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Abstract—Asymmetrical dimethylarginine inhibits nitric oxide synthase, cationic amino acid transport, and endothelial function. Patients with cardiovascular risk factors often have endothelial dysfunction associated with increased plasma asymmetrical dimethylarginine and markers of reactive oxygen species. We tested the hypothesis that reactive oxygen species, generated by nicotinamide adenine dinucleotide phosphate oxidase, enhance cellular asymmetrical dimethylarginine. Incubation of rat preglomerular vascular smooth muscle cells with angiotensin II doubled the activity of nicotinamide adenine dinucleotide phosphate oxidase but decreased the activities of dimethylarginine dimethylaminohydrolase by 35% and of cationic amino acid transport by 20% and doubled cellular (but not medium) asymmetrical dimethylarginine concentrations ($P<0.01$). This was blocked by tempol or candesartan. Cells stably transfected with $p22^{phox}$ had a 50% decreased protein expression and activity of dimethylarginine dimethylaminohydrolase despite increased promoter activity and mRNA. The decreased DDAH protein expression and the increased asymmetrical dimethylarginine concentration in $p22^{phox}$-transfected cells were prevented by proteosomal inhibition. These cells had enhanced protein arginine methylation, a 2-fold increased expression of protein arginine methyltransferase-3 ($P<0.05$) and a 30% reduction in cationic amino acid transport activity ($P<0.05$). Asymmetrical dimethylarginine was increased from 6 ± 1 to 16 ± 3 μmol/L ($P<0.005$) in $p22^{phox}$-transfected cells. Thus, angiotensin II increased cellular asymmetrical dimethylarginine via type 1 receptors and reactive oxygen species. Nicotinamide adenine dinucleotide phosphate oxidase increased cellular asymmetrical dimethylarginine by increasing enzymes that generate it, enhancing the degradation of enzymes that metabolize it, and reducing its cellular transport. This could underlie increases in cellular asymmetrical dimethylarginine during oxidative stress. **(*Hypertension*. 2010;56:498-504.)**

Key Words: dimethylarginine dimethylaminohydrolase (DDAH) ■ protein arginine methyltransferase (PRMT) ■ tempol ■ cationic amino acid transferase (CAT) ■ hypertension

Asymmetrical dimethylarginine (ADMA) inhibits nitric oxide synthase (NOS) and cationic amino acid transport (CAT).[1] ADMA is generated by protein arginine methyltransferases (PRMTs) and, after proteolysis, cellular ADMA is metabolized by dimethylarginine dimethylaminohydrolases (DDAHs) or exported by CATs.[2,3] Angiotensin II (Ang II) can generate reactive oxygen species (ROS) in blood vessels by activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.[4] Patients with early hypertension or kidney disease have elevated plasma levels of ADMA and markers of ROS,[5] which may contribute to endothelial dysfunction and subsequent cardiovascular or renal events. Although increased ADMA occurs in several conditions associated with ROS,[6] it is unclear how ROS increase ADMA. Moreover, infusions of Ang II sufficient to increase ROS have variable effects on plasma ADMA.[7–9]

The present studies were designed to test the hypothesis that NADPH oxidase enhances PRMT and/or reduces DDAH, but that a reduction in CAT activity may limit cellular ADMA export. First, we assessed the effects of Ang II on ROS and ADMA in cultured cells. Thereafter, we investigated the mechanism of NADPH oxidase–induced changes in ADMA directly in cells stably transfected with $p22^{phox}$ which increases NADPH oxidase activity.[10] We selected preglomerular vascular smooth muscle cells (PGVSMCs) because the afferent arteriole is the main resistance vessel in the kidney and generates ROS with Ang II[11] and vascular smooth muscle cells (VSMCs) produce little nitric oxide (NO), which obviates its confounding effects on DDAH activity.[12]

Methods

All animal care and experimental procedures complied with National Institutes of Health guidelines and were approved by Georgetown University Animal Care and Use Committee. Details of the methods

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are available in the online Data Supplement at http://hyper.ahajournals.org.

Cell Culture

PGVSMCs were isolated from 13 to 15 week old male WKY rats purchased from Tacomic Farms (Germantown, NY) as described in detail (see the online Data Supplement).[11]

Measurement of Superoxide Production in PGVSMCs

Cells were seeded into a 96-well plate at densities of 1×10^5 cells per well in 200 μL of the DMEM-F12 medium. After 24 hours, the cells were incubated overnight in serum-free medium, which was replaced 4 hours before incubation with added vehicle or indicated dose of Ang II. Measurement of superoxide ($O_2^{\cdot-}$) was as described in detail previously for these cells using low concentration (5 μmol/L) lucigenin-enhanced chemiluminescence.[11] NADPH oxidase activity was assessed from the increase in superoxide generated after addition of 100 μmol/L of NADPH.

Overexpression of NADPH Oxidase Subunit $p22^{phox}$ in Rat PGVSMCs

A full-length rat $p22^{phox}$ cDNA fragment (709 bp) was cloned into the *Eco*RI/*Xba*I site of pcDNA4 HisMax vector which contained ampicillin and zeocin-resistant genes to allow positive clone (see the online Data Supplement).

DDAH2 Promoter Activity Assay

The mouse DDAH-2 promoter (−924 to −36 bp from transcription start site) that drives a luciferase construct was cloned into pGL3-Basic vector (Promega) as described previously.[13] This was a gift from Dr Satoshi Tanaka (University of Tokyo, Japan) (see the online Data Supplement).

Medium or Cell Lysate ADMA, Symmetrical Dimethylarginine, and L-Arginine

VSMCs were grown to full confluence in 100-mm dishes and cultured for 48 hours in 5 mL of serum-free, phenol red–free medium. Some dishes of cells were directly treated with indicated dose of Ang II for 48 hours and some of them were pretreated for 2 hours with Candesartan (10^{-7} mol/L), Candesartan+PD-123,319 (3×10^{-6} mol/L) or Tempol (10^{-4} mol/L) and then coincubated with vehicle or Ang II (10^{-6} mol/L) for 48 hours. Measurement of ADMA, symmetrical dimethylarginine (SDMA), and L-arginine in the medium and cell lysate was performed using HPLC as previously described (see the online Data Supplement).[14]

Medium H_2O_2

Measurement of H_2O_2 released from intact PGVSMCs was performed using Amplex Red hydrogen peroxide/peroxidase assay kit (Molecular Probes Inc) (see the online Data Supplement).

DDAH Activity

The conversion of [^{14}C]ADMA to [^{14}C]citrulline was used to quantify DDAH activity based on previous assays (see the online Data Supplement).[15]

CAT Activity Assays

CAT activity was assessed in intact cells from 10 mmol/L lysine-inhibitable [^{14}C]ADMA uptake (see the online Data Supplement).[2]

RNA Isolation and Real-Time Quantitative RT-PCR

Real-time quantitative PCR was performed in an ABI Prism 7700 sequence-detection system (Applied Biosystems, Foster City, Calif) as described (see the online Data Supplement).[16]

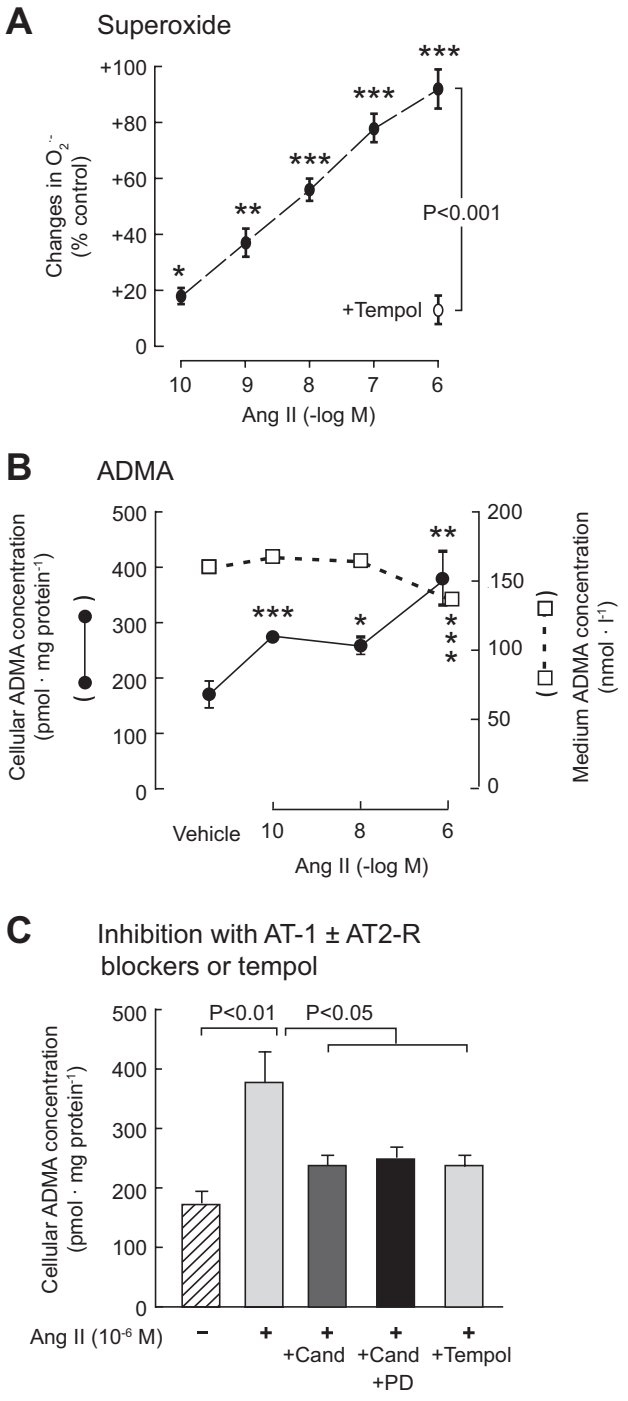

Figure 1. Mean±SEM values for PGVSMCs (n=6 per group) incubated for 4 hours with angiotensin II showing changes in superoxide and the effects of 10^{-4} mol/L tempol (A) on asymmetrical dimethylarginine in the medium (open circles) and cell lysate (close circles) (B) and the effects of coincubation with candesartan (10^{-7} mol/L), PD-123,319 (3×10^{-6} mol/L), or tempol (10^{-4} mol/L). Compared to vehicle: *P<0.05; **P<0.01; ***P<0.005.

Protein Expression

This used Western blot as described (see the online Data Supplement).[16]

Analysis of Protein Carbonylation

The carbonylated proteins from lysed cells were detected with an OxyBlot Oxidized Protein Detection Kit (Millipore, Billerica, Mass) as reported previously (see the online Data Supplement).[17]

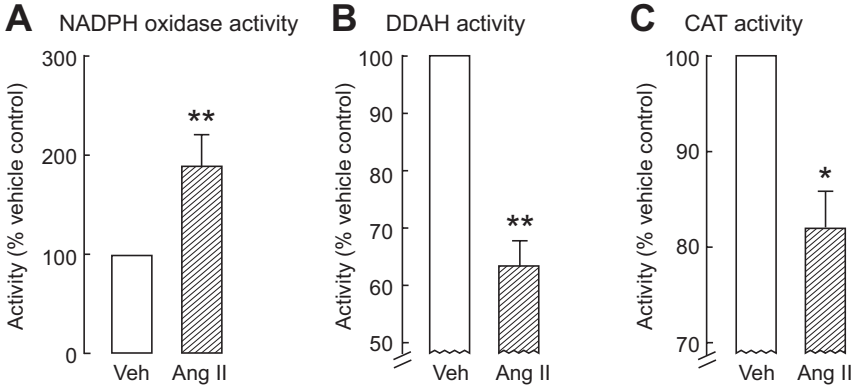

Figure 2. Mean±SEM values (n=3 per group) for cells incubated with a vehicle (open boxes) or angiotensin II (10^{-6} mol/L) (closed boxes) for NADPH oxidase, DDAH, and CAT activities after incubation for 4 hours with vehicle or 10^{-6} mol/L angiotensin II. Compared to vehicle: *P<0.05; **P<0.01.

Materials

Ang II, tempol, and NADPH were obtained from Sigma-Aldrich (St Louis, Mo), DMEM-F12 from Gibco (Carlsband, Calif), and FBS from American Type Culture Collection (Manassas, VA).

Statistical Analysis

Results are expressed as means±SEM. ANOVA was performed, and differences between experimental groups were compared by Student's t test, when appropriate. A value of P<0.05 was considered as statistically significant.

Results

Ang II produced concentration-dependent increases in cellular $O_2^{\cdot-}$ from 10^{-10} mol/L (Figure 1A), confirming prior findings.[11] Cellular ADMA doubled with 10^{-6} mol/L Ang II, but the medium ADMA was modestly reduced (Figure 1B). Coincubation of Ang II–stimulated cells with candesartan or tempol reduced cellular ADMA (Figure 1C), which was not altered by addition of the Ang II type 2 (AT_2) receptor blocker PD-123,319. Ang II increased the activity of NADPH oxidase (2-fold; P<0.01; Figure 2A) but decreased the activities of DDAH by 35% (P<0.01) and CAT by 20% (P<0.05) (Figure 2B and 2C).

Cells stably transfected with $p22^{phox}$ ($S\text{-}p22^{phox}$) had an increased expression of NOX-1 mRNA (2.4±0.37-fold; P<0.02) and protein (2.9±0.1-fold; P<0.003) but no significant changes for NOX-4 (Figure S1 in the online Data Supplement). Transcripts or protein for NOS-1, -2, or -3 were not detected in PGVSMCs (data not shown). The $S\text{-}p22^{phox}$ cells had a 2.5-fold increased $p22^{phox}$ (P<0.01) and a 2-fold increased $O_2^{\cdot-}$ and H_2O_2 (P<0.01; Figure 3). The mRNA for DDAH-1 and DDAH-2 (Figure 4A) and the DDAH-2 promoter activity (Figure 4B) were increased by 2- to 4-fold (P<0.01) in $S\text{-}p22^{phox}$ cells. However, the protein expression for DDAH-1 and DDAH-2 were reduced by 50% (P<0.01; Figure 4C), and the DDAH activity was reduced correspondingly (P<0.01; Figure 4D). $S\text{-}p22^{phox}$ cells had a marked increase in protein carbonyls that was abolished by incubation with catalase and tempol (Figure S2). After incubation of $S\text{-}p22^{phox}$ cells with 1 μmol/L epoxomicin to inhibit proteosomal degradation, DDAH-1 and -2 expression increased by 50% (P<0.05; Figure S3). This was accompanied by a reduction in cellular concentrations of ADMA in $S\text{-}p22^{phox}$ cells to the level measured in wild-type (Wt) cells (Figure S4). The increased mRNA expression for DDAH-1 and -2 in $S\text{-}p22^{phox}$ cells persisted after epoxomicin treatment (DDAH-1 mRNA 1.6±0.1-fold increase; P<0.02 and DDAH-2 2.4±0.3-fold increase; P<0.02; data not shown).

There was a marked increase in asymmetrical dimethylation of proteins in $S\text{-}p22^{phox}$ cells and in cells incubated for 30 minutes with 100 μmol/L H_2O_2 (Figure 5A) accompanied by a doubling of PRMT-3 expression (P<0.05; Figure 5B).

CAT activity, assessed as lysine-inhibitable [^{14}C]ADMA cellular uptake (Figure 6A), was reduced by 30% in $S\text{-}p22^{phox}$ cells (P<0.05; Figure 6B), accompanied by a 70% reduction in CAT-1 mRNA expression (P<0.01) that was mimicked by incubation of Wt cells with H_2O_2 (Figure 6C).

$S\text{-}p22^{phox}$ cells grown in culture medium containing L-arginine had a 10% increase in medium L-arginine concentration (P<0.005), a 60% increase in medium ADMA (P<0.005) and a 5% reduction in medium SDMA (P<0.05; Table). The concentrations in cell water were calculated from the relationship between cell water and cell protein content in rat cultured VSMCs.[18] $S\text{-}p22^{phox}$ cells had a doubling of cellular L-arginine (P<0.005), a 2.6-fold increase in cellular ADMA (P<0.005), and a 3-fold increase in cellular SDMA (P<0.05; Table).

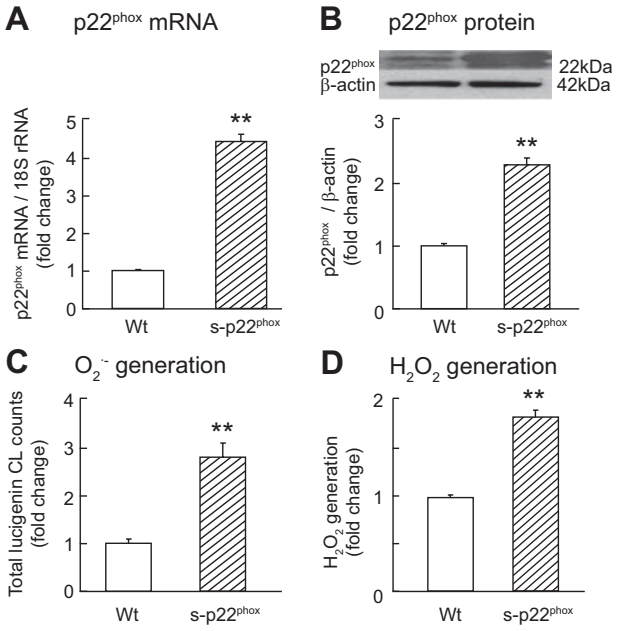

Figure 3. Mean±SEM values (n=3 per group) for cells stably transfected with an empty vector (Wt) (open boxes) or $p22^{phox}$ ($S\text{-}p22^{phox}$) (cross-hatched boxes). Compared to Wt: **P<0.01.

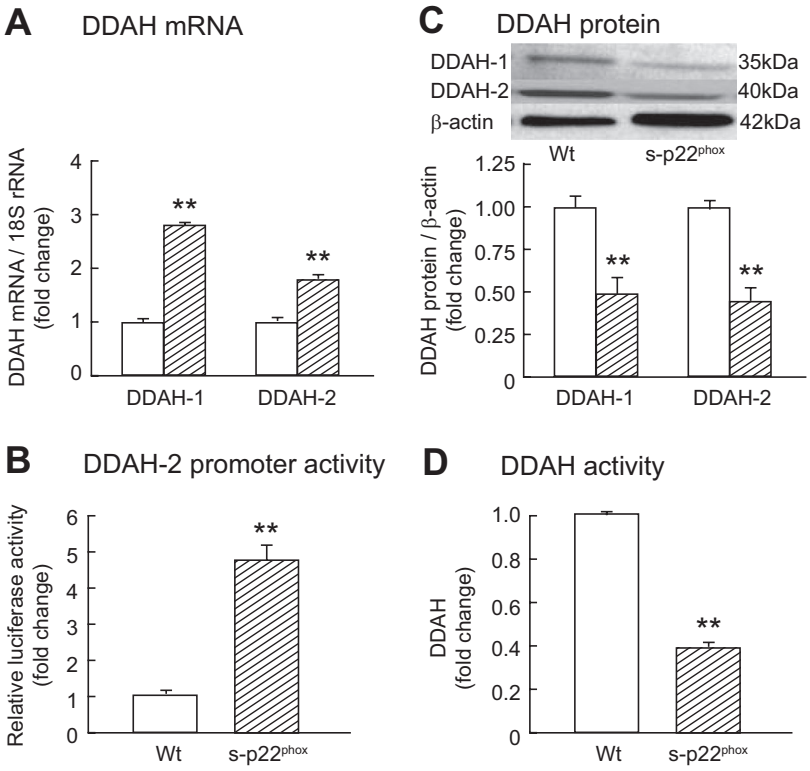

Figure 4. Mean±SEM values (n=3 per group) for cells stably transfected with an empty vector (Wt) (open boxes) or p22^{phox} (S-p22^{phox}) (cross-hatched boxes). Compared to Wt: $^{**}P<0.01$.

The cell:medium concentration ratio for L-arginine, ADMA, and SDMA were all increased significantly ($P<0.05$) in S-p22^{phox} cells (Table).

Discussion

The main new findings are: Incubation of VSMCs with Ang II for 4 hours increased $O_2^{\cdot-}$ and cellular, but not medium, ADMA. Inhibition of AT_1 receptors or ROS blocked the increase in cellular ADMA. Ang II stimulated NADPH oxidase but inhibited DDAH and CAT activities. Stable transfection of cells with p22^{phox} increased mRNA and protein expression for NOX-1 and increased the ROS generation, protein arginine methylation, and PRMT-3 expression but decreased DDAH protein expression and activity, CAT activity and CAT-1 expression. The decrease in DDAH protein and activity occurred despite an increase in DDAH mRNA expression and was prevented by blockade of proteosomal degradation which also reduced cellular ADMA of S-p22^{phox} cells to the level of Wt cells. These changes in S-p22^{phox} cells were accompanied by an increase in medium and a greater increase in cellular levels of ADMA.

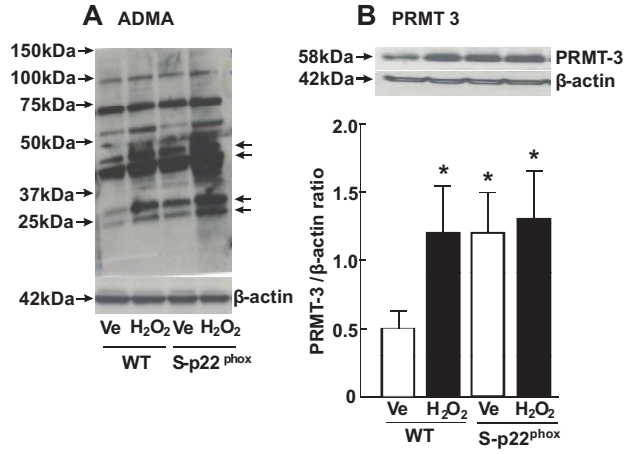

Figure 5. Western blots for asymmetrically demethylated arginine moieties on proteins (A) and PRMT-3 expression in these cells (n=3 per group) (B).

The function of ADMA in VSMCs is not clear. We could not detect expression of mRNA or protein for any NOS isoform in our isolated PGVSMCs. However, ADMA can have adverse vascular effects in endothelial nitric oxide synthase knockout mice.[19] ADMA can stimulate ROS production from some NOS isoforms if arginine is limited or tetrahydrobiopterin is oxidized.[20] Moreover, Vallance and Leiper[1] have shown that ADMA released from 1 cell can inhibit NOS in an adjacent cell and that this could be a mechanism for VSMCs to signal to endothelial cells. Additionally, Ang II doubled the release of ADMA from cultured endothelial cells.[21] Thus, a similar mechanism for angiotensin-induced ADMA generation may occur in VSMCs and endothelial cells, but this remains to be explored.

We found an increase in cellular ADMA with Ang II that was related to AT_1 receptor activation and to ROS. Thus, cellular $O_2^{\cdot-}$ and ADMA increased in parallel from 10^{-10} mol/L Ang II and the increase in ADMA was prevented by candesartan, but not PD-123,319 and by tempol, which prevented Ang II–stimulated increases in $O_2^{\cdot-}$ in these cells.[11] A decreased ADMA metabolism by DDAH during Ang II could contribute to increased cellular ADMA concentrations, whereas a decreased CAT activity could reduce cellular export of ADMA into the medium. Further studies

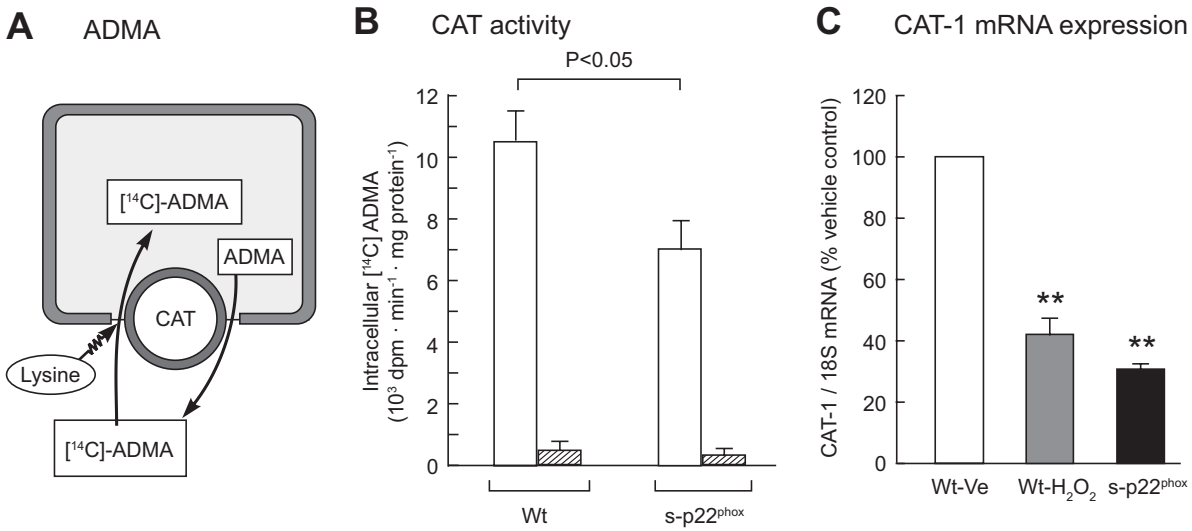

Figure 6. A, Cell diagram of method to measure CAT activity. B, CAT activity showing [^{14}C]ADMA uptake (open bars) and the effects of inhibition with 10 mmol/L lysine (hatched bars). C, CAT-1 mRNA expression in Wt cells treated with vehicle (open bars) or H_2O_2 (cross-hatched bars) and in S-p22^{phox} cells (filled bars) (n=3 per group).

focused on the direct effects of prolonged NADPH oxidase activity on ADMA.

The membrane protein p22^{phox} is a critical component of NADPH oxidase.[10] Smooth muscle specific overexpression of p22^{phox} in mice increased aortic p22^{phox} and NOX-1 proteins and increased $O_2^{\cdot -}$ and H_2O_2 generation,[22] whereas knockdown of p22^{phox} in vivo reduced the protein expression for NOX-1, -2, and -4.[23] Stable transfection of cells with p22^{phox} provided a robust model of cellular oxidative stress with increased $O_2^{\cdot -}$ and H_2O_2 generation and increased NOX-1 expression. In apparent contrast to the finding that Ang II decreased DDAH activity in PGVSMCs, we detected an increase in the mRNA for DDAH-1 and -2 in S-p22^{phox} cells accompanied by an increase in the promoter activity for DDAH-2. However, this was accompanied by a reduction in protein expression for DDAH-1 and -2 and cellular DDAH activity. This discrepancy was related to enhanced proteosomal degradation because the reduced protein expression for DDAH-1 and -2 in S-p22^{phox} cells was mitigated (for DDAH-1) or prevented (for DDAH-2) by inhibition of proteosomal degradation by epoxomicin, which also normalized the increased cellular ADMA concentrations. This extended prior studies in which H_2O_2 enhanced proteosomal degradation of the inositol 1,4,5-triphosphate receptor in VSMCs.[24] Because restoration of DDAH-1 or -2 protein expression with epoxomicin did not correct the increased mRNA in S-p22^{phox} cells, the increased mRNA was not likely a compensation for reduced DDAH expression.

Inhibition of DDAH by oxidative stress has been ascribed to oxidation of a cysteine residue in the catalytic site of the enzyme[1,12] or to downregulation of protein expression.[25] A novel finding was that this also can involve proteosomal degradation of the DDAH protein. We detected increased asymmetrical dimethylarginine in proteins and increased PRMT-3 protein

Table. Concentrations of L-Arginine, ADMA, and SDMA in the Medium and Cell Water of Preglomerular Vascular Smooth Muscle Cells Transfected With p22^{phox} or Wild-Type Cells Transfected With an Empty Vector

Cell Type	Arginine	ADMA	SDMA
Medium			
Wild type	529±5 μmol · L^{-1}	220±7 nmol · L^{-1}	143±6 nmol · L^{-1}
S-p22^{phox}	588±2 nmol · L^{-1}	357±4 nmol · L^{-1}	135±4 nmol · L^{-1}
P	<0.005	<0.005	<0.05
Calculated concentrations in cell water, μmol · L^{-1}*			
Wild type	1645±63	5.9±1.2	1.6±0.7
S-p22^{phox}, μmol · L^{-1}	3222±340	15.7±3.4	4.9±2.7
P	<0.005	<0.005	<0.05
Cell: medium concentration ratio*			
Wild type	3.1±0.06	26.9±3.5	11.5±2.8
S-p22^{phox}	5.5±0.33	51.9±13.9	36.1±12.0
P	<0.005	<0.05	<0.05

Means±SEM (n=6 per group). *Based on a cell water content of cultured vascular smooth muscle cells of 2.53 μL · mg protein^{-1}.[18]

expression in S-p22^{phox} cells, which suggests that increased PRMT activity contributed to increased cellular ADMA. Recently, Chen et al[26] reported that bovine retinal capillary endothelial cells cultured in high-glucose solution that elevated ROS production had increased PRMT-1 expression and decreased DDAH activity and DDAH-2 expression. These were corrected by antioxidants, suggesting that ROS increased PRMT-1 and decreased DDAH, as in our study.

The finding that Ang II or activation of NADPH oxidase reduced cellular CAT activity and that the latter reduced CAT-1 mRNA expression is compatible with prior studies that have reported diminished CAT activity or expression under conditions that induce oxidative stress, for example, exposure to cigarette smoke[27] or homocysteine.[28]

ADMA concentrations in cultured endothelial cells are reported to be ≈10-fold above that in the medium.[29] We measured ADMA concentrations in PGVSMCs to be 27-fold above that in the medium. The calculated intracellular concentrations of ADMA of 6 μmol/L in Wt cells and 16 μmol/L in S-p22^{phox} cells are expected to inhibit NOS in other cell types that express NOS isoforms, but the higher calculated intracellular arginine concentrations would offset this effect. The doubling of intracellular L-arginine in p22^{phox} cells may be secondary to increased cellular proliferation which enhances arginine turnover[30] because p22^{phox} overexpression induces proliferation in VSMCs.[10]

Perspectives

Because Ang II upregulated ROS in many tissues[4,11] and ROS increased cellular ADMA, Ang II should increase ADMA. Indeed, Ang II doubled ADMA release from cultured endothelial cells. However, we found that relatively short-term incubation of VSMCs with Ang II for 4 hours did not change, or even reduced, medium ADMA, despite a rise in cellular ADMA. This may be a consequence of a reduced CAT activity which could slow the cellular export of ADMA. Infusions of Ang II were reported to increase[7] or maintain[8] plasma ADMA or to increase ADMA only at high rates of Ang II infusion.[9] Our findings in isolated cells suggest that there could be a substantial increase in cellular ADMA in conditions that enhance ROS that may not be reflected reliably in plasma levels because of decreased CAT activity. The finding that Ang II and NADPH oxidase expression both reduced CAT activity supports the possibility that in the short term, ADMA export may be diminished. However, in the long term, the increased intracellular ADMA will stress the CAT system and export will increase until a new steady state has been reached where the rate of ADMA production will equal the sum of degradation by DDAH and export by CAT.[2] The main effect of CAT inhibition is that this steady state should be reached at higher intracellular levels of ADMA. ADMA production in S-p22^{phox} cells was increased, as evidenced by increased PRMT expression and increased levels of ADMA moieties on proteins. From the Table, it is apparent that ADMA in the medium increased 1.6-fold compared to wild-type cells. This is less than the 2.7-fold increase in intracellular ADMA, compatible with reduced CAT activity, but clearly shows an increased net export of ADMA. Thus, under steady-state conditions, export of ADMA by VSMCs may be increased by ROS, although this may not hold for other cell types. Uptake of ADMA by neighboring endothelial cells with NOS inhibition is plausible, but remains to be demonstrated experimentally.

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Disclosures

None.

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ONLINE DATA SUPPLEMENT

ANGIOTENSIN II AND NADPH OXIDASE INCREASE ADMA IN VASCULAR SMOOTH MUSCLE CELLS

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Methods

***Cell culture*:** The phenotype of the cells was confirmed from characteristic morphology (hill and valley pattern), Ang II contraction, expression of smooth muscle-specific alpha actin and smooth muscle myosin heavy chain and the absence of mRNA for von Willebrand factor (endothelial cell marker). Experiments were conducted between passages 4 and 12. Cells were cultured in DMEM-F12 medium supplemented with 10% fetal bovine serum, 100 u · ml^{-1} of penicillin, 100 mcg · ml^{-1} of streptomycin and 200 mcg · ml^{-1} of glutamine at 37ºC.

Overexpression of NADPH oxidase subunit $p22^{phox}$ in rat preglomerular vascular smooth muscle cells: Plasmid DNA was purified from an ampicillin-resistant clone. The $p22^{phox}$ insertion was confirmed by electrophoresis and sequencing. The empty pcDNA4 vector alone or vector carrying $p22^{phox}$ cDNA (8µg/each) were suspended in 500µl of Opti-MEM I reduced serum medium (Invitrogen, Carlsbad, CA) and mixed gently with 500µl of 1:25 diluted lipofectamine 2000 (Invitrogen, Carlsbad, CA) to form a nucleic-acid-lipofectamine complex. After incubation for 20 min at room temperature, the complex was added to 60-70% confluent PGVSMCs seeded in a 60mm plate in 5ml of Opti-MEM I reduced serum medium. After incubation at 37ºC for 6h, transfected cells were cultured in DMEM/F-12 medium containing 15% fetal bovine serum. 48 hours later, transfected cells were split 1:4 into 100-mm plates and cultured in regular growth medium with 450µg · ml^{-1} of zeocin (Invitrogen) for clone selection. Zeocin-containing medium was changed every 2 days. Two weeks after selection, zeocin resistant clones were isolated and amplified from the stable transfected cell line (S-$p22^{phox}$). The wild type (Wt) cells with empty vector and S-$p22^{phox}$ cells were used for experiments or stored in -80ºC pending use.

DDAH2 promoter activity assay: The plasmid construction, transfection and DDAH-2 promoter activity assay were described previously [1]. Briefly, both Wt and S-$p22^{phox}$ PGVSMCs were seeded in 12-well plates at a density of 3 x 10^5 cells · cm^{-2} and incubated in culture medium for 24 h. Cells were transiently transfected with the plasmid DNA or promoter lacking pGI3-basic vector using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Cells were incubated for 48 hours and harvested in passive lysis buffer (Promega, Madison, WI). Luciferase activity was measured with luciferase assay substrate and normalized by cellular protein concentrations (protein assay reagent; Bio-Rad Laboratories, Inc., Hercules, CA). Each sample was examined in triplicate, and at least three independent experiments were performed.

Medium or cell lysate ADMA, SDMA, and L-arginine: After 48-hour of cell culture in serum-free, phenol-red-free medium, the conditioned medium was collected in a 15ml sterile polypropylene tube and centrifuged at 4°C for 10 min at 2000g , thereafter the supernatant was transferred to ice cold 1.5 ml tubes and stored at -80 °C. Cells were washed three times with ice-cold PBS, scraped into 5 ml of ice-cold PBS and centrifuged at 500*g* for 5 min. The pellet was resuspended in 250 µl of ice-cold sodium phosphate buffer (100 mM; pH 6.5) on ice for 30 min, sonicated on ice with 3 sets of 10 second pulses using a Sonifier Sonicator 250 (Branson; Danbury CT, USA; output 3.0, duty cycle 30%), mixed with 250 µl of 1.2 M perchloric acid (Sigma, St. Louis, MO) and, after centrifugation (10 minutes at 2,000 g at 4ºC), the supernatant was collected and stored at -80 ºC. Concentrations of L-arginine, ADMA and SDMA in medium

and lysate were measured by HPLC with fluorescence detection as previously described [2] using modified chromatographic separation conditions [3]. Supernatants obtained after deproteinization of cell lysates with perchloric acid were neutralized with two volumes of 0.5 M Na_2HPO_4 to one volume of the supernatant. Cell lysates and medium were spiked with monomethylarginine as an internal standard and purified by solid-phase extraction on polymeric cation-exchange columns. Thereafter, the samples were dried with nitrogen, and derivatized with ortho-phthaldialdehyde reagent containing 3-mercaptopropionic acid. Analytes were separated by isocratic reversed-phase HPLC with fluorescence detection. A human plasma pool was analyzed in all analytical series as a quality control. For all analytes the intra- and inter-assay coefficients of variation were <1.5% and <3.5%, respectively. Lysate concentrations were normalized to protein content.

Medium H_2O_2: Cells were seeded in a 96-well plate and grown in culture medium. 20 h later, 90-95% confluent cells were washed twice with warmed Krebs-Ringer phosphate glucose (KRPG) solution (NaCl 145 mM, sodium phosphate 5.7 mM, KCl 4.86 mM, $CalCl_2$ 0.54 mM, $MgSO_4$ 1.22 mM, glucose 5.5 mM, pH 7.35). 100 µl of reaction mixture containing 50 µM Amplex Red reagent and 0.1 U · ml^{-1} of horseradish peroxidase in KRPG solution was added to each well. The fluorescence was measured at an excitation and emission wavelength of 530/580 nm in a FLUOstar Omega fluorescence reader (BMG Labtech Inc., NC). H_2O_2 concentration was normalized to cellular protein.

DDAH activity: PGSMCs were seeded in 12-well plates at a density of 4 x 10^5 cells · $well^{-1}$ and incubated in culture medium for 24 hours. Cells were washed 3 times in 37ºC-warmed HEPES-buffered Krebs solution (mM: NaCl 131, KCl 5.5, $CaCl_2$ 2.5, $MgCl_2$ 1.0, $NaHCO_3$ 25, NaH_2PO_4 1.0, HEPES 20 and glucose 5.5, pH 7.4). 500 µl of [^{14}C]-labelled ADMA (0.25 µCi/ml, specific activity 52 mCi/mM, radio-labelled at C5 position; custom-synthesized by Moravek Biochemicals Inc, CA.) in HEPES-buffered Krebs solution was added to each well and the culture plates incubated at 37°C in 5% CO_2 for 1 h. The reaction solution was removed and cells were rinsed 3 times with cold HEPES-buffered Krebs solution and lysed in 500 µl of 0.1% (w/v) sodium dodecyl sulphate. 250 µl of cell lysate was assayed by scintillation counting. The remainder was mixed with 1 ml of 50% (v/v) activated dowex 50X8-400 cation exchange resin (Sigma, St. Louis, MO) that binds cationic amino acids including ADMA and arginine, but not citrulline, and centrifuged at 12,000g for 3 min. 400µl of the supernatant containing [^{14}C]-citrulline was taken for scintillation counting. DDAH activity was calculated as [^{14}C]-citrulline generated from [^{14}C]-ADMA.

CAT activity assays: Cells were prepared as for DDAH activity (above) except that cell lysates were not treated with activated dowex 50X8-400 cation exchange resin, but used directly for scintillation counting, which represented total [^{14}C]-ADMA uptake by cells. CAT activity was reflected by lysine-inhibitable [^{14}C]-ADMA uptake into cells.

RNA isolation and real-time quantitative RT-PCR: Total RNA was isolated with RNAqueous-4PCR kit (Ambion Inc., Austin, TX) following the manufacturer's instruction manual**.** Reverse transcription of 1 µg of total RNA / per sample was performed with iScript^{TM}cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Primers and probes for rat $p22^{phox}$ (ID: Rn00377357_m1), DDAH-1, -2 (ID: Rn00574200_m1 and Rn01525776_m1), CAT-1 (ID: Rn01453837_m1) and NOS-1,-2,-3 (ID: Rn00583793_m1, Rn00561646_m1 and

Rn06132634_s1) were purchased for gene expression assays (Applied Biosystems, Foster City, CA) . The comparative [DELTA][DELTA]C_T method was used for relative quantification and statistical analysis.

Protein expression: Cells were cultured in 100mm plates with complete medium (see cell culture above). After confluence, cells were incubated with serum free DMEM/F-12 for 48 hours. Cells were treated with vehicle or reagents for indicated times. Cells were washed 3 times with ice cold PBS and thereafter were lysed with mammalian tissue lysis buffer (Sigma St. Louis, MO), 50-100 µg of lysates were run on 6-12.5% SDS-PAGE gels (Bio-Rad, Hercules, CA), transferred to nitrocellulose membranes, and incubated overnight at 4°C while shaking in 5% Blotto with primary antibodies for DDAH-1 (Abcam Inc, Cambridge, MA), DDAH-2 (Sigma, St Louis, MO), p22phox (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), NOS-1, -2 and -3 (Millipore, Billerica, MA), PRMT-1, -3, -4 and -5 (Sigma, St Louis, MO) at 1:500-2000 dilutions and quantified with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or rabbit anti-mouse or -goat IgG (KPL, Gaithersburg, MD) at 1: 1000-20000 dilution, and probed with β-actin antibody to control for equal loading (Sigma, St Louis, MO). The developed films were scanned with an imagine densitometer and optical densities of protein bands quantified using NIH Image J software (version 1.43, NIH, Bethesda, MD). Determination of protein-incorporated methylated arginine was similar to regular Western Blotting as previously described. The mouse monoclonal primary antibody for ADMA was purchased from Abcam Inc. (Cambridge, MA). The immunogen for this antibody was asymmetric NG-NG-dimethyl arginine (two methyl groups on one of the terminal nitrogen atoms of the guanidinium group) and the antibody reacts with free and bound asymmetric NG-NG-dimethyl arginine. It is not known whether the antibody reacts with symmetric dimethyl arginine (information from manufacturer). Briefly, 100 µg of cell lysates/per lane were run on 12% of SDS-PAGE gels and the transferred membranes were incubated in 5% fat-free milk for 1h at room temperature and then in 1:50 diluted primary antibody overnight at 4°C. After washing 3 times, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse second antibody at 1:3000 dilution for 1 hour with shaking at room temperature. The following procedure was performed as described above.

Analysis of protein carbonylation: Cells were snap-frozen in liquid nitrogen, lysed in mammalian tissue lysis buffer (Sigma St. Louis, MO) and centrifuged at 16,000g for 20 min at 4°C. Carbonyl groups on protein side chains were derivatized with 2,4-dinitrophenylhydrazine (DNPH) to form the 2,4-dinitrophenyl (DNP) hydrazone derivative. 30µg of cell lysates were denatured with 12% sodium dodecylsulfate (SDS), incubated with DNPH for 15 min, and mixed with neutralization solution and ß-mercaptoethanol. For negative control, the same amount of cell lysates was incubated with 1x derivatization-control solution in place of DNPH. The samples were electrophoresed through a 12.5% SDS-PAGE gel (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes, blocked with 1% bovine serum albumin for 1 h at room temperature and incubated overnight at 4°C with rabbit anti-DNP antibody at 1:150 dilution and thereafter with HRP-conjugated goat anti-rabbit IgG at 1:300 dilution for 1 hour at room temperature. The carbonylated proteins were determined by an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

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Supplemental Figures

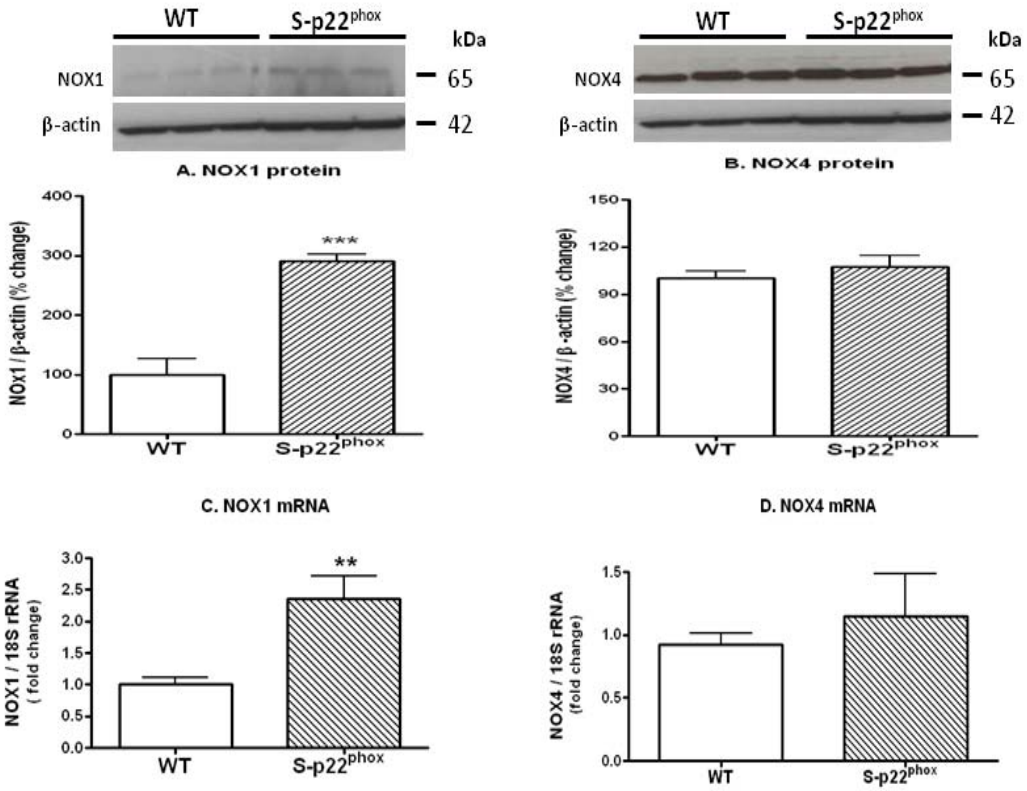

Figure S1: Mean ± SEM values (n=3 per group) for NOX-1 and NOX-4 protein and mRNA expression, comparing values in wild-type, empty vector transfected cells (Wt) with cells stably transfected with $p22^{phox}$ ($S\text{-}p22^{phox}$). Compared to Wt: **, $p<0.01$; ***, $p<0.005$.

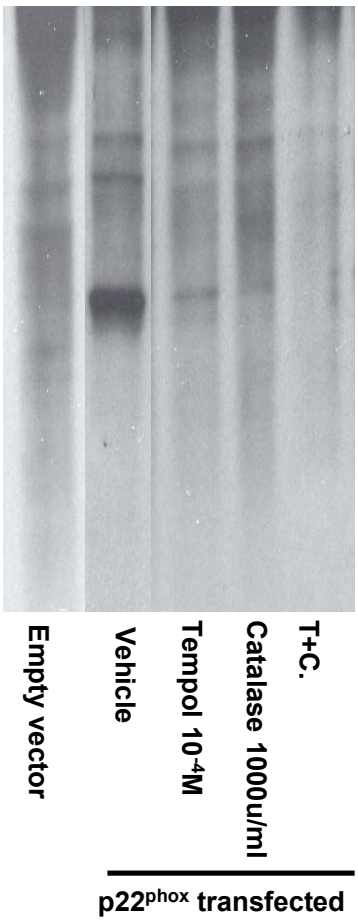

Figure S2: Oxy-blot for protein carbonyls: T+C, tempol plus catalase.

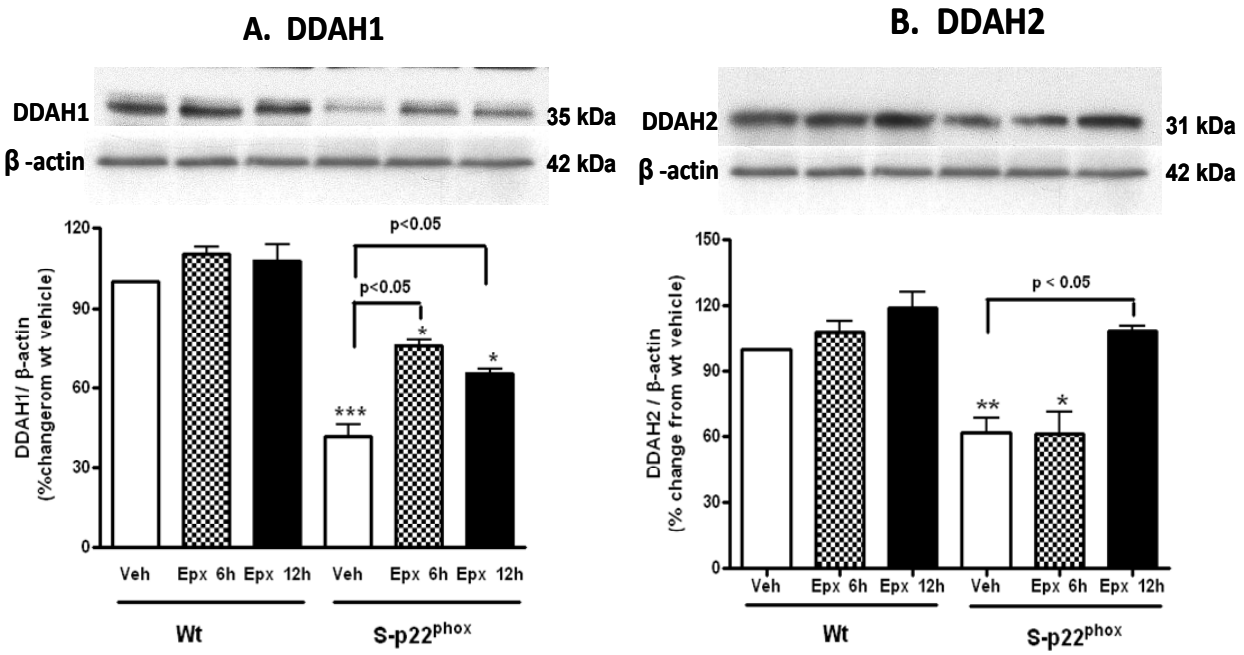

Figure S3: Western analysis for DDAH-1 or -2 from wild type (Wt) and $p22^{phox}$ transfected (S-$p22^{phox}$) cells cultured with a vehicle (Veh) or with 1μM epoxomicin for 6 or 12 hours (n=3 per group). Compared to Wt vehicle: *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

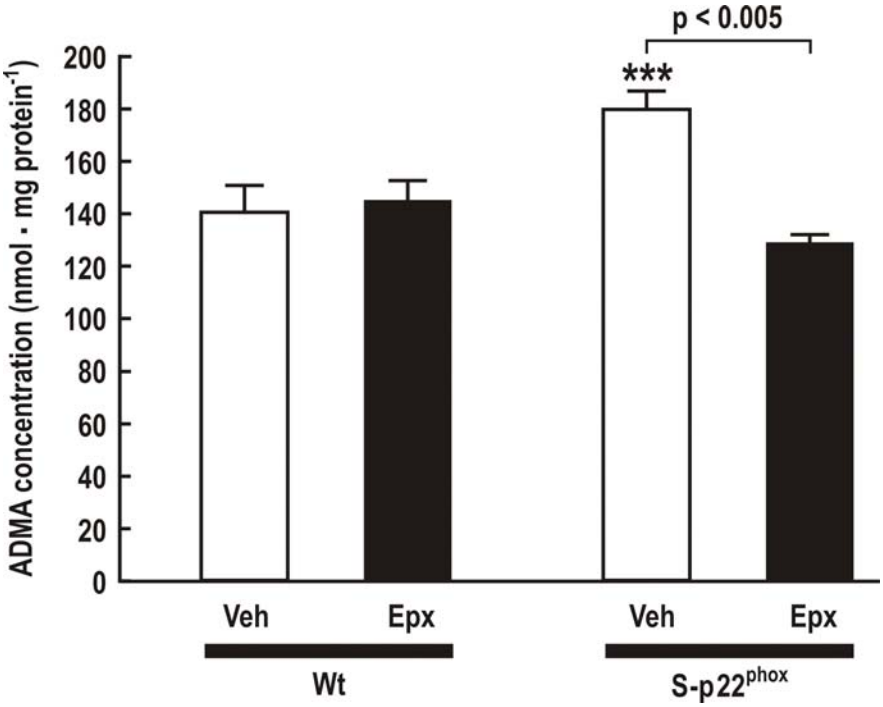

Figure S4: Asymmetric dimethylarginine concentrations in lysates of preglomerular vascular smooth muscle cells (n=5 per group) stably transfected with an empty vector (Wt) or $p22^{phox}$ (S-$p22^{phox}$) and cultured for 12 hours with a vehicle (Veh) or 1 $\mu mol \cdot l^{-1}$ epoxomicin (Epx). Compared to Wt: ***, $p<0.005$.