

Oxidative Stress and Endothelial Dysfunction in Aortas of Aged Spontaneously Hypertensive Rats by NOX1/2 Is Reversed by NADPH Oxidase Inhibition

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Abstract—Arterial hypertension is associated with increased levels of reactive oxygen species, which may scavenge endothelium-derived NO and thereby diminish its vasorelaxant effects. However, the quantitatively relevant source of reactive oxygen species is unclear. Thus, this potential pathomechanism is not yet pharmacologically targetable. Several enzymatic sources of reactive oxygen species have been suggested: uncoupled endothelial NO synthase, xanthine oxidase, and NADPH oxidases. Here we show that increased reactive oxygen species formation in aortas of 12- to 14-month-old spontaneously hypertensive rats versus age-matched Wistar Kyoto rats is inhibited by the specific NADPH oxidase inhibitor VAS2870 but neither by the xanthine oxidase inhibitor oxypurinol nor the NO synthase inhibitor N^G -nitro-L-arginine methyl ester. NADPH oxidase activity, as well as protein expression of its catalytic subunits, NOX1 and NOX2, was increased in the aortas of spontaneously hypertensive rats, whereas the expression of NOX4 protein, the most abundant NOX isoform, was not significantly changed. Impaired acetylcholine-induced relaxation of spontaneously hypertensive rat aortas was significantly improved by VAS2870. In conclusion, NOX1 and NOX2 but not NOX4 proteins are increased in aged spontaneously hypertensive rat aortas. Importantly, these NOX isoforms, in particular, ectopic expression of NOX1 in endothelial cells, appear to affect vascular function in an NADPH oxidase inhibitor-reversible manner. NADPH oxidases may, thus, be a novel target for the treatment of systemic hypertension. (*Hypertension*. 2010;56:490-497.)

Key Words: aged SHR ■ aorta ■ endothelial dysfunction ■ NADPH oxidase ■ NOX ■ oxidative stress

The formation of reactive oxygen species (ROS), resulting in scavenging of NO and reduced NO bioavailability, has been suggested as a hallmark of endothelial dysfunction and a pathomechanism for different cardiovascular diseases, including hypertension.¹ Indeed, increased production of ROS has been observed in human hypertension,^{2,3} as well as in animal models, such as angiotensin II⁴ or aldosterone plus salt-induced⁵ or genetically defined hypertension, for example, in spontaneously hypertensive rats (SHRs).⁶

With respect to the molecular source of these ROS, several enzymes have been suggested, including uncoupled endothelial NO synthase (NOS)⁷ and xanthine oxidase (XOD).^{8,9} More recently, NADPH oxidases have been added as a major source of ROS¹⁰ in resistance and conduit arteries.¹⁻³ At least 3 catalytic NADPH oxidase subunits (NOXs) are expressed in the rodent vasculature, NOX1, NOX2, and NOX4, of

which the latter appears to have the highest expression levels.^{11,12} However, no quantitative protein expression data of all of the rodent vascular NOX isoforms are available. The expression of other noncatalytic, regulatory subunits of NADPH oxidases correlate with hypertension,¹³ for example, p47phox⁴ and p22phox.³ Nevertheless, proof of principle for the role of individual vascular NOX isoforms or NADPH oxidase in hypertension and for their therapeutic targeting is rare. Data in NOX1^{-/-} mice suggest a role of NOX1 in angiotensin II-induced hypertension.¹⁴ However, data in rats or other species are missing, as are data derived from specific pharmacological validation and parallel investigations of all 3 of the rodent vascular NOX isoforms, NOX1, NOX2, and NOX4. This knowledge gap is mainly attributed to the lack of potent and specific NADPH oxidase inhibitors^{14,15} and antibodies. Most widely used inhibitors, such as apocynin or diphenylene iodonium (DPI), are nonspecific.^{16,17}

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An excellent animal model to investigate the possible role of NADPH oxidases as a quantitative relevant source of oxidative stress and endothelial dysfunction in hypertension is the aged SHR, because ROS generation and endothelial dysfunction in these rats is even more pronounced than in younger SHRs.^{18,19} Therefore, we used this model to investigate the protein expression of the vascular NOX isoforms, NOX1, NOX2, and NOX4, in the aortic wall of aged SHRs in comparison with normotensive, age-matched Wistar Kyoto (WKY) control rats. We demonstrate increased protein levels of NOX1 and NOX2 but not NOX4. In contrast to NOS and XOD inhibitors, the apparently specific NADPH oxidase inhibitor 3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo[4,5-di]pyrimidine (VAS2870)^{20–22} reduced vascular ROS production and regenerated endothelium-dependent relaxation in aortas of aged SHRs.

Methods

For a more detailed Methods description, as well as a chemical and reagent section, please see the online Data Supplement at <http://hyper.ahajournals.org>.

Animals and Tissue Preparation

All of the procedures were performed according to the recommendations of the Federation of European Laboratory Animals Science Association and approved by the local ethics committee. Male SHRs and age-matched male WKY rats were maintained in animal facilities and fed standard rodent chow and water ad libitum. Twelve- to 14-month-old rats were killed by CO₂ inhalation, and thoracic aortas were carefully excised, dissected, and placed in chilled Krebs-Henseleit buffer (pH 7.4) consisting of 118.00 mmol/L of NaCl, 4.70 mmol/L of KCl, 2.50 mmol/L of CaCl₂, 1.18 mmol/L of MgSO₄, 1.18 mmol/L of KH₂PO₄, 24.90 mmol/L of NaHCO₃, and 5.50 mmol/L of glucose. Adherent tissues, as well as contaminating blood, were carefully removed.

Measurement of ROS Generation by Dihydroethidium Fluorescence

Unfixed frozen cross-sections (5 μm) were incubated with dihydroethidium (DHE; 5 μmol/L). Serial sections were treated with tiron (1 mmol/L), PEG-SOD (200 U/mL), DPI (10 μmol/L), VAS2870 (10 μmol/L), apocynin (1 mmol/L), oxypurinol (100 μmol/L), or N^G-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L) for 30 minutes before incubation with DHE. Images were obtained with a DM 6000 B fluorescence microscope (Leica). For a more detailed method, please see the online Data Supplements.

Measurement of NADPH Oxidase Activity

NADPH oxidase activity was measured in the aortic homogenates using a lucigenin assay (5 μmol/L) in the absence and presence of apocynin (100 μmol/L), DPI (10 μmol/L), VAS2870 (10 μmol/L), the NOS inhibitor L-NAME (100 μmol/L), the XOD inhibitor oxypurinol (1 mmol/L), or the superoxide dismutase mimetic tiron (1 mmol/L). For a more detailed method, see the online Data Supplement.

Quantitative Western Blot Analysis

For quantitative analysis of NOX1, NOX2, and NOX4 expression, Western blots were performed on aortic homogenates. For a more detailed method, see the online Data Supplement.

Immunofluorescence Detection

Aortic segments were incubated with the NOX1 or NOX2 antibodies followed by FITC- or Cy3-coupled secondary antibodies. To localize

the NOX signal, double fluorescence labeling was performed with an FITC-conjugated α-smooth muscle actin antibody and/or a RECA-1 antibody. For colocalization experiments with aortic ROS production, DHE staining was performed as described above followed by immunostaining. For a more detailed description, see the online Data Supplement.

Endothelium-Dependent Relaxation

Endothelium-dependent relaxation of rat aorta to acetylcholine (ACh) was performed as described previously.^{23,24} For a detailed description see the online Data Supplement.

Statistical Analysis

All data are presented as mean ± SEM. Statistical comparisons were performed with ANOVA using Bonferroni correction for multiple and Student *t* test for single comparisons. *P* values <0.05 were considered to be significant. All of the statistical tests were carried out using the Prism software package (version 4, GraphPad).

Results

Increased In Situ Aortic ROS Levels in SHRs Are Reversed by NADPH Oxidase Inhibition but not by NOS nor XOD Inhibition

Vascular ROS production was determined in situ in rat aortic sections using the redox-sensitive dye, DHE. Figure 1A shows representative images and Figure 1B the semiquantitative analysis. Aortic sections of SHRs showed a 5.7 ± 1.3-fold (*P* < 0.001) increase in signal intensity compared with age-matched WKY control aortas. Inhibition of the signal by tiron and polyethylene glycol-superoxide dismutase identified superoxide as the main ROS in the aortas of SHRs. Preincubating SHR aortic segments with the NADPH oxidase inhibitors DPI, VAS2870, or apocynin significantly attenuated the signal intensity to levels measured in aortas of WKY rats. In contrast, the NOS inhibitor L-NAME and the XOD inhibitor oxypurinol had no significant effect on aortic ROS production in SHRs. Importantly, none of the inhibitors showed any significant effect on the lower basal superoxide levels in the aortic sections of WKY rats (data not shown).

Elevated NADPH Oxidase Activity in SHR Aortas

Having shown that the enhanced ROS-derived DHE signal in the SHR aortas depends on NADPH oxidases but not on NOS or XOD, we sought to confirm this observation by measuring NADPH oxidase activity in homogenates using lucigenin chemiluminescence (see Figure 2). Similar to the DHE signal, this activity was 1.8 ± 0.1-fold higher in aortic homogenates of SHRs compared with that of WKY rats (*P* < 0.05). Furthermore, this ROS signal was suppressed by tiron, suggesting again a prominent contribution of superoxide. Again, the NADPH oxidase inhibitors DPI, VAS2870, and apocynin, but neither the NOS inhibitor L-NAME nor the XOD inhibitor oxypurinol, effectively blocked this increased signal in the SHR aorta. None of the compounds resulted in a significant reduction of the signal in homogenates of WKY rat aortas (data not shown).

Increased Protein Levels of NOX1 and NOX2, but not NOX4 in SHR Aortas

To investigate whether increased NADPH oxidase activity in SHR aortas can at least in part be explained by increased

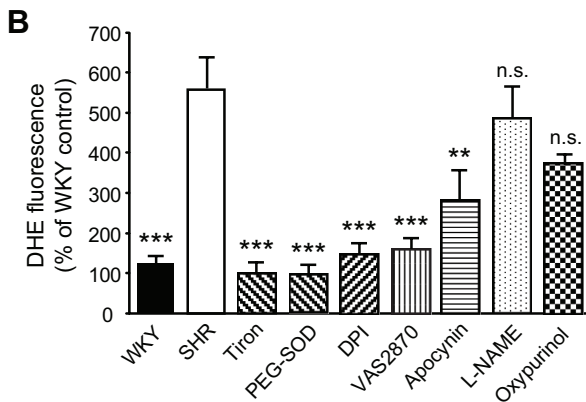
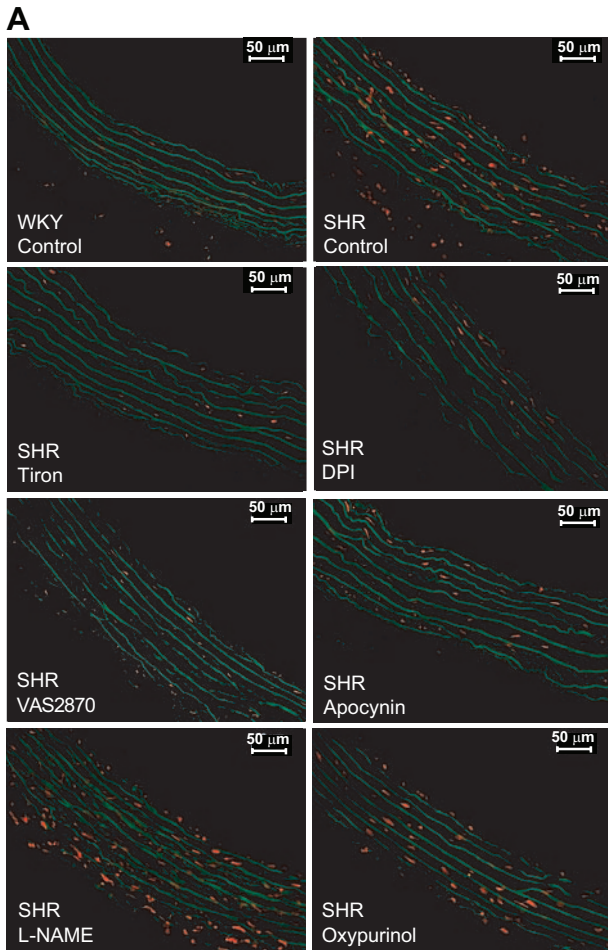


Figure 1. ROS generation in aortic sections measured by DHE fluorescence. Representative images of the assay (A). Images are shown for WKY and SHR aortic sections in the absence of any inhibitor and for SHR aortic sections in the presence of the superoxide scavenger tiron (1 mol/L) or PEG-SOD (200 U/mL); the NADPH oxidase inhibitors DPI (10 μmol/L), VAS2870 (10 μmol/L), or apocynin (1 mmol/L); the NOS inhibitor L-NAME (100 μmol/L); or the XOD inhibitor oxyipurinol (100 μmol/L) for 30 minutes before incubation with DHE (5 μmol/L). Sections were then incubated for 30 minutes with DHE. All of the values were normalized to the WKY control. Semiquantitative analysis of fluorescence intensities (B). n=6 for each group; ***P*<0.01, ****P*<0.001 vs SHR control. n.s. indicates not significant.

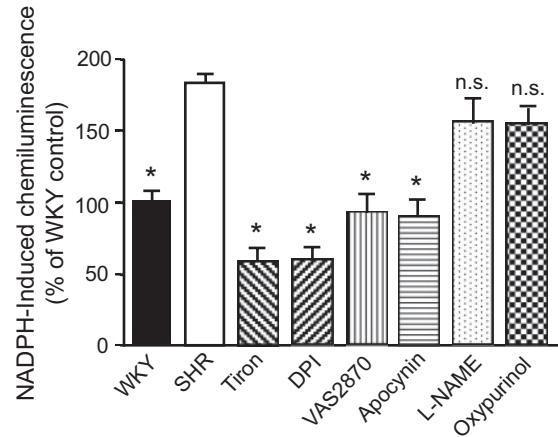


Figure 2. NADPH oxidase activity in aortic homogenates (50 μg per well). The graph shows the quantitative analysis of NADPH-derived lucigenin (5 μmol/L) chemiluminescence measured in WKY aortas and in SHR aortas in the absence or presence of tiron (1 mmol/L), DPI (10 μmol/L), VAS2870 (10 μmol/L), apocynin (1 mmol/L), L-NAME (100 μmol/L), or oxyipurinol (100 μmol/L). After a 20-minute incubation with the inhibitors, NADPH (100 μmol/L) was added and measurements were taken over 10 minutes (intervals of 10 seconds). All of the values were normalized to the WKY control (n≥3 for each group; **P*<0.05 vs SHR control). n.s. indicates not significant.

NOX expression, quantitative Western blot analysis was performed. Figure 3 shows quantitative analysis of Western blots of the NOX1, NOX2, and NOX4 immunoreactive proteins after normalization to β-actin levels. Representative Western blots are shown in Figure S1 (see the online Data Supplement at <http://hyper.ahajournals.org>). NOX1 and NOX2 proteins were significantly upregulated in SHR aortas (3.4x±0.6, *P*<0.05 and 1.6x±0.1, *P*<0.05 compared with WKY rat aortas, respectively), whereas no statistical difference could be detected for the NOX4 immunoreactive band. To elucidate the cell types in which NOX1 and NOX2 inductions occur, we performed immunofluorescence analysis. NOX1 protein was mainly located in the media of the aortic wall with strong signals in the luminal cell layers

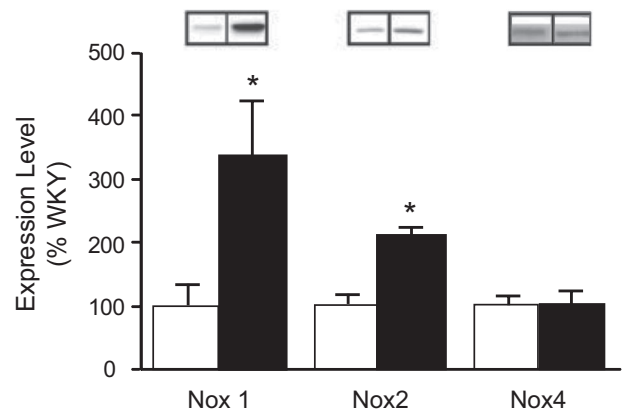


Figure 3. Western blot analysis of NOX isoforms in aortic homogenates of WKY rats and SHRs. Densitometric analysis of NOX1, NOX2, and NOX4 protein expression in aortic homogenates of WKY and SHR aortas. Representative blots are shown in Figure S1. Bar graphs represent mean±SEM from n=5 to 6 animals per group; **P*<0.05.

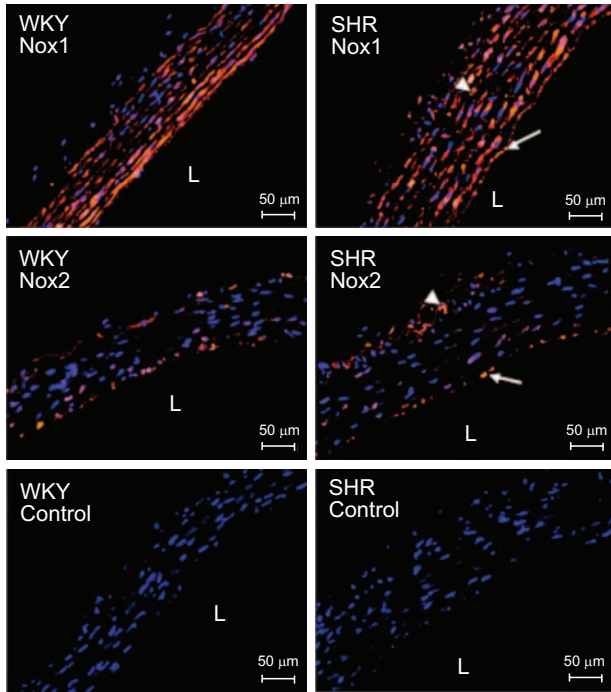


Figure 4. Distribution of NOX1 and NOX2 in WKY rat and SHR aortas. NOX1 immunofluorescence was mainly detected in the media and less pronounced in the intima of WKY rat aortas. In contrast, NOX1 immune signals in SHR aortas were found in the media (arrowhead) and were increased in the intima (arrow). NOX2 protein was detected in the adventitia and the intima of WKY rat aortas. The same expression pattern for NOX2 was found in SHR aortas, where the adventitia (arrowhead) and the intima (arrow) were labeled. Control experiments without the primary antibody are shown for WKY rats and SHRs. Antigen-antibody complexes were visualized with a Cy3-coupled secondary antibody leading to a red-orange fluorescence. Nuclei were counterstained with Hoechst 33342 (blue). L indicates lumen. Images are representative for $n=6$ animals per group.

(Figure 4). Importantly, in SHR aortas, the intima showed an additional strong NOX1 immunofluorescence signal, whereas the corresponding cell layer of WKY rat aortas was negative to only weakly positive. Further cytological characterization (Figures 5 and S3) showed that NOX1 protein was located in the α -smooth muscle actin negative intimal layer of SHR aortas. At a cellular level, NOX1 colocalized with RECA-1 endothelial cell-specific immunofluorescence and the DHE-dependent *in situ* ROS signal. NOX2 was present in the adventitia and the intima of aortic sections, whereas the media appeared to be devoid of NOX2 protein. Both rat strains displayed the same distribution of NOX2, which is in line with the expression of NOX2 in fibroblasts and endothelial cells but not in vascular smooth muscle cells of conduit arteries. These data suggested that NOX1 is induced in endothelial cells, which physiologically express none or much lower levels of this isoform, and that NOX expression and ROS formation were likely to affect endothelium-derived NO.

NADPH Oxidase Inhibition Improves Endothelial Function

We next tested the possibility that the increased NOX1 and NOX2 protein levels affected endothelium-dependent relax-

ation in an NADPH oxidase-dependent manner. As expected, aortic endothelial function, as indicated by the maximal relaxation response to ACh, was significantly impaired in SHRs versus WKY rats (SHR: $56.2 \pm 1.1\%$ versus WKY: $67.9 \pm 2.7\%$; see Figure 6). The NADPH oxidase inhibitors VAS2870 (10 $\mu\text{mol/L}$) and apocynin (100 $\mu\text{mol/L}$) improved the relaxation in aortas of WKY rats ($79.4 \pm 2.2\%$ and $80.2 \pm 2.6\%$, respectively) and even more pronounced in SHR aortas ($80.8 \pm 3.6\%$ and $77.8 \pm 4.9\%$, respectively; see Figure S2) resulting in now similar relaxations of SHR and WKY aortas.

Discussion

We here for the first time provide evidence for a role of induced NADPH oxidases, specifically NOX1 and, to a lesser degree, NOX2, in hypertension-induced endothelial dysfunction using a nontransgenic rodent hypertensive model and investigating both the protein levels of all rodent vascular NOX isoforms and the effects of specific pharmacological inhibition. Increased vascular levels of ROS accompany hypertension. This leads to reduced bioavailability of NO, resulting in a loss of its vasoprotective effects.¹ The contribution of different vascular enzymatic sources of ROS to this is unclear. NADPH oxidase, the only known enzyme family solely dedicated to ROS production, may be a key player. However, definitive evidence for a functional role of NOX isoforms in genetic hypertension is lacking. A key problem in this field is the lack of specific inhibitors and antibodies. Thus, although previous studies have shown increased NOX mRNA expression,^{25,26} as well as increased NOX2 protein levels,²⁷ data on the aortic protein levels of all rodent vascular NOX isoforms in hypertensive animal models are missing. In addition, because of the lack of specific NADPH oxidase inhibitors, reliable pharmacological evidence that NADPH oxidases indeed play a functional role in this setting has not yet been possible.

To investigate this potential role of NADPH oxidases in hypertension, we used aged SHRs, an established model of chronic hypertension. For the first time, we determined protein levels of NOX1, NOX2, and NOX4, and the localization of induced NOX1 and NOX2. We combined this with measurement and localization of ROS production in aortas of aged SHRs, as well as the vasodilator function in these vessels. To provide a definitive link we applied both the commonly used, yet unspecific NADPH oxidase inhibitors DPI and apocynin, as well as the more novel and apparently specific NADPH oxidase inhibitor, VAS2870. We also excluded alternative non-NADPH oxidase sources of ROS.

With respect to the pharmacology of NADPH oxidases, DPI is a generic flavoenzyme inhibitor and, thus, by definition, unspecific.¹⁶ In addition, data obtained with apocynin have to be taken with caution. Indeed, apocynin blocks NADPH oxidase activity by preventing the translocation of p47phox and p67phox to the membrane²⁸ and also inhibits the expression of these subunits in SHRs.²⁹ However, it also directly scavenges hydrogen peroxide²⁸ and has paradoxical stimulatory effects on ROS production in nonphagocytic cells.^{30,31} It also inhibits Rho kinase,³² a mechanism which may account for a large part of its vasodilator actions.³²

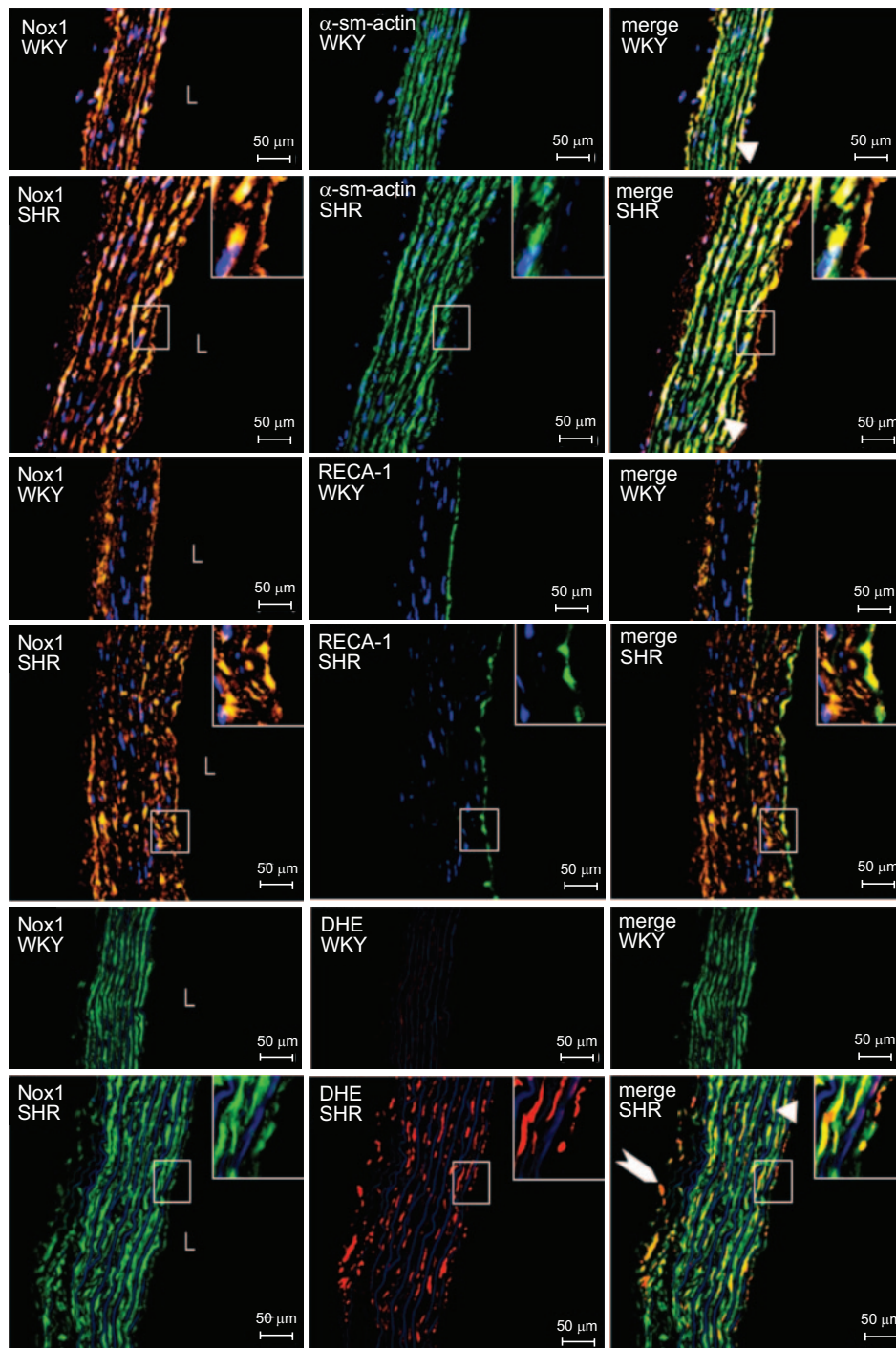


Figure 5. Colocalization of NOX1 protein with α -smooth muscle actin, RECA-1, and oxidized DHE in WKY and SHR aortas. Images on the left show the detection of NOX1 (red-orange fluorescence and green fluorescence, respectively), images in the middle represent α -smooth muscle actin or RECA-1 labeling (green fluorescence) or the red fluorescence of oxidized DHE. Images on the right show the merge of the stainings. NOX1 and α -smooth muscle actin colocalization in WKY aorta are shown in the top row. NOX1 signals matched with α -smooth muscle actin in the medial layer (arrowhead). This colocalization was also found in SHR aorta (arrowhead). In addition, the intima showed a strong NOX1 fluorescence, which did not match with α -smooth muscle actin (inset). NOX1 and RECA-1 colocalization in WKY aorta is shown in the third row. Colocalization experiments in SHR aorta showed that the strong intimal NOX1 signal matches with the RECA-1 fluorescence (inset). Colabeling of NOX1 and oxidized DHE was not visible in WKY aortas (fifth row), because the signal of oxidized DHE was too weak. In contrast, SHRs showed a strong DHE fluorescence, which was colocalized with NOX1 immunosignals. Immunofluorescence and DHE signals matched in the media (arrowhead) and partly in the intima (inset) of SHR aortas. However, there were also ROS-generating cells showing no NOX1 immunofluorescence, mainly in the adventitia (chevron) and to some extent in the intima of SHR aortas. L indicates lumen. Images are representative for $n=6$ animals per group and were consistently reproducible between animals.

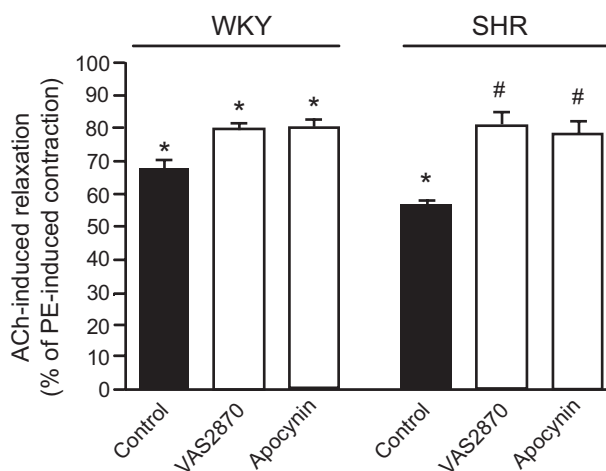


Figure 6. ACh induced vasorelaxation in the absence and presence of NADPH oxidase inhibitors. ACh-induced relaxation was significantly impaired in aortic rings of SHR in comparison with WKY rats (as depicted in the control columns, which represent relaxation in the absence of any compound). The NADPH oxidase inhibitors VAS2870 (10 $\mu\text{mol/L}$) and apocynin (100 $\mu\text{mol/L}$) compensated for the impaired relaxation response to ACh in SHR (see also Figure S2) and also resulted in an enhanced maximal relaxation of WKY aortic rings. Values are mean \pm SEM, $n \geq 5$, * $P < 0.05$ vs WKY control, # $P < 0.05$ vs SHR control.

Therefore, the use of apocynin has potentially lead to an overestimation of the relevance of NADPH oxidases^{32–34} and may need to be revisited or reinterpreted. Nevertheless, we included apocynin in our experiments to allow a direct comparison of its efficacy to that of VAS2870.

VAS2870 has been identified by high-throughput screening for NADPH oxidase inhibitors, where it suppressed the PMA (phorbol 12-myristate 13-acetate)-stimulated oxidative burst in HL60 cells.²⁰ Its potency to inhibit NADPH oxidase activity has been confirmed in cell-free assays; however, there is no evidence as yet for any NOX isoform selectivity. Specificity of VAS2870 for NOX was demonstrated with xanthine/XOD assays, where antioxidative effects and flavoenzyme inhibition were excluded. In addition, VAS2870 inhibits platelet-derived growth factor-stimulated NADPH oxidase activity in rat primary vascular smooth muscle cells²¹ and oxidized low-density lipoprotein-mediated ROS formation in HUVECs,²² indicating that VAS2870 indeed specifically inhibits vascular NADPH oxidases. Although VAS2870 does not inhibit the non-NOX sources, XOD and uncoupled endothelial NOS (unpublished observations), we cannot completely exclude an effect of VAS2870 on ROS production via the mitochondrial electron transport chain or other sources. Indeed, others have demonstrated effects of mitochondria-derived ROS on vascular diseases, including in deoxycorticosterone acetate salt hypertensive rats,³⁵ and in angiotensinergic signaling in neurons, associated with hypertension and heart failure.³⁶ In addition, decreased mitochondrial ROS formation reduces atherogenesis.³⁷ However, the contribution of mitochondria to total ROS production may be overestimated.³⁸ Importantly, NOX4 is present in mitochondria, where it contributes to ROS production.³⁹ Thus, the precise relationship among ROS, mitochondria, and NADPH oxidases as well as their contribution to ROS production in different

vascular disorders warrants further investigations. In our model, the production of ROS is primarily derived from NADPH oxidases. However, we cannot exclude a small amount of ROS released by mitochondria.

XOD and uncoupled NOS did not appear to contribute to total ROS production in the aortas of SHR, because the specific inhibitors, oxypurinol and L-NAME, respectively, had no effects. In contrast, 3 putative NADPH oxidase inhibitors, DPI, apocynin, and VAS2870, reduced in situ ROS formation and inhibited NADPH oxidase activity, supporting the hypothesis that NADPH oxidases are indeed a major source for vascular oxidative stress in SHR. This finding differs from other blood vessels or different models of hypertension, such as deoxycorticosterone acetate salt-treated rats, where increased ROS in mesenteric arteries originate from XOD.³⁵

Importantly, we also validated the functional relevance of our biochemical observations. Impaired endothelium-dependent relaxation in aortas of aged SHR was significantly improved in the presence of VAS2870 (and apocynin), as was, to a smaller extent, the relaxation response in aortas from aged-matched WKY rats, despite the fact that no effect of VAS2870 or apocynin on ROS production could be observed in WKY rat aortas. We hypothesize that DHE staining and the lucigenin assay may not be sensitive enough to pick up small changes in ROS production in the WKY rat aortas and that a bioassay such as endothelium-dependent vasorelaxation responds to more subtle or localized (see below for the discussion of NOX1 in endothelial cells of SHR) differences.

Thus, vascular NADPH oxidases are not only a major source of vascular ROS but also functionally contribute to the impairment of endothelium-dependent relaxation. Despite the fact that ACh-induced relaxation of aorta represents a commonly used model to investigate vascular function and endothelial dysfunction, these data are not necessarily translatable into blood pressure effects, and further in vivo studies are needed once the pharmacokinetics of VAS2870 have been established.

Given that NADPH oxidases play a role in endothelial dysfunction of SHR, it is of interest to better define this target, that is, which catalytic subunit, NOX1, NOX2, or NOX4, is relevant. Morawietz et al²⁶ demonstrated an increase of NOX2 mRNA in the aortas of aged SHR. Others¹⁷ reported increased mRNA levels of NOX1, NOX2, and NOX4 in aortas of SHR (unspecified age) compared with WKY rats. In our study, Western blot analysis revealed an upregulation of NOX1 and NOX2 proteins in SHR aortas, whereas NOX4 levels were unchanged. This was somewhat surprising, because its high vascular mRNA expression levels¹² make NOX4 a prime candidate to mediate vascular oxidative stress. However, NOX4 may have protective, anti-proliferative effects.⁴⁰ Similar patterns of NOX1 and NOX2 upregulation and no effect on NOX4 were observed after angiotensin II infusion^{27,41} and in diabetic versus nondiabetic rats.^{42,43} The high basal expression in endothelial cells⁴⁴ and vascular smooth muscle cells¹¹ suggests that NOX4 plays a more physiological role in maintaining basal levels of ROS,

whereas induced NOX1 and, to a lesser extent, NOX2 mediate pathophysiological stress responses.

With respect to the role of NOX2 in blood pressure regulation, studies in knockout mice have yielded conflicting data.^{45,46} In acute and chronic angiotensin II–induced hypertension, no role for NOX2 was observed. Deletion of NOX2 prevents vascular hypertrophy on acutely (6 days infusion) elevated angiotensin II levels⁴⁵ but not after chronic upregulation of angiotensin II in TTRhRen transgenic/NOX2-deficient mice.⁴⁶

With respect to NOX1, recent publications highlight a possible role for this isoform in angiotensin II–mediated hypertension,^{14,47,48} and our study supports this. In transgenic mice overexpressing NOX1 in smooth muscle cells, oxidative pressor and hypertrophic responses to angiotensin II were increased.⁴⁷ Furthermore, NOX1-deficient mice exhibited decreased pressure responses to angiotensin II infusion associated with increased NO bioavailability.⁴⁸ This was confirmed in a different line of NOX1-deficient mice.¹⁴ Despite discrepancies in hypertrophic responses and basal blood pressures in these 2 lines of NOX1-deficient mice, it can be concluded that NOX1 is involved in endothelial dysfunction and hypertension after angiotensin II infusion in mice. However, information on the possible roles of other NOX isoforms in mice and other species is lacking. Nevertheless, our findings are compatible with increased NADPH oxidase activity in angiotensin II–induced hypertension,⁴ as well as in aging.¹⁸

The cellular localization of NOX1 appears also to be of relevance. Our immunofluorescence data suggest a physiological expression in the media of the aortic wall and a pathological upregulation in the intima of SHR aortas compared with WKY rat aortas. Furthermore, the increased NOX1 expression was linked to enhanced ROS production in SHRs, as indicated by the cellular colocalization of NOX1 immunofluorescence and the DHE ROS stain to endothelial cells. It is noteworthy that we are reporting cellular and not subcellular localization here, which, by definition, nuclear DHE stain would not be able to detect. There has also been conjecture in the literature on the general validity of the DHE stain. It is not suitable (and we certainly do not want to imply this) to localize superoxide formation in tissues.⁴⁹ There is only one superoxide-specific product from DHE, 2-hydroxyethidium, which cannot be specifically assessed using fluorescent microscopy. However, the nature of the NOX products (superoxide or H₂O₂) is unclear, in particular in vivo, and there may be an array of oxidative byproducts (eg, peroxynitrite). Nevertheless, based on the strong inhibition of the DHE stain by the specific NOX inhibitor VAS2870, we believe that this method can be used to localize sources of ROS in general.

Importantly, human hypertension is a complex disease, with different not-yet-understood molecular disease mechanisms. Therefore, the final proof of a role for NOX1 and NOX2 in human hypertension will have to be provided in humans.

In conclusion, induced and ectopic expressions of NOX1 and elevated levels of NOX2, but not NOX4, contribute to increased levels of ROS in the aorta of aged SHRs. Inhibition of NADPH oxidase activity reverses endothelial dysfunction.

Thus, NOX may be a novel pharmacological target to treat hypertension, warranting testing in more models of hypertension and, importantly, also in vivo and in humans.

Perspectives

This study provides further evidence for NADPH oxidase being a target for the treatment of hypertension. Inhibitors such as VAS2870 may be prototype compounds and replace the so far unsuccessful use of antioxidants to overcome oxidative stress. NADPH oxidase inhibition and, thus, preventing oxidative stress may become a powerful new antihypertensive therapeutic strategy.

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Disclosures

H.H.H.W.S. declares that he holds shares in vasopharm GmbH (Würzburg, Germany), which pharmaceutically develops VAS2870. H.H.H.W.S. and K.W. are coinventors on a patent on VAS2870.

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Oxidative Stress and Endothelial Dysfunction in Aortae of Aged Spontaneously Hypertensive Rats by NOX1/2 is Reversed by NADPH Oxidase Inhibition

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Supplementary Methods

Chemicals and Reagents

Phenylephrine hydrochloride, acetylcholine chloride, 4,5-Dihydroxy-1,3-benzene-disulfonic acid (tiron), diphenylene iodonium (DPI), N^G-nitro-L-arginine methyl ester (L-NAME), oxypurinol, dihydroethidium (DHE), N,N-dimethyl-9,9-biacridinium dinitrate (lucigenin) were obtained from Sigma (Deisenhofen, Germany); apocynin from Calbiochem (Darmstadt, Germany); and VAS2870 (3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo[4,5-d]pyrimidine) from vasopharm GmbH (Würzburg, Germany)¹. The NOX1 antibody has been generated by our group² and used previously on Western blots of rat aortic smooth muscle cells³ and in transgenic NOX1 overexpressing mice⁴; the NOX2 antibody is commercially available (rabbit, polyclonal, Upstate Biotechnology, USA); the NOX4 antibody was provided by Ajay Shah's laboratory and has been extensively characterised by Western blot and immunofluorescence⁵.

Details of Measuring ROS Generation by DHE Fluorescence

DHE is able to permeate cells. In the presence of superoxide and other reactive species, it is oxidized to 2-Hydroxyethidium and ethidium, respectively, which are trapped by intercalation with DNA resulting in bright red fluorescence (excitation: 488 nm; emission: 610 nm)⁶. Thoracic aortae of SHR (n=6) and WKY rats (n=6) were embedded in Tissue Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA). Unfixed frozen cross sections (5 µm) were incubated with DHE (5 µmol/L; Molecular Probes) in a light-protected moist chamber at 37°C for 30 min. Serial sections were treated with either tiron (1 mmol/L), DPI (10 µmol/L), VAS2870 (10 µmol/L), apocynin (1 mmol/L), or L-NAME (100 µmol/L) for 30 min before incubation with DHE. Images were obtained with a DM 6000 B fluorescence microscope (Leica, Wetzlar, Germany) using the same imaging settings in each case. For semi-quantitative analysis of ROS production, three to six images were acquired from three sections per aortic ring for each experimental condition. Images were analyzed with the FW4000 software (Leica, Wetzlar, Germany), and changes in total fluorescence intensity were calculated as percent of SHR control.

Details of Measuring NADPH Oxidase Activity

NADPH-induced superoxide production was measured in aortic homogenates using a chemiluminescence based assay containing 5 µM lucigenin, a concentration that does not appear to be involved in redox cycling⁷. Briefly, aortae were snap frozen and minced in liquid nitrogen. Homogenates were collected in 0.5 ml Krebs-HEPES buffer (pH 7.4) consisting of 118 mmol/L NaCl, 4 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.18 mmol/L MgSO₄, 1.18 mmol/L KH₂PO₄, 24.9 mmol/L NaHCO₃, 11 mmol/L glucose, 0.03 mmol/L EDTA, 20 mmol/L HEPES and protease inhibitor cocktail (Roche, Germany). The homogenates were subjected to 1000g (4°C, 10 min) to remove cell debris, and the protein contents of the supernatants were determined by the method described by Lowry⁸. The chemiluminescent probe lucigenin was added resulting in a final concentration of 5 µmol/L. After an incubation period of 20 min the reaction was started by addition of substrate NADPH (100 µmol/L). NADPH oxidase activity in the homogenates was measured in a total volume of 100 µl (50 µg protein/well) using a luminescence plate reader (Fluoroskan Ascent FL, Thermo labsystems, Vantaa, Finland) in the absence and presence of apocynin (100 µmol/L), VAS2870 (10 µmol/L), the NO synthase inhibitor L-NAME (100 µmol/L), the xanthine oxidase inhibitor oxypurinol (1 mmol/L) or the SOD mimetic tiron (1 mmol/L).

Details of Immunofluorescence Detection

Aortic segments were embedded in Tissue Tec O.C.T. Compound (Sakura Finetek, Torrance, USA) and stored at -80°C until use. Tissues were sectioned to an average thickness of $7\ \mu\text{m}$ with a Leica 1900 cryostat (Bensheim, Germany). After fixing with ice-cold acetone (10 min), sections were blocked with 10% normal goat serum (DAKO, Hamburg, Germany) containing 5% bovine serum albumin (Sigma, Deisenhofen, Germany). Incubations with the immuno-purified primary antibodies anti-NOX1 (rabbit, polyclonal, raised against NOX1-aa-545-561: RYSSLDPRKVQFYC) or anti-NOX2 (mouse, polyclonal, BD biosciences, Heidelberg, Germany) were performed at 4°C for 24 h followed by incubation with FITC- (Sigma, Deisenhofen, Germany) or Cy3-coupled (Chemicon, Hofheim, Germany) secondary antibodies at RT for 2 h. Immunoreactivity was visualized by fluorescence microscopy. To localise the NOX signal, double fluorescence labelling was performed with a FITC-conjugated α -smooth muscle actin antibody (Sigma, Deisenhofen, Germany) and/or a RECA-1 antibody (Serotec, Duesseldorf, Germany). For nuclear staining, the Hoechst dye 33342 (Molecular probes, Eugene, USA) was used. Serial sections were treated with the secondary antibodies alone as control for non-specific staining. For co-localization experiments with aortic ROS production, DHE staining was performed as mentioned above, sections were then fixed, then followed by immunostaining as described above.

Details of Quantitative Western Blot Analysis

Western blot analysis of NOX1, NOX2 and NOX4 was performed with aortic homogenates, which were produced by mincing the tissue in liquid nitrogen followed by lysis in a buffer consisting of 20 mmol/L TRIS, 150 mmol/L NaCl, 10 mmol/L Na-pyrophosphate, 1% TritonX100, 2mmol/L orthovanadate, 10 nmol/L ocadeic acid, 230 $\mu\text{mol/L}$ PMSF and a protease inhibitor mix of Roche[®] for 10 min on ice (NOX1 and NOX2) or Laemelli buffer containing 7.5% Glycerin, 3.75% Mercaptoethanol, 2.25% SDS, 0.075mol/L Tris/HCl (pH 8.0) and 0.75mg/mL Bromophenol Blue (NOX4). After determination of protein content⁸, 40 μg of protein from each sample were separated by SDS-PAGE, transferred to nitrocellulose membranes (NOX1 and NOX2) or a PVDF membrane (NOX4), blocked and incubated overnight at 4°C with the NOX1 (1:25000) and NOX4 (1:2000) antibody or a commercial NOX2 antibody (1:2000, rabbit, polyclonal, Upstate biotechnology, USA). After incubation with the HRP-conjugated secondary antibody (DAKO, Hamburg, Germany), immune complexes were detected using the Advanced ECL Immunodetection Kit (Amersham Pharmacia Biotech, Germany). Luminescent signals were scanned and quantified with the Image Station IS440CF (Kodak, USA) or a Bio-Rad Gel-Doc Imaging System (Bio-Rad, Hercules, CA, USA) and normalized to the housekeeping gene β -actin using an antibody from Oncogene (USA).

Details of Measuring Endothelium-Dependent Relaxations

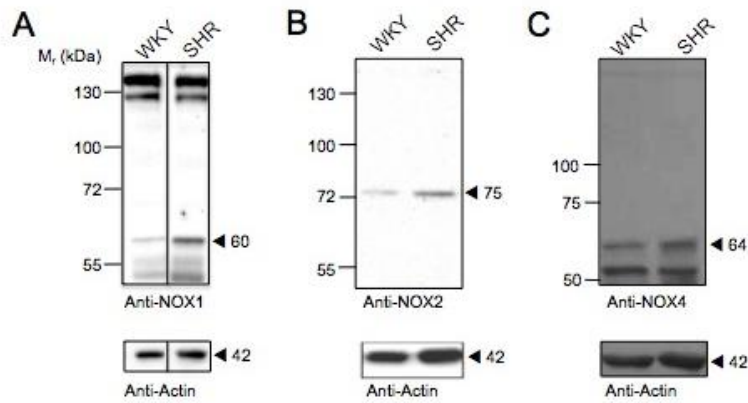
Aortae were cut into rings (2-3 mm) and mounted in organ baths (FMI Föhr Medical Instrument, Seeheim, Germany) containing 5 ml Krebs Henseleit buffer (pH 7.4). The solution was continuously oxygenated with a 95% O_2 – 5% CO_2 mixture and maintained at 37°C . During an equilibration period of 90 min the resting tension was gradually increased to 20 mN and the buffer was exchanged every 15 min. The aortic rings were then challenged with 120 mmol/L potassium chloride to activate the vessels. A second activation was performed after an equilibration period of 30 min with 10 $\mu\text{mol/L}$ phenylephrine (PE). Aortic rings from WKY and SHR were contracted submaximally (60-80%) with phenylephrine (PE) before concentration-response curves for the endothelium-dependent vasodilator acetylcholine (ACh) (1 nmol/L-10 $\mu\text{mol/L}$) were generated in the absence and presence of apocynin (100 $\mu\text{mol/L}$) or VAS2870 (10 $\mu\text{mol/L}$). Dose-response curves were analyzed (see Supplementary Figure 2), and the maximal relaxation value (E_{max}) in percent of the PE induced contraction for each ring was calculated.

Supplementary References

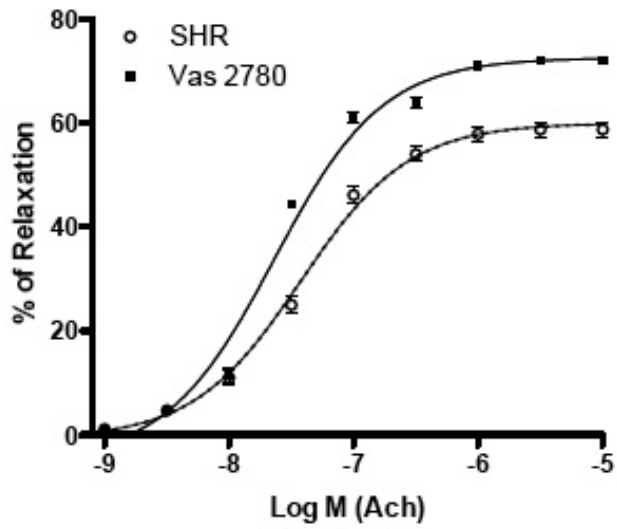
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Supplementary Figures and Legends

S1. NOX1 (A), NOX2 (B), and NOX4 (C) protein expression in aortic homogenates of WKY and SHR aortae. NOX1 was detected at 60 kDa, NOX2 at 75 kDa, and NOX4 at 64 kDa. The bands at approximately 128 kDa and 138 kDa and bands below 60 kDa in (A) are non specific, as is the band below 64 kDa in (C). The NOX1 and NOX4 bands were all blocked with NOX1 or NOX4 antibody specific peptides, respectively. Representative immunoblots of n=5-11.



S2. Concentration response curves of ACh in SHR aortae without (SHR) and after preincubation with VAS287. Preincubation with 10 μ M for 30 min VAS2870 resulted in an increased E_{max} of ACh. For experimental details see Methods section.



S3. Control sections for co-localisation of NOX1 and NOX2 proteins with DHE. Images on the left show the detection of either NOX1 or NOX2 (green fluorescence). Image in the middle show oxidized DHE (red). Images on the right show the merge of the stainings.

