibitor, 10 μmol/L), GKT136901 (Nox1/4 inhibitor, 10 μmol/L) 4-Phenylbutyric acid (4-PBA) (ER stress inhibitor, 1 mmol/L), tauroursodeoxycholic acid (Tudca, ER stress inhibitor , 200μM) and mito-Tempo (mitochondria-targeted antioxidant, 50nM). In some experiments, cells werethen stimulated with Ang II (100 nmol/L, 5min). Lucigenin-enhanced chemiluminescence assay was used to detect NADPH-dependent superoxide anion (O_2^-) production in VSMC as we previously described (1). Briefly cells were homogenized in lysis buffer and incubated with lucigenin (5 μmol/L) and NADPH (0.1 mmol/L). Luminescence was measured for 29 cycles of 1.8 seconds each by a luminometer (Lumistar Galaxy; BMG Labtechnologies, Germany). Basal readings were recorded prior to the addition of NADPH as the substrate, and were subtracted from the NADPH-dependent luminescence signal. ROS production was expressed as relative luminescence unit (RLU)/μg protein.
Hydrogen peroxide (H_2O_2) levels in VSMC lysates were assessed using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Life Technologies, Carlsbad, USA) according to manufacturer´s instructions. H_2O_2 levels were calculated based on standard H_2O_2 curves and normalised to the protein concentration for each sample. The results are expressed in arbitrary units/milligram protein.

Immunoblotting. VSMCs were scraped in lysis buffer and centrifuged at 16,000 x g for 4 min, at 4°C. Supernatants were collected and protein concentration was determined using the Bio-Rad DC Protein Assay kit (BioRad Laboratories Ltd, Hertfordshire, UK). Equal amounts of protein were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked with either non-fat dry milk or BSA, before overnight incubation in protein-specific primary antibodies. Secondary fluorescence-coupled antibodies (LICOR) were incubated for 1 h and were visualized by an infrared laser scanner (Odyssey Clx, LICOR). Western blotting images were quantified using the software Image Studio™ Lite. Protein expression levels were normalized to loading controls and expressed as absolute values or percentage (%) of the control.

Assessment of protein sulfenylation. To assess one of the first reversible cysteine oxidative modifications in proteins, sulfenylation, we used two different probes that specifically binds the sulfenic acid groups in proteins. (DCP-Bio, Kerafast Boston, MA, USA) is a biotin-tagged dimedone-based probe that specifically binds sulfenic acid groups (SOH) in proteins[2]. Cells were scraped in lysis buffer supplemented with DCP-Bio1 (1mmol/L), N-methylmalemide (10mmol/L), catalase (200U) and protease inhibitors (1mmol/L PMSF and 1 μg/mL of aprotinin, leupeptin and pepstatin). Samples were kept on ice for 30 min and centrifuged at 16,000 x g for 4min at 4˚C. Supernatants were collected and DCP-Bio1 excess was removed by acetone precipitation. Pellets were re-suspended in lysis buffer and prepared for western blot.

Additionally, we used BCN-E-BCN, a cell permeable probe composed of two symmetrical strained cyclooctynes connected by a short ethylenediamine-derived linker[3]. VSMCs were grown to 70% confluence. Cells were incubated in starving media (DMEM 0.5% FBS) overnight prior to labelling. BCN-E-BCN was synthesised by the literature procedure (3). BCN-E-BCN (100μmol/L) was reconstituted in DMSO and added to cells for 10 min. Cells were washed in PBS and lysed on ice for 10 minutes in lysis buffer and centrifuged at 16,000 x g for 4 minutes at 4°C. BCN-E-BCN was conjugated with biotin using a copper-free click

reaction. For this, lysates were incubated with azide-PEG3-biotin (1 mmol/L, Sigma). Excess azide-PEG3-biotin was removed by precipitating lysates in 100% ice cold acetone. Pellets were re-suspended in lysis buffer and prepared for western blot.

Affinity capture of sulfenylated proteins. After experimental treatments, cells were scraped in lysis buffer supplemented with DCP-Bio1 (1mmol/L) and submitted to the same procedures as described previously for protein sulfenylation. After removing the excess of DCP-Bio1, protein levels were determined using the Bio-Rad DC Protein Assay kit (BioRad Laboratories Ltd, Hertfordshire, UK). Equal amounts of protein (500µg) were added to a 50 µl slurry of nonliganded support beads (sepharose CL-4B beads, Sigma-Aldrich, Seelze, Germany) to remove proteins with a tendency to bind nonspecifically and incubated for 2h at 4˚C with constant rotation. Beads were centrifuged at 1,000 x g for 2 min, the supernatant was collected and incubated with streptavidin beads (High Capacity Streptavidin–Agarose Resin, Thermo Scientific, Illinois, USA) overnight at 4˚C with constant rotation. After the incubation steps beads were centrifuged at 1,000 x g for 2 min and washed with PBS three times. Proteins were then eluted in 50 µl of 2 x sample buffer for western blotting and boiled (5 min).

Determination of irreversible PTP and Prx oxidation. VSMCs were lysed and analyzed by immunoblotting using specific antibodies, which recognize the hyperoxidized catalytic centre (-SO_3H) on PTPs (ox-PTP, R&D Systems, Michigan, USA) and Prx (Prx-SO3, Abcam, Cambridge, UK).

Proliferation assay. The fluorescein-based dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (CellTrace™ CFSE Cell Proliferation, Thermo Fisher) was used to assess proliferation of cultured VSMCs. CSFE is a fluorescent cell staining dye that can be used to monitor cell proliferation due to the progressive halving of CFSE fluorescence within daughter cells following each cell division. VSMCs were trypsinized and ressuspended in PBS containing 1% FBS. Cells were then incubated with 5 µmol/L CFSE in 1 mL of PBS for 30 min at 37°C. After the incubation, cells were resuspended in media (DMEM plus FBS 10%) and washed twice. CFSE labelled VSMCs were plated at 30% confluence and cultured for 1 day in growth media (DMEM plus FBS 10%) followed by incubation in DMEM supplemented with FBS 5% for 72h. During this period, cells were treated with NoxA1ds (Nox1 inhibitor, 10µmol/L), GKT136901 (Nox1/4 inhibitor, 10µmol/L),4-PBA (ER stress inhibitor, 1mmol/L), Tudca (ER stress inhibitor , 200µM) and STF-083010 (disruptor of Ire1-XBP1 pathway, 60µM) added to the media and replaced every day. After 72h, cells were trypsinized and resuspended in in PBS containing 1% FBS. Flow cytometry analysis with Ex/Em 492⁄517 filters was performed using the FACS CANTO II system (BD Biosciences), using acquisition software FACS DIVA (BD Biosciences). Data were analysed using the FlowJo X software (TreeStar, Ashland, OR, USA).

Vascular function assessed by wire myography. Mesenteric arteries from WKY and SHRSP were cut into 2 mm ring segments and mounted on a wire myograph filled with 5 mL of physiological solution and continuously gassed with a mixture of 95% O_2 and 5% CO_2 at a temperature of 37º C. After 30 min of stabilization, the contractile ability of the preparations was assessed by adding KCl (120 mmol/L) to the organ baths. Endothelial integrity was verified by relaxation induced by acetylcholine (10 µmol/L; ACh) in vessels pre-contracted with phenylephrine (10 µmol/L). Endothelium-dependent and independent relaxation were assessed by concentration-response curves to ACh (1 nmol/L - 100 µmol/Land noradrenaline (NA; 1 nmol/L - 100 µmol/L) in mesenteric arteries from all animal groups. In some experiments, vascular preparations were incubated with the ER stress inhibitor 4-PBA (1mmol/L).

Reagents and antibodies

Lucigenin, angiotensin II, N-ethylmaleimide (NEM), sodium orthovanadate (Na_3VO_4), catalase, elastase, soybean trypsin inhibitor, PMSF, aprotinin, leupeptin and pepstatin were from Sigma-Aldrich (Seelze, Germany). Collagenase type-1 was from Wothrington Biochemical Corporation (Lakewood, UK). Primary antibody used for Nox1 detection was purchased from Sigma-Aldrich (Seelze, Germany). Antibodies towards Nox2, Nox4, Na/K ATPaseand α-tubulin were from Abcam (Cambridge, UK). Antibodies to PERK (protein kinase RNA-like endoplasmic reticulum kinase) and its phosphorylated form (Thr980), VDAC3(voltage-dependent anion-selective channel protein 3), HDAC3 (histone deacetylase 3), CHOP (CCAAT-enhancer-binding protein homologous protein), and PTP1B (protein tyrosine phosphatase 1B) and spliced XBP1 were from Cell Signalling Technologies (Danvers, MA, USA). Phosphorylated (Ser724) and total IRE1α (inositol-requiring enzyme 1) antibodies were from Thermo Fischer Scientific (Illinois, USA). BiP (immunoglobulin heavy-chain-binding protein) antibody was from R&D Systems (Michigan, USA). PCNA (proliferating cell nuclear antigen), Trx and Calreticulin antibodies were from Santa Cruz Biotecnology (Heidelberg, Germany).

Statistical Analysis

All results are reported as mean±SE. For comparisons between two groups t-test was used. For multiple comparisons one-way analysis of variance (ANOVA) followed by Bonferroni's post-test was conducted, as appropriate. Graphs were plotted in GraphPad Prism 5 software and values of $p<0.05$ were considered significant.

References

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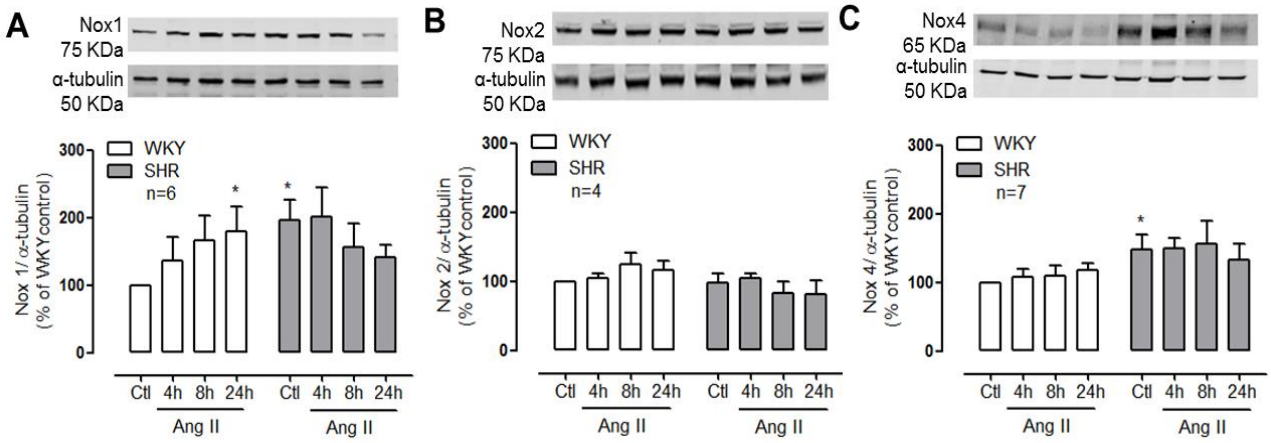
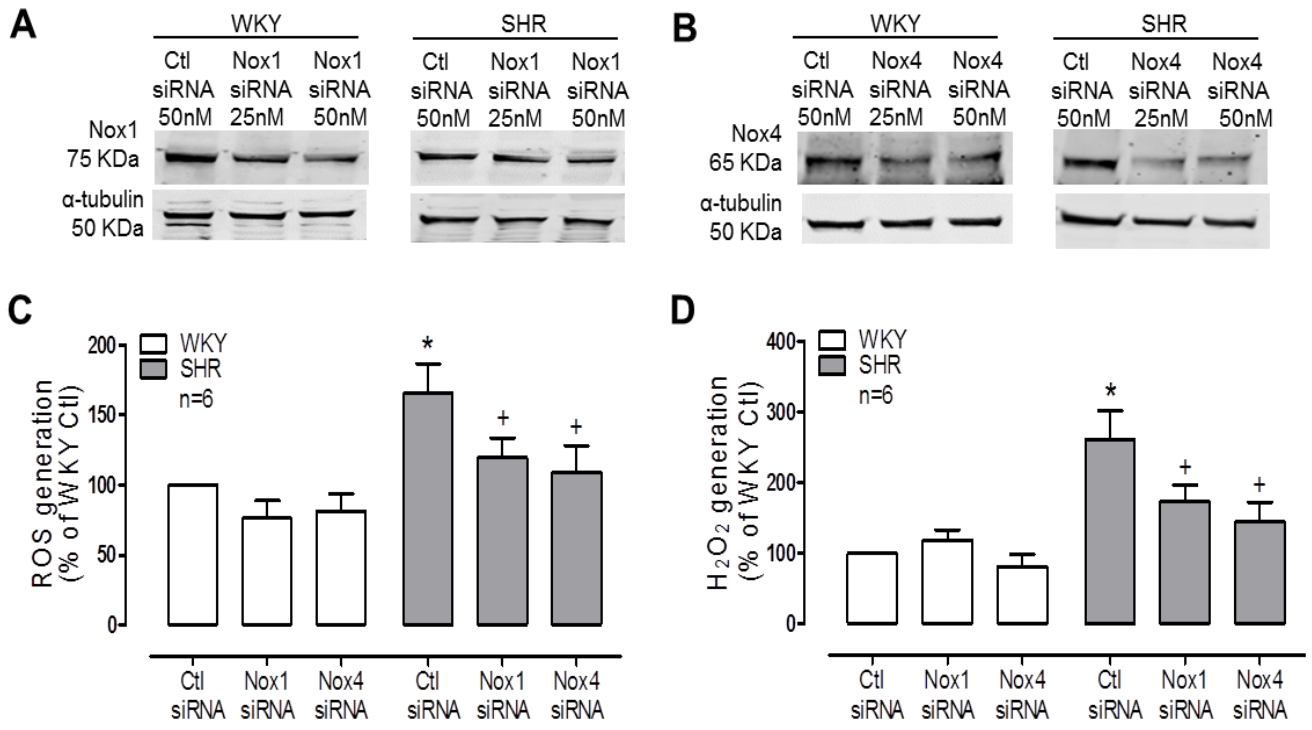
2. Nelson KJ, Klomsiri C, Codreanu SG, et al. Use of dimedone-based chemical probes for sulfenic acid detection methods to visualize and identify labeled proteins. *Methods Enzymol*. 2010;473:95-115
3. McGarry DJ, Shchepinova MM, Lilla S, Hartley RC, Olson MF. A cell-permeable biscyclooctyne as a novel probe for the identification of protein sulfenic acids. *ACS Chem Biol*. 2016;11:3300-3304

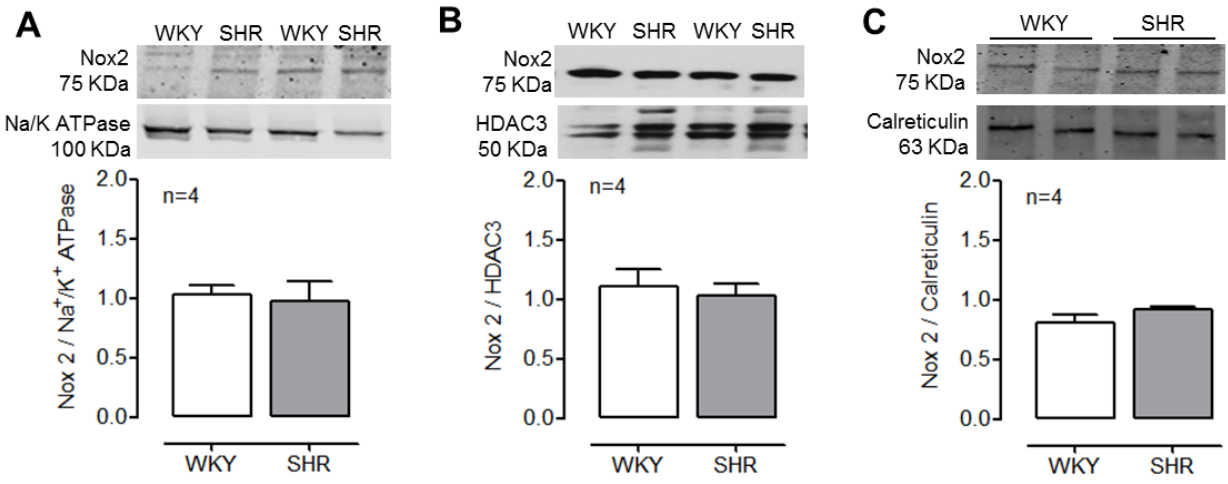
Supplementary Figures



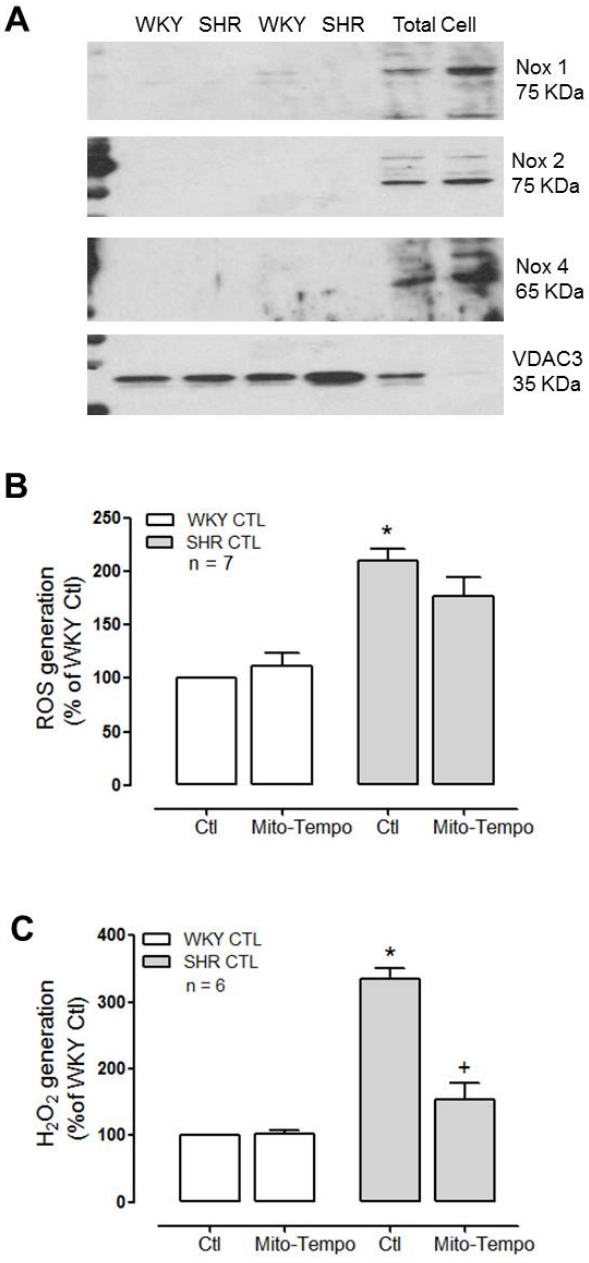
S1. Expression of Nox1 and Nox4 is increased in VSMC from hypertensive rats. Expression of Nox1 (A), Nox2 (B) and Nox4 (C) in VSMCs from SHR and WKY rats detected by Western blot. Cells were treated with Ang II (100nmol/L) for the indicated time points. α-Tubulin was used as loading control. Results are expressed as mean ±SEM of 4-7 separate experiments.*p<0.05 vs WKY Ctl.



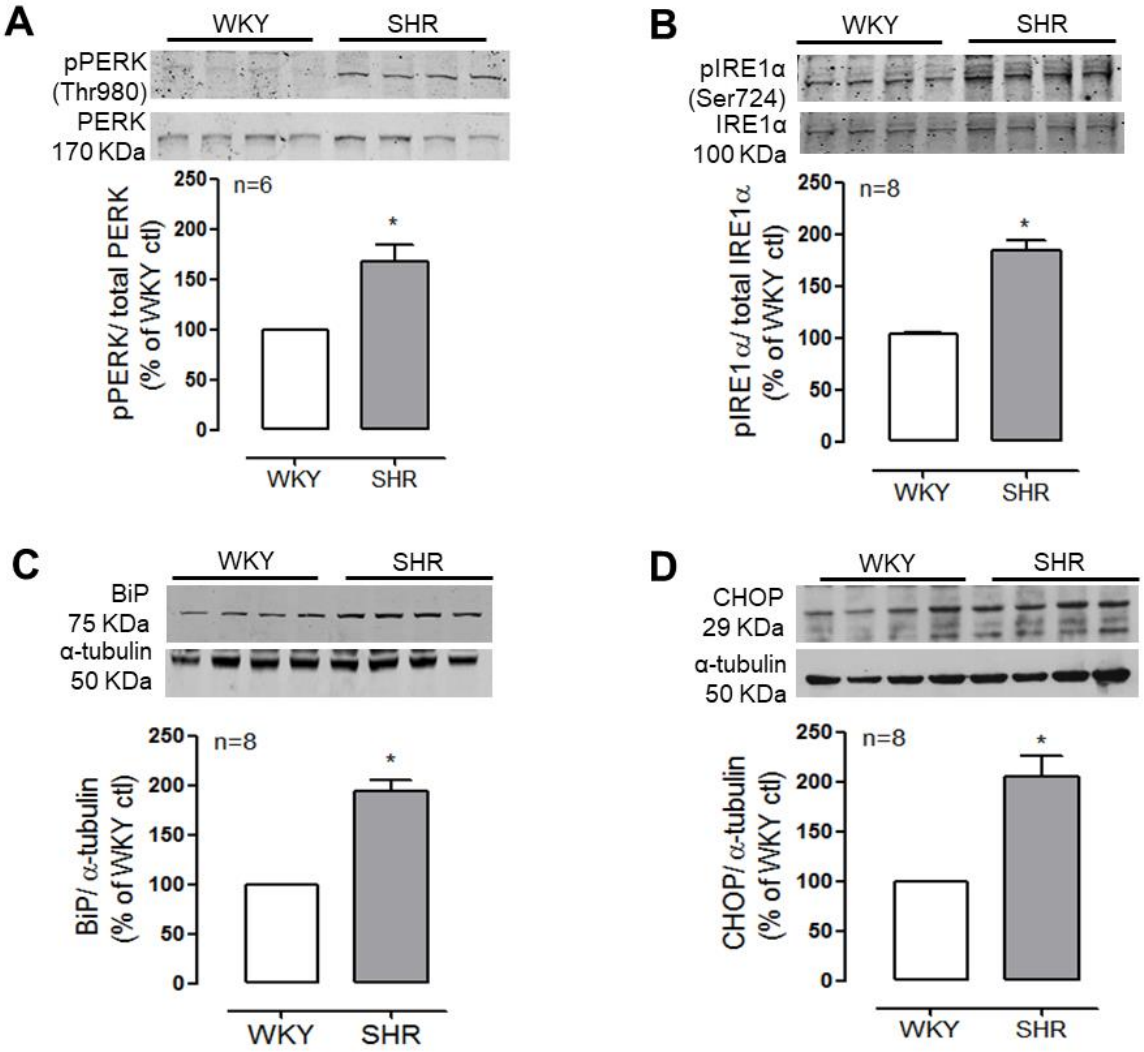
S2. Nox1 and Nox4 are involved in basal ROS generation in SHR VSMCs. Nox1 (A) and Nox4 (B) siRNA was used to decrease Nox 1 and Nox4 expression in WKY and SHR VSMCs. ROS was assessed by chemiluminescence (C) and amplex red (D) in cells transfected with Nox1 and Nox4 siRNA (50 nM). Protein quantification was normalized by α-tubulin. Results are expressed as mean±SEM of 6 separate experiments. *$p<0.05$ vs WKY Ctl and $^{+}$ $p<0.05$ vs SHR Ctl.



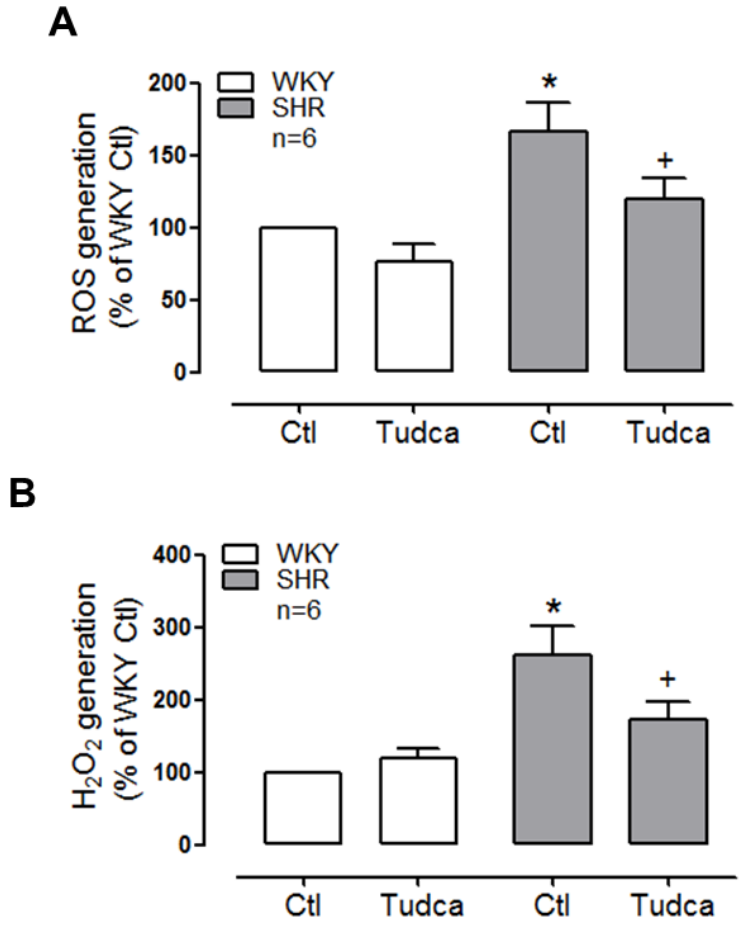
S3. Subcellular localization of Nox2 in VSMCs from WKY and SHR rats. Expression of Nox2 in isolated plasma membrane (A), nuclear/endoplasmic reticulum fraction (B) and isolated endoplasmic reticulum (C). Results are expressed as mean±SEM of 4 separate experiments and were normalized by Na/K ATPase (plasma membrane marker), HDAC3 (Histone deacetylase 3; nuclear marker) or Calreticulin (ER marker).*$p<0.05$ vs WKY.



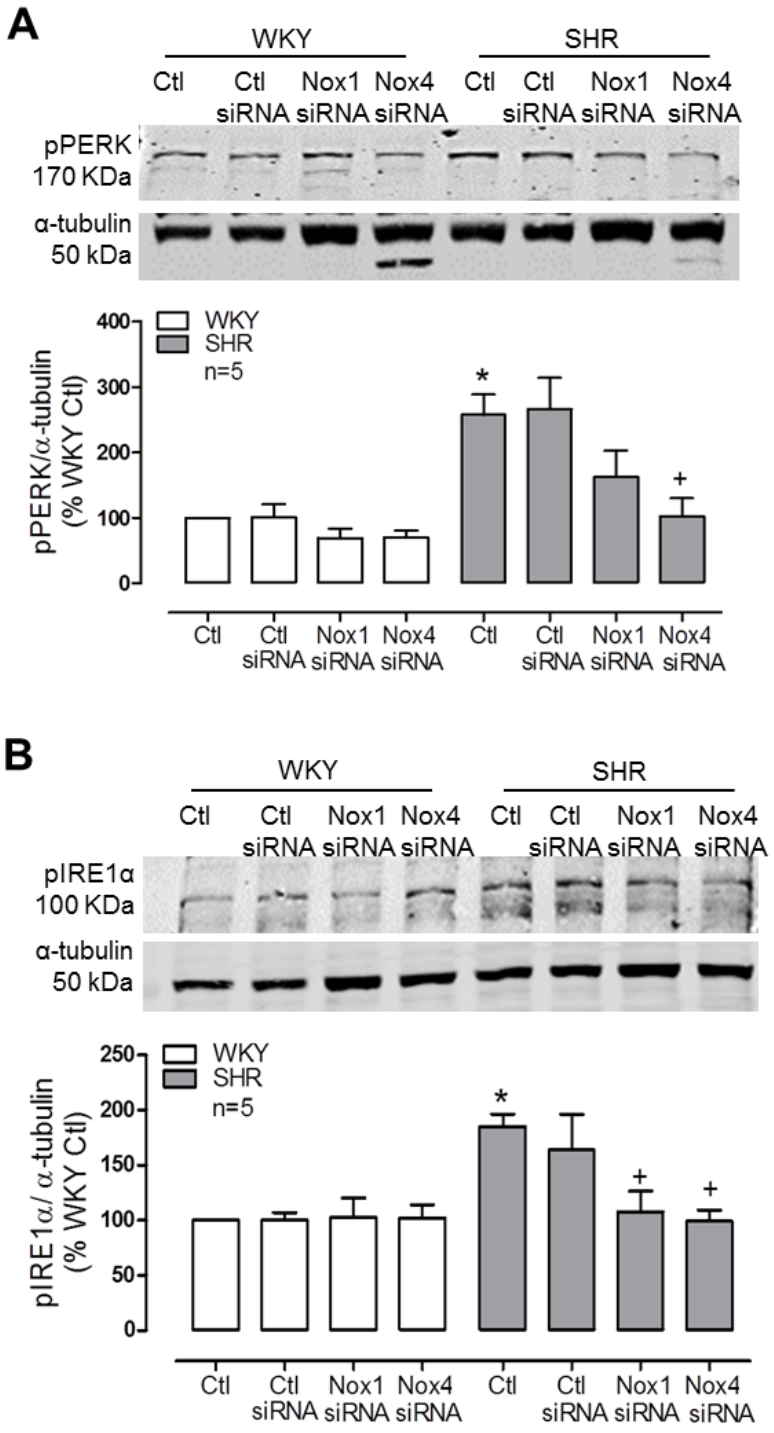
S4. Mitochondrial ROS is involved in basal hydrogen peroxide generation in hypertension independently of NADPH oxidases. A) Nox1, Nox2 and Nox4 protein was not detected in mitochondria isolated with magnetic bead. Total cell lysate from WKY and SHR rats was used as a positive control. VDAC3 (voltage-dependent anion channel) was used as mitochondrial marker.. B) ROS generation measured by lucigenin derived chemiluminescence (B) and amplex red (C) in the presence of the antioxidant targeted to the mitochondria mitoTEMPO (50 nmol/L). *$p<0.05$ vs WKY Ctl and $^{+}p<0.05$ vs SHR Ctl.



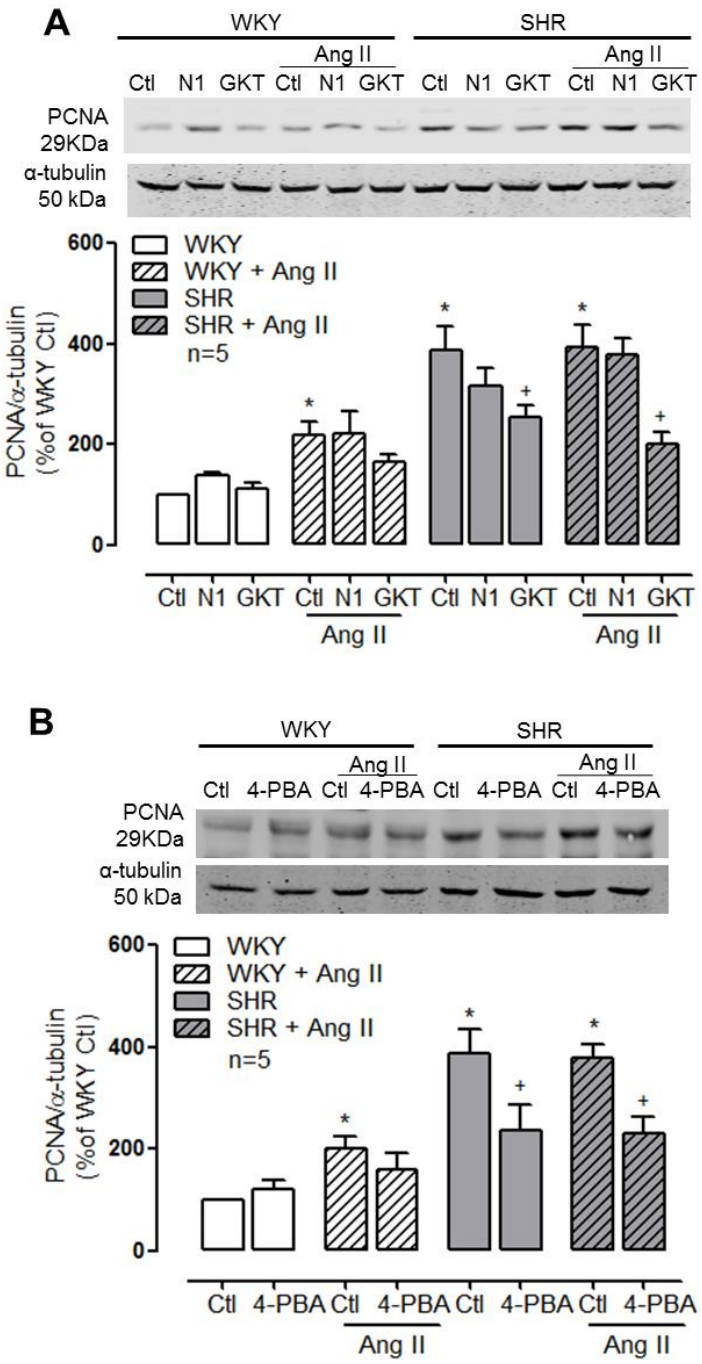
S5. ER stress response is activated in SHR VSMCs. Phosphorylation of ER stress activators PERK (A) and IRE1α (B), and expression of BIP (C) and CHOP (D) were detected by western blot in cells from WKY and SHR rats. Protein quantification was normalized by total PERK, total IRE1a or α- tubulin. Results are expressed as mean±SEM of 6-8 separate experiments. *p<0.05 vs WKY Ctl.



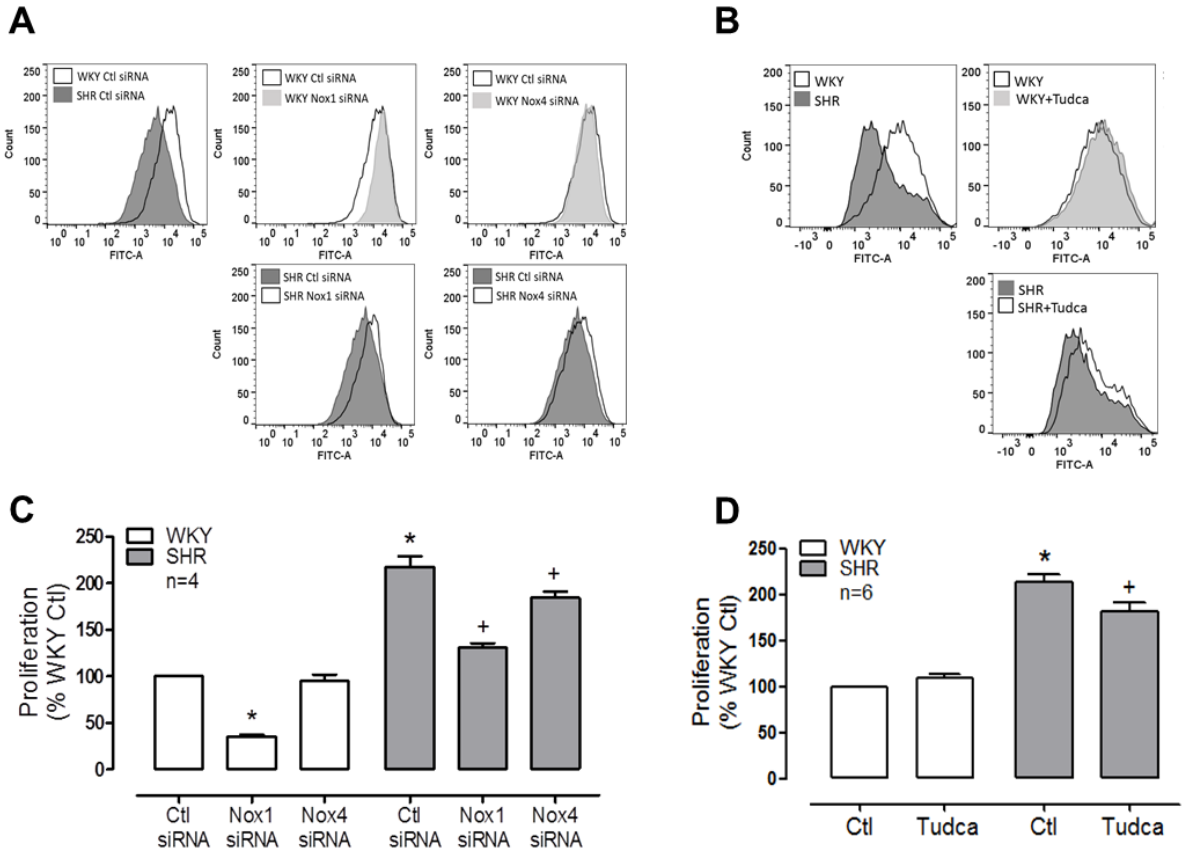
S6. ER stress is involved in increased ROS generation and proliferation in VSMCs from SHR. ROS was assessed by chemiluminescence (A) and amplex red (B) in the presence of tauroursodeoxycholic acid (Tudca, 200µM, 24h). Results are expressed as mean±SEM of 6 separate experiments. *$p<0.05$ vs WKY Ctl and and $^{+}p<0.05$ vs SHR Ctl



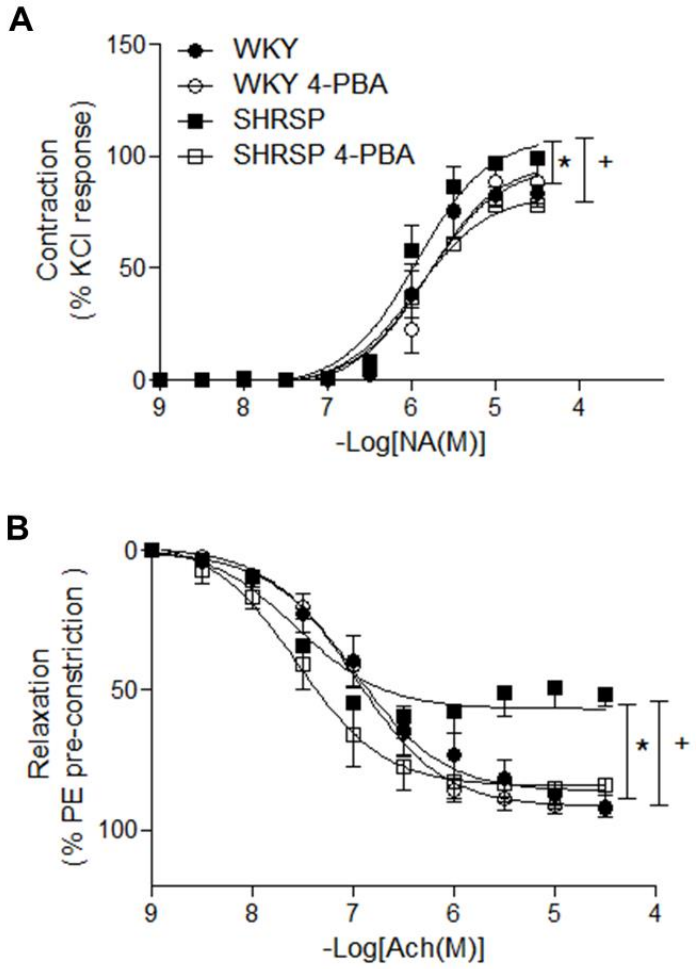
S7. Nox1 and Nox4 are involved in ER stress activation in SHR VSMCs.. Phosphorylation of ER stress activators PERK (A) and IRE1α (B) was detected by western blot after Nox1 and Nox4 siRNA transfection. Non transfected cells were used as control (Ctl) and a control siRNA was used as control for transfection (Ctl siRNA). Protein quantification was normalized by α- tubulin. Results are expressed as mean±SEM of 5-6 separate experiments. *p<0.05 vs WKY Ctl and $^{+}$ p<0.05 vs SHR Ctl.



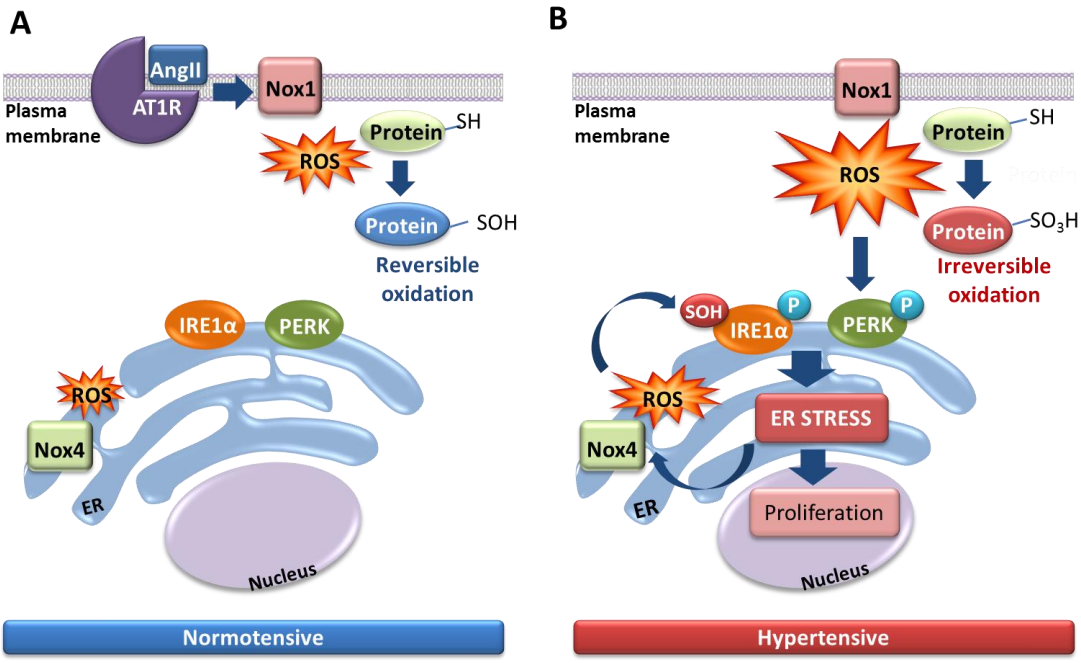
S8. Nox4 and ER stress inhibitors decreased molecular proliferation markers in VSMCs from SHR. PCNA expression was assessed by western blot in VSMCs from WKY and SHR stimulated with Ang II for 24h in the presence of Nox inhibitors (A) or ER stress inhibitor (B). Protein quantification was normalized by α- tubulin. Results are expressed as mean±SEM of 5 separate experiments. *$p<0.05$ vs WKY Ctl and and $^{+}p<0.05$ vs SHR Ctl.



S9. Nox1, Nox4 and ER stress are involved in increased proliferation in VSMCs from SHR. A) Proliferation was assessed using the CSFE assay in cells transfected with Nox1 or Nox4 siRNA. B) Proliferation assay in the presence of tauroursodeoxycholic acid (Tudca, 200µM). Quantification of CSFE fluorescence in cells transfected with Nox1 or Nox4 siRNA (C) and Tudca treated cells (D). Results are expressed as mean±SEM of 4-6 separate experiments. *$p<0.05$ vs WKY Ctl and and $^{+}p<0.05$ vs SHR Ctl



S10. ER stress is involved in vascular dysfunction in hypertension. Isolated mesenteric arteries from normotensive (WKY) and hypertensive (SHRSP) rats were used to assess vascular function by wire myography. Concentration-response curves to noradrenaline (NA) (A) and to acetylcholine (Ach) (B) were performed in the presence or absence of 4-PBA (1mmol/L, 3h pre-incubation). NA responses were normalized by E_{max} of KCl 120 mmol/L, and ACh concentration–response curve was performed in vessels precontracted with phenylephrine (10 µmol/L). Curves represent the mean±SEM (n=5). *p<0.05 vs WKY and $^{+}$p<0.05 vs SHR.



S11. Schematic demonstrating alterations in redox profile in hypertension. A) In cells from normotensive rats ROS generation by Nox1 is controled by factors such as Ang II and induces reversible protein oxidation for signalling purposes. Nox 4 may be active in the ER, however the UPR signalling is not activated. B) In cells from hypertensive rats Nox1 and Nox4 are constitutively active resulting in increased irreversible protein oxidation and activation of ER stress. Nox1 induced the PERK arm of the UPR response, while Nox4 promotes IRE1α sulfenylation (SOH). ER stress and Nox4 play a role in the hyperproliferative phenotype of cells from hypertensive rats.