

## Increased NADPH oxidase activity mediates spontaneous aortic tone in genetically hypertensive rats

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Received 6 February 2006; received in revised form 22 May 2006; accepted 12 June 2006

Available online 18 June 2006

### Abstract

NADPH oxidase is critically involved in increased blood pressure, vascular hypertrophy, inflammation and endothelial dysfunction in experimental and clinical hypertension. We hypothesized that NADPH oxidase might also play a role in the development of spontaneous aortic tone in spontaneously hypertensive rats (SHR). Wistar Kyoto rats (WKY) were used as normotensive controls. Tone was recorded under isometric conditions. NADPH oxidase activity was measured by both lucigenin luminescence and dihydroethidium fluorescence. p47<sup>phox</sup> protein was localized by immunohistochemistry. SHR (but not WKY rat) aortae showed spontaneous tone in the absence of exogenous vasoconstrictors as evidenced by a stronger relaxant effect of Ca<sup>2+</sup>-free sodium nitroprusside solution. This tone was enhanced in endothelium-denuded arteries and was inhibited by superoxide dismutase, apocynin, diphenylene iodonium and quercetin. Aortic NADPH oxidase activity, measured by both lucigenin luminescence and dihydroethidium fluorescence, was increased in SHR compared with WKY rats. Immunohistochemical analysis revealed a strong increase in p47<sup>phox</sup> expression in the medial layer in SHR. Taken together, the present results indicate that enhanced NADPH oxidase activity and, hence, NADPH driven O<sub>2</sub><sup>-</sup> production, is involved in the spontaneous aortic tone in SHR. This was associated with an increased expression of p47<sup>phox</sup> in the medial layer of the aorta.

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**Keywords:** p47<sup>phox</sup>; SHR (spontaneously hypertensive rat); Spontaneous tone; Superoxide

### 1. Introduction

Spontaneous arterial tone, also referred to as myogenic, intrinsic, basal or resting tone, is a state of partial contraction of isolated arteries when stretched or pressurized without exposure to exogenous vasoconstrictor stimuli and is a typical feature of resistance arteries (Folkow, 1989). Spontaneous tone is an intrinsic property of vascular smooth muscle cells (i.e. myogenic) even though it can be modulated by endothelial factors (Sunano et al., 1996; Veerareddy et al., 2004) and involves depolarization and subsequent opening of voltage-

operated Ca<sup>2+</sup> channels (Laher and Bevan, 1989) as well as changes in Ca<sup>2+</sup> sensitivity (Matchkov et al., 2002).

Conductance arteries from healthy individuals do not show spontaneous tone. However, spontaneous tone can be drastically altered by hypertension and diabetes mellitus (Sunano et al., 1996; Falcone et al., 1993; Frisbee et al., 2002). Thus, several studies have demonstrated enhanced spontaneous tone in aortae from different rodent models of experimental hypertension, including spontaneously hypertensive rats (SHR) (Sunano et al., 1996), deoxycorticosterone salt hypertensive rats (Ghosh et al., 2004) and hypertensive rats with aortic coarctation (Peral de Bruno et al., 1999; Pucci et al., 1994). Although the aorta does not contribute to the control of blood pressure, it is not just a passive vessel, and changes in aortic structure and function are essential features of hypertension (Laurent et al., 2001). The

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development of tone in large arteries from hypertensives is thus expected to impact on systemic hemodynamics.

Increased superoxide anion ( $O_2^-$ ) produced under conditions of high oxidative stress is known to produce significant changes in vascular function (Cai and Harrison, 2000).  $O_2^-$  inactivates the potent vasodilator nitric oxide (NO) impairing endothelium-dependent vasorelaxation (Cai and Harrison, 2000) and may also produce a direct vasoconstrictor effect (Jin et al., 2004). Increased myogenic tone has also been associated recently with an increased oxidative stress (Frisbee et al., 2002). In fact, small mesenteric arteries from superoxide dismutase (SOD) knockout mice (SOD<sup>-/-</sup>) exhibited enhanced myogenic tone (Veerareddy et al., 2004). NADPH oxidase is a multi-subunit enzymatic complex which comprises membrane-bound flavocytochrome b558 (formed by gp91<sup>phox</sup> (nox 2) or gp91<sup>phox</sup> homologues (nox 1 and nox 4) and p22<sup>phox</sup>) and 3 cytoplasmic subunits, p47<sup>phox</sup>, p67<sup>phox</sup>, and p40<sup>phox</sup> (Babior, 1999). This complex is considered to be the most important source of  $O_2^-$  in the vessel wall (Griendling et al., 2000; Souza et al., 2001). Interestingly, excess  $O_2^-$  synthesized by NADPH oxidase is critically involved in increased blood pressure, vascular hypertrophy, inflammation and endothelial dysfunction in experimental and clinical hypertension (Cai and Harrison, 2000; Calo et al., 2005; Li et al., 2004; Viridis et al., 2004; Zalba et al., 2000).

In the present study, we hypothesized that  $O_2^-$  derived from NADPH oxidase might play a role in the development of spontaneous aortic tone in SHR. Wistar Kyoto (WKY) rats were used as normotensive controls.

## 2. Materials and methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by our Institutional Committee for the ethical care of animals. Twenty week old male SHR and WKY rats were obtained from Harlan Laboratories (Barcelona, Spain) and killed with a lethal dose of pentobarbitone. The descending thoracic and the suprarenal segment of the abdominal aortae were dissected.

### 2.1. Contractile tension recording

Thoracic aortic rings (~3 mm) were mounted in organ chambers for isometric force recording (Duarte et al., 1993; Lopez-Lopez et al., 2004). Rings were stretched to 2 g of tension and equilibrated for 90–120 min. During this period, Krebs solution was changed every 30 min and rings were re-stretched as needed to maintain a final tension of 2 g. In some experiments, the endothelium was removed by rubbing the luminal surface with a metal rod (Lopez-Lopez et al., 2004). After equilibration, the bathing Krebs solution was replaced with a  $Ca^{2+}$ -free Krebs solution containing 2 mM EGTA and  $10^{-5}$  M sodium nitroprusside and changes in contractile tone were recorded for 20 min. Parallel experiments in which the solution was changed to a  $Ca^{2+}$ -containing Krebs solution served as time controls. In other sets of experiments, after equilibration, rings were exposed

to apocynin ( $3 \times 10^{-4}$  M), diphenylene iodonium (DPI,  $10^{-5}$  M), quercetin ( $10^{-5}$  M), SOD (100 U/ml), catalase (100 U/ml), the prostanoid TP receptor antagonist 1S-[1 $\alpha$ ,2 $\alpha$ (Z),3 $\alpha$ ,4 $\alpha$ ]-7-[3[[2-[(phenylamino)carbonyl[hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ29548,  $10^{-6}$  M) or vehicle (0.1% dimethylsulfoxide, DMSO) and tension was recorded for 30 min.

### 2.2. NADPH oxidase activity

NADPH enhanced  $O_2^-$  release in intact thoracic aortic rings and in homogenates from cultured aortic smooth muscle cells was quantified by lucigenin-enhanced chemiluminescence as previously described (Lopez-Lopez et al., 2004). Cell culture was obtained from aortic explants. Briefly, aortas were cleared of connective tissue, cut into pieces and cultured in Dulbecco's modified Earle's medium (DMEM) containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml) and non-essential amino acids. At subconfluence, cells (passages 5–10) were placed in serum-free medium overnight, washed in ice-cold PBS, and incubated in lysis buffer (20 mmol/L monobasic potassium phosphate, 1 mmol/L EGTA, 10  $\mu$ g/ml aprotinin, 0.5  $\mu$ g/ml leupeptin, 0.75  $\mu$ g/ml pepstatin and 0.5 mmol/L phenylmethylsulfonyl fluoride) for 10–20 min at 4 °C. Protein content was measured by using the bicinchoninic acid (BCA) method. NADPH (100  $\mu$ mol/L) was added to the buffer containing the aortic rings or to the aortic homogenate suspension (30  $\mu$ g protein in 500  $\mu$ L) and lucigenin was injected automatically, at 5  $\mu$ M to avoid known artifacts when used at higher concentrations (Janiszewski et al., 2002). NADPH oxidase activity was calculated by subtracting the basal values from those in the presence of NADPH. In order to analyze the effects of quercetin on NADPH oxidase-derived  $O_2^-$ , NADPH-stimulated lucigenin luminescence was analyzed in aortic rings from Wistar rats.

### 2.3. In situ detection of vascular $O_2^-$ production

Dihydroethidium (DHE) enters the cells and is oxidized by  $O_2^-$  to yield ethidium, which binds to DNA to produce bright red fluorescence. Unfixed abdominal aortic rings were cryopreserved (phosphate buffer solution 0.1 M [PBS], plus 30% sucrose for 1–2 h), placed in OCT, frozen ( $-80$  °C), and 10  $\mu$ m cross sections were obtained in a cryostat (Microm International Model HM500 OM). Sections were incubated in a humidified chamber for 30 min in Hepes buffered solution (in mM: NaCl 130, KCl 5,  $MgCl_2$  1.2, glucose 10, and HEPES 10, pH 7.3 with NaOH) at 37 °C, and then incubated for 30 min in Hepes solution containing DHE ( $10^{-5}$  M) in the dark in the absence or presence of DPI ( $10^{-5}$  M) and/or NADPH ( $10^{-4}$  M), the preferred NADPH oxidase substrate in vascular smooth muscle. Then preparations were counterstained with the nuclear stain 4,6-diamidin-2-phenylindol dichlorohydrate (DAPI,  $3 \times 10^{-7}$  M) for 5 min at 37 °C and mounted with a coverslip. Four sections of each preparation were examined on a fluorescence microscope (Leica DM IRB, Wetzlar, Germany) using a 40 $\times$  objective with the following pairs of excitation/emission filters: 340–380 nm/425 nm long pass to

visualize DAPI blue fluorescence, BP 450–490 nm/515 nm long pass to visualize the green autofluorescence of elastin and 545–30 nm/610–75 nm long pass to visualize ethidium red fluorescence. Sections were photographed with a Leica DC300F color digital camera and images were saved for off-line analysis. Microscope and camera settings were kept constant for all preparations. Ethidium and DAPI fluorescence were quantified using ImageJ (ver 1.32j, NIH, <http://rsb.info.nih/ij/>).  $O_2^-$  production was estimated from the ratio of ethidium/DAPI fluorescence.

#### 2.4. Immunohistochemistry

Sections (10  $\mu$ m) of abdominal aorta were prepared as described above for DHE fluorescence, fixed with paraformaldehyde 4%, blocked with 0.1 M PBS+0.3% Tween 20+5% bovine serum albumin for 1 h at 37 °C in a humidified chamber, incubated with rabbit anti-p47<sup>phox</sup> polyclonal antibodies (1:50 dilution, SantaCruz Biotechnology), then gently washed in 0.1 M PBS+0.3% Tween 20 and incubated with secondary Cy3 conjugated goat anti-rabbit antibody (1:200, Jackson Immuno-research Laboratories, Cambridgeshire, UK), counterstained with DAPI and examined, photographed and quantified as described above for DHE fluorescence.

#### 2.5. Drugs

All drugs and reagents were from Sigma, except DAPI from Calbiochem. DPI and quercetin were initially dissolved in DMSO and all other drugs in distilled water.

#### 2.6. Statistical analysis

Results are expressed as the mean $\pm$ S.E.M. and *n* reflects the number of experiments in tissues from different animals. Statistically significant differences between experimental groups were calculated by Student's *t* test for paired or unpaired observations. For multiple comparisons a linear univariant analysis (multiple way ANOVA, using SPSS software) was carried out and when differences were significant, pairwise comparisons were carried out.  $P < 0.05$  was considered statistically significant.

### 3. Results

#### 3.1. Aortic rings from SHR show spontaneous tone

During equilibration, aortae were stretched as needed to maintain a final tension of 2 g. The relaxant response induced by a  $Ca^{2+}$ -free Krebs containing sodium nitroprusside ( $10^{-5}$  M) allowed us to estimate the previous active spontaneous tone. Changing the medium to fresh  $Ca^{2+}$ -containing Krebs (time control) had no significant effect on tone while the  $Ca^{2+}$ -free nitroprusside solution induced a relaxant response in intact aortic rings from SHR but not from WKY rats (Fig. 1). This relaxant response was greater in endothelium-denuded compared with endothelium-intact SHR arteries indicating that SHR aortae show endothelium-independent tone in the absence of exogenous vasoconstrictors. This intrinsic tone in endothelium-

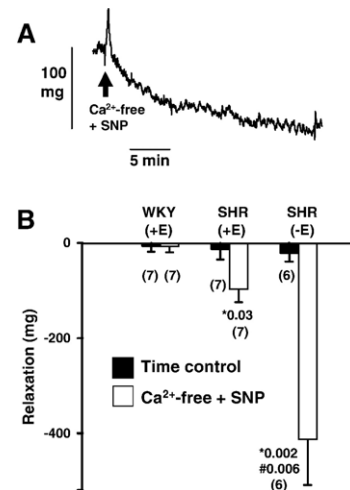


Fig. 1. Spontaneous tone in aortic rings from SHR and endothelial dependence. Panel A shows an original recording in endothelium-intact aortic ring from SHR. Replacement of the Krebs bathing solution with a  $Ca^{2+}$ -free (2 mM EGTA) Krebs containing sodium nitroprusside (SNP,  $10^{-5}$  M) is indicated by the arrow. Panel B shows the average changes in tone in SHR and WKY rats in endothelium-intact (+E) or endothelium-denuded (-E) aorta. Time controls were performed by changing the medium with fresh  $Ca^{2+}$ -containing Krebs. Results are expressed as the means $\pm$ S.E.M., the number of experiments is shown in parenthesis, measurements were taken 20 min after bath solution replacement. \**P* vs. time-control and #*P* vs. endothelium-intact (unpaired Student's *t* test).

intact rings ( $77 \pm 9$  mg) represented  $\sim 10\%$  of the maximal constrictor response to noradrenaline ( $10^{-6}$  M) obtained in parallel experimental rings.

#### 3.2. SOD, quercetin and the NADPH oxidase inhibitors apocynin and DPI inhibit the spontaneous tone in SHR

Inhibition of NADPH oxidase with apocynin ( $3 \times 10^{-4}$  M) or DPI ( $10^{-5}$  M) or addition of quercetin ( $10^{-5}$  M) or SOD (100 U/ml) induced a relaxant response in endothelium-intact SHR (Fig. 2A). These effects were of a similar magnitude to those of  $Ca^{2+}$ -free nitroprusside solution ( $P < 0.05$  vs. data in Fig. 1) indicating that NADPH oxidase inhibitors produced maximal or near-maximal inhibition of spontaneous tone. Fig. 2B shows that, in SHR, the effects of quercetin were increased in endothelium-denuded arteries or in arteries treated with the NO synthase inhibitor L-NAME ( $10^{-4}$  M, Fig. 2B). Furthermore, in arteries relaxed by apocynin ( $-108 \pm 36$  mg,  $n = 5$ ), which inhibits the endogenous source of  $O_2^-$ , addition of an external source of  $O_2^-$  ( $10^{-4}$  M hypoxanthine plus 16  $\mu$ M/ml xanthine oxidase) was able to restore a contractile tone which reached values above the initial ones ( $537 \pm 121$  mg at steady state after 30 min).

Addition of catalase (100 U/ml) induced a contraction in both endothelium-intact and denuded vessels from SHR (Fig. 2C). However, in arteries which were previously relaxed by apocynin ( $-104 \pm 41$  mg,  $n = 5$ ), catalase failed to induce a significant contractile response. The thromboxane  $A_2$ /prostaglandin endoperoxide receptor (prostanoid TP receptor) antagonist SQ29548 ( $10^{-6}$  M) had no significant effect ( $-24 \pm 22$  mg,  $n = 5$ ) in endothelium-denuded vessels.

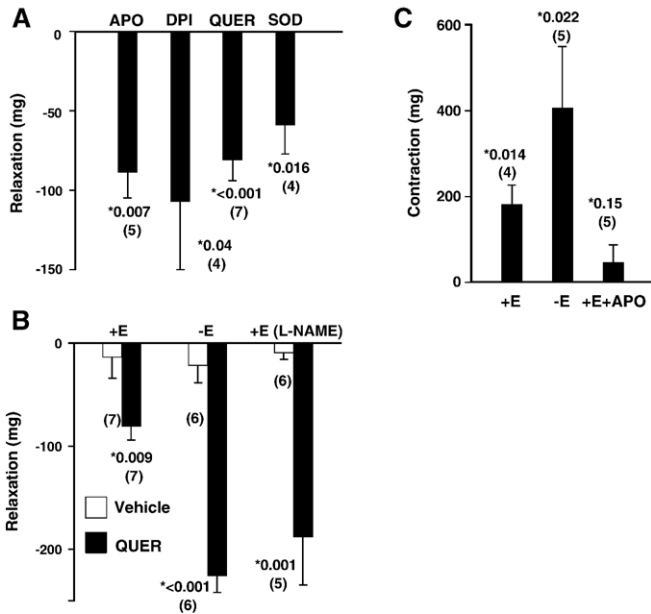


Fig. 2. Effects of apocynin (APO,  $3 \times 10^{-4}$  M), DPI ( $10^{-5}$  M), quercetin (QUER,  $10^{-5}$  M), SOD (100 U/ml) and catalase (100 U/ml) on spontaneous tone. A) Effects of apocynin, DPI, quercetin and SOD in endothelium-intact aortic rings from SHR. \**P* vs. baseline (paired Student's *t* test). B) Effects of quercetin and vehicle (0.1% DMSO) in endothelium-intact (+E), endothelium-denuded (-E) and endothelium-intact L-NAME-treated ( $10^{-4}$  M) rings from SHR. \**P* vs. vehicle (unpaired Student's *t* test). C) Contractile effects of catalase in SHR aorta +E, -E and +E after treatment with apocynin. \**P* vs. baseline (paired Student's *t* test). Results are expressed as the means  $\pm$  S.E.M., the number of experiments is shown in parenthesis, measurements were taken 20 min after drug addition.

### 3.3. NADPH oxidase activity is increased in aortic rings from SHR

NADPH stimulated production of  $O_2^-$ , measured by lucigenin luminescence in endothelium-intact aortic rings was significantly enhanced in SHR compared to WKY rats (Fig. 3A). NADPH stimulated production of  $O_2^-$  was significantly inhibited by  $10^{-5}$  M quercetin (by  $51 \pm 7\%$ ,  $n=5$ ) and  $10^{-5}$  M DPI (by  $95 \pm 1\%$ ,  $n=5$ ) in aortic rings. NADPH oxidase activity was also measured in homogenates from vascular smooth muscle cells in culture obtained from WKY rat and SHR aorta. Again, the activity was significantly higher in homogenates from SHR than those from WKY (Fig. 3B).

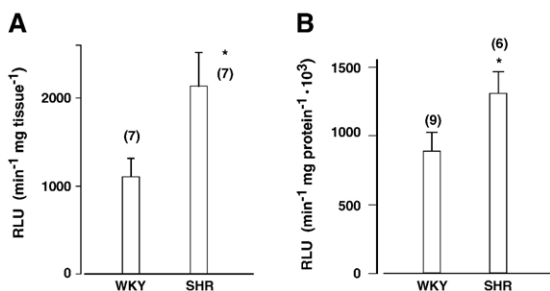


Fig. 3. NADPH oxidase activity measured by lucigenin luminescence in (A) aortic rings from SHR and WKY rats (B) homogenates from cultured aortic smooth muscle cells from SHR and WKY rats. Results are the means  $\pm$  S.E.M., the number of experiments is shown in parenthesis, \**P* vs. WKY rats (unpaired Student's *t* test).

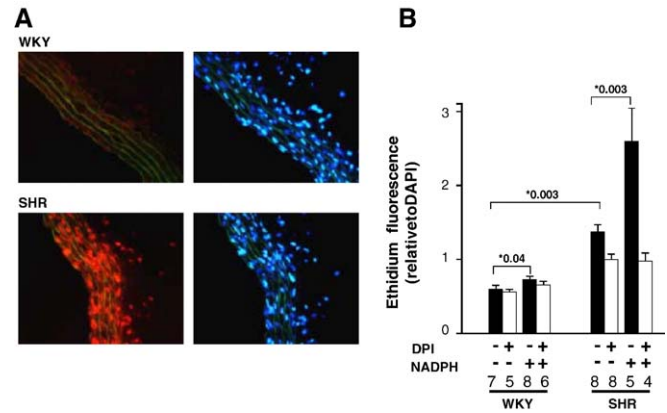


Fig. 4. *In situ* localization of  $O_2^-$  production in aortic rings. A. Left pictures show arteries incubated in the presence of DHE which produces a red fluorescence when oxidized to ethidium by  $O_2^-$  merged with green elastin autofluorescence. Right pictures show blue fluorescence of the nuclear stain DAPI merged with green elastin autofluorescence. B. Averaged values ( $\pm$  S.E.M.) of red ethidium fluorescence normalized to blue DAPI in arteries incubated in the absence (as in panel A) or presence of DPI ( $10^{-5}$  M), NADPH ( $10^{-4}$  M) or both. Numbers at the bottom indicate the number of sections analyzed. Overall significance was  $<0.001$  in univariate analysis (ANOVA) using as fixed factors strain, NADPH and DPI. All individual fixed factors were significant ( $P=0.003$ ,  $0.046$  and  $0.007$ , respectively) as well as the interaction NADPH  $\times$  DPI ( $P=0.005$ ). In DPI untreated sections, the interaction strain  $\times$  NADPH was also significant ( $P=0.004$ ). \**P*  $< 0.05$  and \*\**P*  $< 0.01$ .

### 3.4. In situ detection of $O_2^-$ production in SHR and WKY rats

To further characterize and localize  $O_2^-$  production within the vascular wall, ethidium red fluorescence was analyzed in sections of aorta incubated with DHE. Positive red nuclei were observed in adventitial, medial and endothelial cells (Fig. 4A). Red fluorescence was quantified and the data were normalized by the blue fluorescence of the nuclear stain DAPI (Fig. 4B). Rings from SHR showed markedly increased staining in adventitial, medial and endothelial cells as compared with those from WKY rats. Addition of NADPH ( $10^{-4}$  M) significantly increased the fluorescence in both strains, although this increase was much weaker in WKY rats. DPI ( $10^{-5}$  M) prevented NADPH-induced increased fluorescence in both strains.

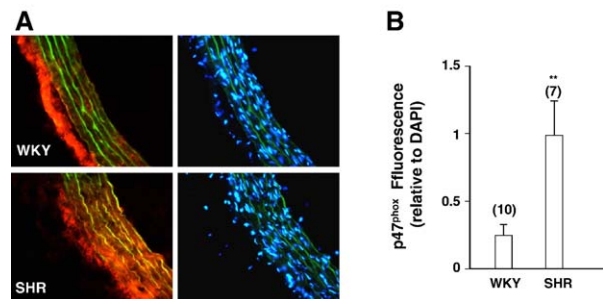


Fig. 5. Localization of p47<sup>phox</sup> protein. (A) Immunohistochemistry of p47<sup>phox</sup> in aortic sections from SHR and WKY rats. Left pictures show p47<sup>phox</sup> protein (red fluorescence) merged with green elastin autofluorescence. Right pictures show blue fluorescence of the nuclear stain DAPI merged with green elastin autofluorescence. (B) Averaged values ( $\pm$  S.E.M.), of red fluorescence normalized to blue DAPI. The number of experiments is shown in parenthesis, \**P*  $< 0.05$  vs. WKY rats (unpaired Student's *t* test).

### 3.5. p47<sup>phox</sup> expression is increased in the medial layer in SHR

p47<sup>phox</sup> is a cytosolic sub-unit of NADPH oxidase. When phosphorylated, it translocates to the membrane and interacts with flavocytochrome b558 to yield the active form of membrane NADPH oxidase. We have recently shown that the expression of p47<sup>phox</sup> in both the cytosolic and the membrane fractions was significantly increased in SHR compared with WKY rats as measured by Western blot (Sanchez et al., 2006). Herein, we analyzed its localization in aortic cross sections. p47<sup>phox</sup> was localized by immunohistochemistry mainly to the adventitial layer and weaker staining was observed in the medial and intimal layers. A strong increase in medial staining was observed in SHR as compared to WKY rats (Fig. 5).

## 4. Discussion

The main novel finding of the present study is that NADPH oxidase activity plays a key role in the development of spontaneous aortic tone in SHR. This is based on the following evidences: a) tone was abolished by inhibitors of NADPH oxidase, b) the presence of spontaneous tone in SHR was associated with increased basal and/or NADPH stimulated O<sub>2</sub><sup>-</sup> production and with increased medial expression of the NADPH oxidase subunit p47<sup>phox</sup>.

To analyze the spontaneous tone we measured the relaxation induced by a Ca<sup>2+</sup>-free solution containing sodium nitroprusside to inhibit both Ca<sup>2+</sup> entry through voltage-operated Ca<sup>2+</sup> channels (Laher and Bevan, 1989) and non-electromechanical coupling mechanisms (Matchkov et al., 2002). Consistent with previous reports in SHR (Sunano et al., 1996), endothelium is not essential for spontaneous tone. In fact, spontaneous tone was greater in endothelium-denuded arteries, indicating that endothelial-derived vasodilators limit spontaneous tone development.

Recent evidence suggests that myogenic tone in resistance arteries is enhanced in the presence of oxidative stress (Matchkov et al., 2002; Veerareddy et al., 2004). Because hypertension is associated with increased oxidative stress, we hypothesized that spontaneous tone in the aorta from SHR could be related to enhanced O<sub>2</sub><sup>-</sup> production. The O<sub>2</sub><sup>-</sup> scavengers SOD and quercetin (Duarte et al., 2001; Lopez-Lopez et al., 2004) inhibited spontaneous tone, suggesting that O<sub>2</sub><sup>-</sup> is involved in the genesis of this spontaneous tone. Furthermore, because SOD is not very membrane permeable, part of the spontaneous tone must have been due to extracellular O<sub>2</sub><sup>-</sup>. This free radical may induce a contractile response by activating RhoA/Rho kinase pathway, which is a known mechanism for inducing Ca<sup>2+</sup>-sensitization (Jin et al., 2004), and for increasing Ca<sup>2+</sup> entry (Tabet et al., 2004). The endothelium was not required for the relaxant effect of quercetin. In fact, quercetin-induced relaxation was increased in endothelium-denuded arteries (i.e. an effect similar to that induced by the Ca<sup>2+</sup>-free plus nitroprusside solution) and in L-NAME-treated arteries.

As the major source of O<sub>2</sub><sup>-</sup> in the vessel wall of hypertensive animals is NADPH oxidase (Cai and Harrison, 2000), we tested the effects of DPI, an inhibitor of flavin containing enzymes including NADPH oxidase, and apocynin, a specific inhibitor of

NADPH oxidase. The inhibitory effect of apocynin and DPI, together with the inhibitory effects of SOD and quercetin, strongly suggest that spontaneous tone is due to increased NADPH oxidase-derived O<sub>2</sub><sup>-</sup>. Furthermore, in aorta treated with apocynin, i.e. under conditions in which NADPH oxidase-derived O<sub>2</sub><sup>-</sup> production and spontaneous tone are inhibited, exogenous addition of O<sub>2</sub><sup>-</sup> was able to restore tone.

NADPH-induced O<sub>2</sub><sup>-</sup> production, as measured by lucigenin luminescence, was increased in aortic rings from SHR compared with those from WKY rats. A similar difference was observed when analyzing NADPH oxidase activity in homogenates from vascular smooth cells in culture. Likewise, NADPH produced a greater increase in O<sub>2</sub><sup>-</sup> production measured by ethidium fluorescence in aortae from SHR than in those from WKY rats and this increase was abolished by DPI in both strains, indicating that NADPH oxidase activity is enhanced in SHR. Stretching or pressurization is known to trigger the release of O<sub>2</sub><sup>-</sup> in vascular tissues via activation of NADPH oxidase (Ghosh et al., 2004; Ungvari et al., 2003). O<sub>2</sub><sup>-</sup> production measurements in the present study were performed in un-stretched arteries and are probably an underestimation of the values in stretched arteries in the tissue bath. In fact, DPI produced a weak inhibitory effect on O<sub>2</sub><sup>-</sup> production in the absence of exogenous NADPH in SHR.

Several recent studies have suggested a pivotal role of the p47<sup>phox</sup> subunit of NADPH oxidase in the O<sub>2</sub><sup>-</sup> production and blood pressure response to angiotensin II (Lavigne et al., 2001; Chabrashvili et al., 2002; Calo et al., 2005). In SHR aortae we found higher protein levels of p47<sup>phox</sup> than in WKY rat aortae (Sanchez et al., 2006). Increased p47<sup>phox</sup> expression has also been found in SHR kidney (Chabrashvili et al., 2002). Immunohistochemical staining indicated that NADPH oxidase proteins p22<sup>phox</sup>, p47<sup>phox</sup>, and p67<sup>phox</sup> were almost exclusively localized in the adventitia of the Wistar rat aorta (Wang et al., 1998). Likewise, we found that p47<sup>phox</sup> was mainly localized in the adventitial layer of WKY rat aorta. However, the aortae from SHR also showed strong p47<sup>phox</sup> expression in the medial layer, which is consistent with increased production of O<sub>2</sub><sup>-</sup> in the vascular smooth muscle cells from the media as measured by ethidium fluorescence and with the increase p47<sup>phox</sup> expression induced by angiotensin II in vascular smooth muscle cells in culture (Touyz et al., 2002). Therefore, the increased p47<sup>phox</sup> protein expression in the media, together with increased expression of other NADPH subunits such as the p22<sup>phox</sup> (Zalba et al., 2000), explains the increased O<sub>2</sub><sup>-</sup> production stimulated by NADPH and the inhibitory effects of NADPH inhibitors on spontaneous tone in SHR aortae. In the SHR, p47<sup>phox</sup> is downregulated by angiotensin II receptor antagonists (Izuhara et al., 2005). Therefore, we speculate that spontaneous tone in the SHR aorta is due to a chronic systemic elevation of the renin angiotensin system leading to the overexpression of p47<sup>phox</sup> and other NADPH oxidase subunits.

O<sub>2</sub><sup>-</sup> is rapidly dismutated by the activity of SOD into H<sub>2</sub>O<sub>2</sub> which may produce both contractile or vasodilator effects (Lucchesi et al., 2005). The possibility that H<sub>2</sub>O<sub>2</sub> might act as a downstream effector of O<sub>2</sub><sup>-</sup> to induce spontaneous tone was analyzed using catalase, a scavenger of H<sub>2</sub>O<sub>2</sub>. Catalase induced a

further contractile response, an effect consistent with that observed in deoxycorticosterone acetate salt hypertensive rats (Ghosh et al., 2004). These results suggest that endogenous  $H_2O_2$  is not responsible for the spontaneous tone, but rather that it acts as a vasodilator and limits spontaneous tone development. This suggestion is consistent with the increased myogenic tone observed in  $SOD^{-/-}$  mice (Veerareddy et al., 2004) which, in addition to increased  $O_2^-$ , would be expected to show reduced  $H_2O_2$  levels. Furthermore, the contractile effect of catalase was prevented by pretreatment with apocynin, indicating that the endogenous source of  $H_2O_2$  is also NADPH oxidase. It should be noted that in some vessels such as rat cerebral arteries the vasodilator effect of NADPH oxidase-derived  $H_2O_2$  is predominant and, therefore, cerebral SHR arteries show greater NADPH-induced relaxant responses than WKY rats (Paravicini et al., 2004). A possible role of COX-derived eicosanoids activating the prostanoid TP receptors (Hibino et al., 1999) on the spontaneous tone in SHR was ruled out since the prostanoid TP receptor antagonist SQ29548 had no effect on tone.

In summary, recent evidence suggests that increased oxidative stress derived from membrane NADPH oxidase plays a pathophysiological role in cardiovascular disease, being involved in the increased blood pressure, vascular hypertrophy, inflammation and endothelial dysfunction associated with hypertension. Our present study indicates that an increased expression of p47<sup>phox</sup> in the medial layer of the aorta and enhanced NADPH oxidase activity are also involved in the spontaneous aortic tone in SHR.

## Acknowledgements

This work was supported by grants from the Comisión Interministerial de Ciencia y Tecnología (AGL2004-06685/ALI and SAF 2005/03770) and from the Comunidad Autónoma de Madrid (GR/SAL/0594/2004). F. Lodi, A. Cogolludo and L. Moreno are supported by CSIC (I3P pre-doctoral grant), RECAVA (Red Temática de Investigación Cardiovascular) and Ministerio de Educación y ciencia (FPU), respectively. A. Coviello was supported by a grant from the Ministerio de Educación, Cultura y Deporte of Spain.

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