Iranian Journal of Basic Medical Sciences
Vol. 13, No. 2, Spring 2010, 48-56
Received: Oct 24, 2009; Accepted: Feb 1, 2010

IJBMS

Original article

Role of NADPH Oxidase in the Endothelial Dysfunction and Oxidative Stress in Aorta of Aged Spontaneous Hypertensive Rats

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Abstract

Objective(s)
Increased reactive oxygen species (ROS) production is implicated in the pathogenesis of arterial hypertension and the development of endothelial dysfunction. NADPH oxidase type enzyme family has been suggested to form ROS and to interfere with endothelium-dependent relaxation. However, the specific isoform of NADPH oxidases that may predominantly contribute to these events remains to be clarified.
Materials and Methods
Here we investigated the expressional regulation of NADPH oxidase isoforms (NOX_1, NOX_2 and NOX_4) in aorta of aged spontaneously hypertensive rats (SHR) in comparison to age matched Wistar Kyoto rats (WKY). Moreover, we examined the effect of *in vitro* inhibition of NADPH oxidase by apocynin or the novel NADPH oxidase inhibitor, VAS2870 on the vascular reactivity and ROS production.
Results
Our results showed that ROS formation was largely increased in aorta of SHR as measured by dihydroethidine (DHE) fluorescence and inhibited by apocynin or VAS2870. NADPH oxidase activity, measured by lucigenin-enhanced chemiluminescence and of NOX_1 and NOX_2 protein levels were increased in aortic homogenates from SHR compared to WKY. However, NOX_4 protein expression was not significantly changed. Furthermore, the impaired acetylcholine-induced relaxation of SHR aorta was significantly improved in the presence of either apocynin or VAS2870.
Conclusion
Collectively, our data suggest that NADPH oxidases, particularly NOX_1 and NOX_2 are relevant sources of ROS in the aorta of aged SHR thereby cause endothelial dysfunction, and VAS2870 is effective as apocynin in reversing these consequences.

Keywords: Aorta, Endothelial dysfunction, Oxidative stress, Spontaneously hypertensive rats

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Introduction

Inactivation of nitric oxide (NO) by reactive oxygen species (ROS) leads to reduced NO bioavailability, which is the hallmark of endothelial dysfunction, a phenomenon that has been implicated in many cardiovascular diseases (1, 2). Overproduction of superoxide and other ROS, also referred to as oxidative stress, has been demonstrated in humans (3, 4). In addition, increased ROS production has been reported in several animal models of hypertension, such as angiotensin II-induced hypertension (5, 6), aldosterone/salt-induced hypertension (7) or the genetic hypertension of spontaneously hypertensive rats (SHR) (8). In particular in aged SHR, ROS generation and endothelial dysfunction are more pronounced compared to younger rats (9, 10) making this model ideal for investigating the source(s) of oxidative stress.

It is well established that NADPH oxidase is the major source of superoxide anion in the vasculature (11-13). In addition, uncoupled endothelial NO synthase (14) and XOD (15, 16) are suggested to contribute to amplified ROS generation under pathophysiological conditions. However, the exact contribution of each of these sources of superoxide remains elusive. The important contribution of NADPH oxidase to the oxidative stress in hypertension is underlined by the findings that SHR exhibit increased NADPH oxidase activity in resistance as well as conduit arteries (10, 17, 18), and inhibition of these enzyme complexes improves endothelial function (19). The vascular NADPH oxidases appear to be structurally and functionally different from the neutrophil oxidase. Recently, it has been shown that in addition to NOX_2 (gp91phox) two isoforms of the catalytic subunit, namely NOX_1 and NOX_4, are expressed in the vasculature (20, 21). Several studies showed the involvement of p47phox (6) and p22phox (10) in oxidative stress during hypertension, but the role of the individual vascular NOX homologues has not been clarified yet.

Inhibition of NADPH oxidases promises to be of immense therapeutic potential. Compounds used to target NADPH oxidases are either non-specific and/or mechanistically poorly understood (22, 23). The present study aimed to investigate the expressional regulation of NADPH oxidase isoforms (NOX_1, NOX_2 and NOX_4) in aorta of aged SHR in comparison to age matched WKY. Moreover, we examined the effectiveness of *in vitro* inhibition of NADPH oxidase by apocynin or the novel NADPH oxidase inhibitor, 3-benzyl-7-(2-benzoxazolyl) thio-1, 2, 3-triazolo [4,5-d] pyrimidine (VAS2870) on the vascular ROS production and endothelial function in this model of arterial hypertension.

Materials and Methods

Chemicals

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) (all from Sigma, Germany), apocynin (Calbiochem, Germany). VAS2870 (3-benzyl-7-(2-benzoxazolyl) thio-1,2,3-triazolo[4,5-d]pyrimidine) was provided by vasopharm Biotech GmbH, Würzburg, Germany. VAS2870 was characterized by NMR and mass spectrometry (^{1}HNMR (DMSO-D6): δ 5.85 (s, 2H, CH_2), 7.3-7.4 (m, 5H, Ph), 7.5-7.6 (m, 2H, Ar), 7.85 (d, 1H, Ar), 7.95 (d, 1H, Ar), 8.95 (s, 1H, H-5). ms: (+APCl) m/z 361 $[M+H]^+$). VAS2870 has been identified by NADPH oxidase specific high-throughout screening.

Animals and tissue preparation

Aortae were obtained from 12-14 month old male SHR and age-matched male WKY. Animals were maintained in animal facilities and fed standard rodent chow and water *ad libitum*. Rats were killed by CO_2 inhalation, thoracic aortae were carefully excised, dissected and placed in chilled Krebs-Henseleit buffer pH 7.4 consisting of 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 5.5 mM glucose. Adherent tissues as well as contaminating blood were carefully removed. All procedures were performed according to the recommendations of the Federation of European Laboratory Animals Science Association (FELASA).

Measurement of superoxide generation by DHE fluorescence

DHE was able to permeate into cells and, in the presence of superoxide, it is oxidized to a product similar to ethidium bromide (24), which was trapped by intercalation with DNA resulting in bright red fluorescence (excitation: 488 nm; emission: 610 nm). Thoracic aortae of SHR (n= 6) and WKY (n= 6) were embedded in Tissue Tek OCT Compound (Sakura Finetek, Torrance, CA, USA). Unfixed frozen cross sections (5 μm) were incubated with DHE (5 μM; Molecular Probes) in a light-protected moist chamber at 37 °C for 30 min. Serial sections were treated with either tiron (1 mM), DPI (10 μM), VAS2870 (10 μM), apocynin (1 mM) or L-NAME (100 μM) 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 semiquantitative analysis of superoxide production, three to six images were acquired from three sections per aortic ring and sampled for each experimental condition. Images were analyzed with the FW4000 software (Leica, Wetzlar, Germany) and changes in total fluorescence intensity were calculated as percentage of SHR control.

Measurement of NADPH oxidase activity

NADPH-induced $O_2^{\cdot-}$ 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 cycling (25). Briefly, aortic homogenates minced in liquid nitrogen were collected in 0.5 ml Krebs-HEPES buffer consisting of 118 mM NaCl, 4 mM KCl, 2.5 mM $CaCl_2$, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 11 mM glucose, 0.03 mM EDTA, 20 mM HEPES and protease inhibitor cocktail (Roche, Germany), pH 7.4. The homogenate was subjected to 1000×*g* (4°C, 10 min) to remove deprives and then the protein contents of the supernatants were determined by means of Lowry. Following addition of substrate NADPH (100 μM), NADPH oxidase activity in the homogenates in a total volume of 100 μl (50 μg protein/well) was measured, using a luminescence plate reader (Fluoroskan Ascent FL, Thermo labsystems, Vantaa, Finland) in the absence and presence of apocynin (100 μM), VAS2870 (10 μM) the NO synthase inhibitor L-NAME (100 μM), the XOD inhibitor oxypurinol (1 mM) and the SOD mimetic tiron (1 mM), respectively.

Quantitative Western blot analysis

Western blot analysis of NOX_1, NOX_2 and Nox_4 was performed as previously described (21). Briefly, equal amounts of protein from each sample were separated by SDS-PAGE, transferred to nitrocellulose membranes, blocked and incubated overnight at 4 °C with the NOX_1 and NOX_4 antibodies mentioned above or a commercial Nox_2 antibody (rabbit, polyclonal, upstate biotechnology, USA). After incubation with the HRP-conjugated secondary antibody (DAKO, Hamburg, Germany) immune complexes were detected using the Advanced ECL (Enhanced-chemiluminescence Light) Immunodetection Kit (Amersham Pharmacia Biotech, Germany). To correct for differences in protein loading, the membranes were washed and reprobed with 1:1000 dilution monoclonal antibody to human ß-actin (Abcam). Luminescent signals were scanned and quantified with a Kodak Image Station IS440CF and normalized to the housekeeping gene β-actin (Oncogene, USA).

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 (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 for 90 min the resting tension was gradually increased to 20 mN and the buffer was exchanged every 15 min. Then the aortic rings were challenged with 120 mM potassium chloride to contract the vessels. A second contraction was performed after an equilibration period of 30 min with 10 μM 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 nM-10 μM) were generated in the absence and presence of apocynin (100 μM) or VAS2870 (10 μM). Dose-response curves were analyzed, and the maximal relaxation value (E_{max}) in percentage of the PE induced contraction for each ring was calculated.

Statistical analysis

All data are presented as means±SEM. Statistical comparisons were performed with ANOVA, using Bonferroni's test for multiple groups and Student´s t test for simple comparisons. *P*-values< 0.05 were considered significant. All statistical tests were carried out, using the Prism software package, (version 4, GraphPad, USA).

Results

Increased DHE fluorescence in aorta of SHR was reversed by NADPH oxidase inhibition

ROS production within the vascular wall was determined, using the redox sensitive dye DHE. Figure 1A shows representative images of the assay, the semi-quantitative analysis is shown in Figure 1B. Aortic sections of SHR showed a 5.7±1.3 fold (*P*< 0.001) higher intensity than their age matched WKY controls. Inhibition of the DHE fluorescence by tiron and PEG-SOD indicated superoxide as the main ROS responsible for oxidative stress in the aorta of SHR. Preincubation the SHR aortic segments with the NADPH oxidase inhibitors DPI, VAS2870 and apocynin significantly attenuated the signal intensity, whereas the eNOS inhibitor L-NAME and the XOD inhibitor oxypurinol had no significant effect on aortic ROS production in SHR. The mentioned substances did not show any statistical significant effect on superoxide generation in aortic sections of WKY.

Elevated NADPH oxidase activity in aorta SHR

NADPH-derived lucigenin chemiluminescence was 1.8±0.1 (*P*< 0.001) fold higher in aortic homogenates of SHR compared to WKY. The response was suppressed by tiron, DPI, VAS2870 and apocynin but not by the eNOS inhibitor, L-NAME or the XOD inhibitor, oxypurinol (Figure 2). None of the mentioned substances showed any significant suppression of the response in aortic homogenates of WKY.

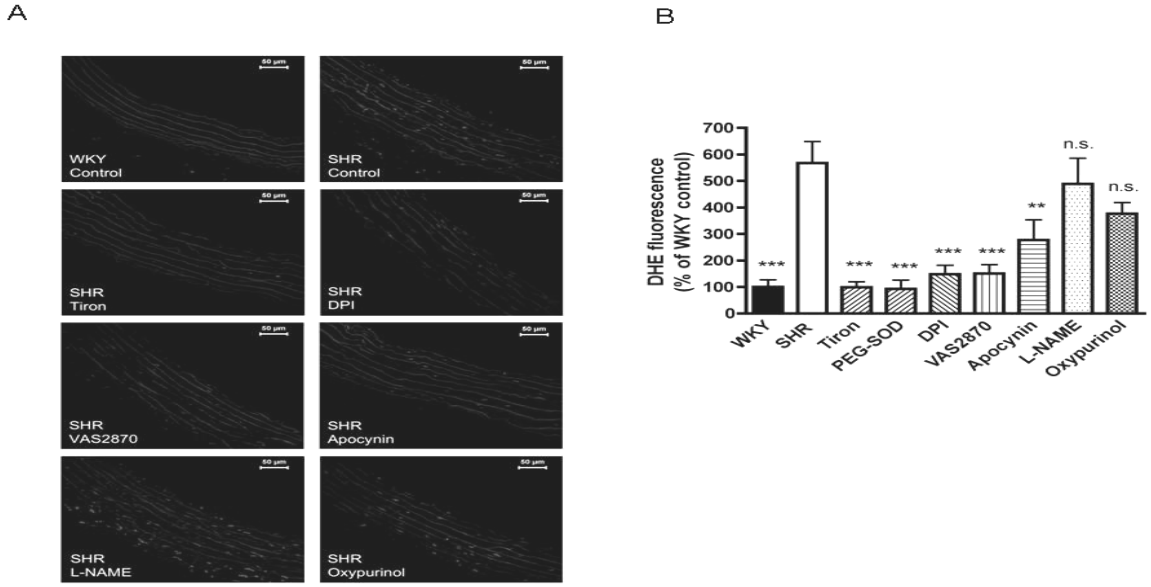

Figure 1. ROS production in aortic sections from WKY and SHR, determined using DHE fluorescence. A. Representative images for effects of several ROS-generating enzyme inhibitors in aortic segments of WKY and SHR. B. Semiquantitative analysis of DHE fluorescent intensities examined in the images in B. Results revealed increased superoxide generation in SHR versus WKY aorta, which could be suppressed by the superoxide scavengers tiron (1 mM) and PEG-SOD (250 U/ml) as well as by the NADPH oxidase inhibitors DPI (10 μM), VAS2870 (10 μM) and apocynin (1 mM). Neither the eNOS inhibitor, L-NAME (100 μM) or the XOD inhibitor, oxypurinol (100 μM) was able to produce a significant effect, n= 6 for each group **P*< 0.01, ****P*< 0.001 in comparison to SHR control.

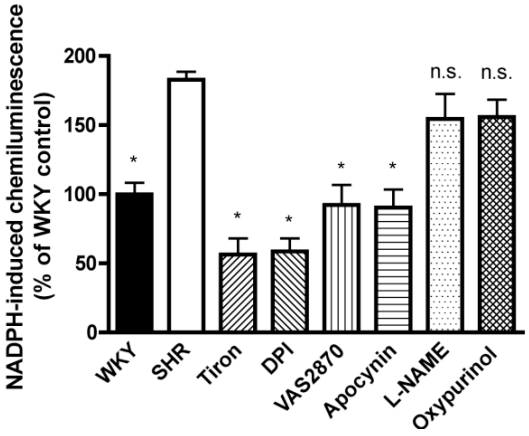

Figure 2. NADPH oxidase activity in aortic homogenates of WKY and SHR. The graph shows the quantitative analysis of NADPH-derived lucigenin chemiluminescence (5 μM) measured in the aortic homogenates of WKY and SHR in the absence or presence of tiron (1 mM), DPI (10 μM), VAS2870 (10 μM), apocynin (1 mM), L-NAME (100 μM), or oxypurinol (100 μM). ($n \geq 3$ for each group, **P*< 0.05 in comparison to SHR control).

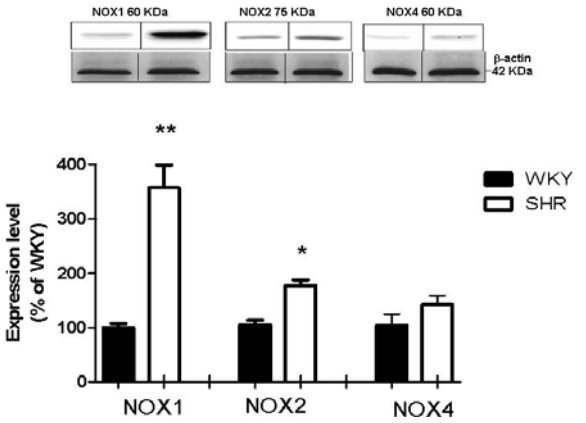

Figure 3. Quantitative western blot analysis of NOX isoforms in aortic homogenates of WKY and SHR. Representative immunoblots and densitometric analysis of NOX_1, NOX_2 and NOX_4 protein expression in aortic homogenates of WKY and SHR, normalized with β-actin. NOX_1 was detected at 60 kDa, NOX_2 at 75 kDa and NOX_4 at 60 kDa. Bar graphs are expressed as % of WKY, values are means±SEM, n=6, ***P*< 0.01 and **P*< 0.05 for NOX_1 and NOX_2, respectively, in SHR compared to WKY.

Increased NOX_1 and NOX_2 protein levels in SHR aorta

Quantitative Western blot analysis was performed to determine if the increased oxidative stress in SHR aorta is accompanied by a change in protein expression of the catalytic NOX isoforms NOX_1, NOX_2 and NOX_4. Figure 3 shows representative Western blots of the NOX proteins and the quantitative analysis after densitometric calculation of the bands and normalization to actin. NOX_1 and NOX_2 proteins were significantly upregulated in the SHR aorta (3.4x±0.6, *P*< 0.01 and 1.6x±0.1, *P*< 0.01 higher expression than the levels measured in WKY aorta, respectively), while no statistical difference could be detected with the NOX_4 antibody.

NADPH oxidase inhibition improved the eendothelial function

Aortic endothelial function as indicated by the maximal relaxation response to acetylcholine was significantly impaired in SHR compared to WKY (SHR: 56.2±1.1% *versus* WKY: 67.9±2.7%). The NADPH oxidase inhibitors VAS2870 (10 μM) and apocynin (100 μM) were able to improve the relaxation in WKY (79.4±2.2% and 80.2±2.6%, respectively) as well as in SHR (80.8±3.6% and 77.8±4.9%, respectively) to a similar relaxation (Figure 4).

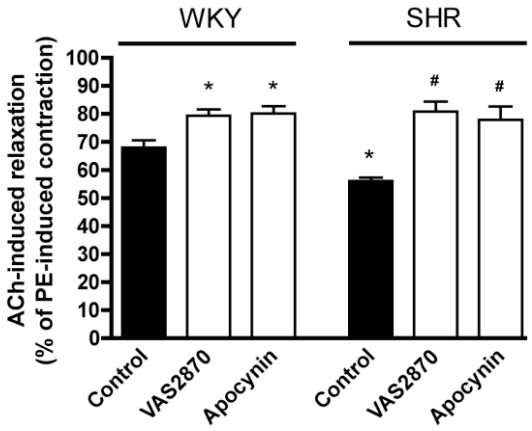

Figure 4. Acetylcholine (ACh) induced vasorelaxation in the absence and presence of NADPH. oxidase inhibitors. ACh-induced relaxation was significantly impaired in aortic rings of SHR in comparison to WKY (as depicted in the control columns, which represent relaxation in the absence of any compound). The NADPH oxidase inhibitors VAS2870 (10 μM) and apocynin (100 μM) improved the impaired relaxation response to ACh in SHR, and also resulted in enhanced maximal relaxation of WKY aortic rings. Values are means±SEM, n= 5, **P*< 0.05 versus WKY control, #*P*< 0.05 versus SHR control.

Discussion

Hypertension is accompanied with oxidative stress in the vessel wall and NADPH oxidases, uncoupled eNOS and XOD are discussed as relevant sources of ROS generation (1). The exact contribution of these sources is still a

matter of debate. Therefore, we tested the involvement of these enzymes in the aortic oxidative stress in an established model of chronic hypertension, i.e. aged SHR. Surprisingly, we could not detect any significant effect of the specific XOD inhibitor oxypurinol and the eNOS inhibitor L-NAME on superoxide production in aorta of 12-14 month old SHR, suggesting a rather secondary role of these enzymes in oxidative stress generation. In contrast, increased NADPH oxidase activity measured as well as the effectiveness of the NADPH oxidase inhibitors DPI, apocynin and the new inhibitor VAS2870 in suppressing the increased vascular ROS production support the hypothesis that NADPH oxidases are the major source for oxidative stress in this genetic model of hypertension. This is in line with several observations, where increased NADPH oxidase activity is shown in angiotensin II mediated hypertension (6, 26, 27) as well as genetic hypertension and aging (9). Furthermore, the NADPH oxidase-mediated oxidative stress is associated with an impaired endothelium-dependent relaxation, which was significantly improved in the presence of NADPH oxidase inhibitors apocynin and the novel substance VAS2870. Thus, vascular NADPH oxidases are indeed the main source of vascular superoxide in aged SHR and significantly contribute to the endothelial dysfunction of these rats.

Vascular NADPH oxidases can consist of the NOX homologues NOX_1, NOX_2 or NOX_4 (28) which all require p22phox to gain functional activity (29). In aged SHR, p22phox expression was upregulated on mRNA (10) as well as on protein level (9). A very recent study demonstrated increased mRNA levels of NOX_1, NOX_2, NOX_4 and p22phox in SHR aorta when compared with WKY, however, there was no information about the age of rats (30). In the present study, western blot analysis revealed an upregulation of NOX_1 and NOX_2 proteins in SHR, while NOX_4 levels were not statistically different. Several studies are in harmony with such an observation: angiotensin II infusion causes a marked upregulation of NOX_2 and NOX_1, whereas NOX_4 exhibits minor changes (31, 32). Considering the high basal expression of NOX_4 in endothelial cells (33) and VSMC (21), it is tempting to suggest a rather physiological role for NOX_4 in maintaining steady state levels of ROS, whereas NOX_1 and NOX_2 preferably mediate stress responses under pathophysiological conditions.

Studies in NOX_2-deficient mice could not clarify the role of this catalytic subunit in hypertension. The effect on basal blood pressure is contradictory. In addition, a role in mediating angiotensin II-induced hypertension could not be demonstrated under acute as well as chronic conditions and deletion of NOX_2 prevented vascular hypertrophy in acute but not in chronic settings of angiotensin II upregulation in mice (34, 35). Recent reports highlighted the role of NOX_1 in angiotensin II mediated hypertension: Dikalova and colleagues could show that in transgenic mice overexpressing NOX_1 in smooth muscle cells oxidative, pressor and hypertrophic responses to angiotensin II were increased (36). Furthermore, NOX_1-deficient mice exhibit decreased pressure responses to angiotensin II infusion due to an increased NO bioavailability (37). Although there were some discrepancies concerning hypertrophic responses and basal blood pressure in NOX_1-deficient mice between the two studies, it can be concluded that NOX_1 is involved in endothelial dysfunction and hypertension after angiotensin II infusion. Based on these findings, the present study indicates that in 12-14 month old SHR the same mechanism of NO- scavenging by NOX_1-derived superoxide is involved in endothelial dysfunction. In contrast, the role of NOX_2 mediated ROS formation remains to be elucidated. In addition, further studies have to clarify the possible causal role of NOX_1 in the development of hypertension in SHR. A putative therapeutic relevance of targeting NOX_1 in vascular diseases was indicated in a recent publication linking the antioxidative effect of atorvastatin to decreased expression levels of NOX_1 and p22phox in SHR (38).

We demonstrated that the NADPH oxidase inhibitor apocynin as well as the novel

inhibitor VAS2870 improve endothelial function and inhibit superoxide generation and NADPH oxidase activity in aged SHR. Interestingly, these compounds also improved the endothelial function in age-matched WKY rats. An explanation for this could be a NOX-mediated, age-related development of endothelial dysfunction also in non-hypertensive rats, but less pronounced than in SHR. Another possibility is that NADPH oxidases contribute to the maintenance of vascular tension via counterbalancing the NO production under physiological conditions. However, these interpretations remain speculative, since no significant suppression of ROS generation in WKY aorta could be detected, using apocynin as well as VAS2870. A reason for this might be the limitation of our assays: Although being specific for superoxide, the DHE fluorescence method is semiquantitative and the lucigenin assay in a 96-well format is probably not as sensitive as other chemiluminescence based methods.

Considering the role of NADPH oxidases in hypertension and other cardiovascular diseases, specific inhibitors promise to be of immense therapeutic relevance (28). Among the identified NADPH oxidases inhibitors, DPI, a flavoenzyme inhibitor, is unspecific and should be rather limited to *in vitro* assays (22). Apocynin was shown to block NADPH oxidase activity by preventing the translocation of p47phox and p67phox to the membrane (39). Because of its low toxicity and oral bioavailability apocynin is frequently used as specific NADPH oxidase inhibitor *in vitro* and *in vivo*. A recent study has shown that chronic apocynin treatment decreased production of ROS and improved nitric oxide bioavailability in SHR (40). In addition, apocynin has even stimulatory effects on ROS production in non phagocytic cells (41, 42), showing that its effects are by far not clarified and that its application should be taken with caution. Considering the quest for specific inhibitors, we investigated the novel NADPH oxidase inhibitor VAS2870 in the present study. VAS2870 has been identified in a cell-based high-throughout screening for NADPH oxidase inhibitors, where it suppressed the PMA stimulated oxidative burst in HL60 cells (43). Its potency to inhibit NADPH oxidase activity has been confirmed by means of a cell-free assay and specificity was demonstrated by means of a xanthine/XOD assay, where antioxidative effects and flavoenzyme inhibition could be excluded. In addition, it has been shown to inhibit β-PDGF-stimulated NADPH oxidase activity in rat primary vascular smooth muscle cells (44) and oxidised low density lipoprotein-mediated ROS formation in HUVEC (45) indicating that VAS2870 is able to inhibit vascular NADPH oxidases.

Conclusion

Our data demonstrate that NADPH oxidases, particularly NOX_1 and NOX_2 are relevant sources of ROS in the aorta of aged SHR thereby causing endothelial dysfunction. Additionally, the novel NADPH oxidase inhibitor, VAS2870 is effective as apocynin in reversing these consequences. However, further studies have to show the mechanism of action of VAS2870 and to establish it as a tool in experimental as well as clinical pharmacology of NADPH oxidases.

Acknowledgment

The authors thank Petra Kronich and Helmut Müller for their excellent technical assistances. This work was funded by SFB547/Teilprojekt C7.

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