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RESEARCH PAPER

Role of NADPH oxidase and iNOS in vasoconstrictor responses of vessels from hypertensive and normotensive rats

Y Álvarez[1], AM Briones[1], R Hernanz[2], JV Pérez-Girón[2], MJ Alonso[1,2] and M Salaices[1]

[1]*Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, Madrid, Spain and* [2]*Departamento de Ciencias de la Salud III, Facultad de Ciencias de la Salud, Universidad Rey Juan Carlos, Madrid, Spain*

Background and purpose: To analyse the influence of hypertension in the modulation induced by inducible NOS (iNOS)-derived NO and superoxide anion ($O_2^{\bullet-}$) of vasoconstrictor responses and the sources of $O_2^{\bullet-}$ implicated.
Experimental approach: Vascular reactivity experiments were performed in segments of aorta from normotensive, Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR); protein and mRNA expressions were respectively measured by western blot and quantitative reverse transcription-polymerase chain reaction and $O_2^{\bullet-}$ production was evaluated by ethidium fluorescence.
Key results: The contractile responses to phenylephrine (1 nM–30 μM) and 5-hydroxytryptamine (0.1–100 μM) were greater in aortic segments from SHR than WKY. The selective iNOS inhibitor, 1400W (10 μM), increased the phenylephrine contraction only in WKY segments; however, iNOS protein and mRNA expressions were greater in aorta from SHR than WKY. Superoxide dismutase (SOD, 150 U ml^{-1}) reduced phenylephrine and 5-hydroxytryptamine responses only in aorta from SHR; the NAD(P)H oxidase inhibitor apocynin (0.3 mM) decreased phenylephrine and 5-hydroxytryptamine responses more in vessels from SHR than WKY. Co-incubation with SOD plus 1400W potentiated the phenylephrine and 5-hydroxytryptamine responses more in segments from SHR than WKY. $O_2^{\bullet-}$ production was greater in aorta from SHR than WKY; apocynin abolished this difference.
Conclusions and implications: Increased $O_2^{\bullet-}$ formation from NADP(H) oxidase in vessels from hypertensive rats contributes to the vasoconstrictor responses and counteract the increase of NO from iNOS and the consequent modulation of these responses.
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Keywords: iNOS; superoxide anion; NAD(P)H oxidase; aorta; hypertension

Abbreviations: 1400W, *N*-(3-(aminomethyl)benzyl)acetamidine hydrochloride; E_{max}, maximum response; iNOS, inducible nitric oxide synthase; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); $O_2^{\bullet-}$, superoxide anion; SHR, spontaneously hypertensive rat; SOD, superoxide dismutase; WKY, Wistar Kyoto rat

Introduction

Hypertension is associated with elevated levels of circulating proinflammatory cytokines (Savoia and Schiffrin, 2006; Vaziri and Rodríguez-Iturbe, 2006), which may alter the vascular expression of enzymes like inducible nitric oxide synthase (iNOS) and modify the regulation of vascular tone during this pathology. Indeed, increased vascular iNOS activity and/or protein expression (Chou *et al.*, 1998; Vaziri *et al.*, 1998; Briones *et al.*, 2000, 2002a) have been described in hypertension. The role of iNOS-derived NO in vasoconstrictor and endothelium-dependent vasodilator responses has been previously analysed by our group and others in lipopolysaccharide or interleukin-1β-stimulated arteries (Hernanz *et al.*, 2003; Vo *et al.*, 2005; Jiménez-Altayó *et al.*, 2006). However, the participation of iNOS-derived NO in vasoconstrictor responses in unstimulated vessels is not well studied.

Oxidative stress can affect vascular reactivity by different mechanisms. Reactive oxygen species function as second messengers, activating numerous signalling molecules and play an important role in vascular physiopathology (Paravicini and Touyz, 2006; Vaziri and Rodríguez-Iturbe, 2006). Several sources of superoxide anion ($O_2^{\bullet-}$) within vessels have been described. Among them, xanthine oxidase, uncoupled NOS and COX can produce $O_2^{\bullet-}$ in different

Correspondence: Dr M Salaices, Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, C/Arzobispo Morcillo 4, Madrid 28029, Spain.
E-mail: mercedes.salaices@uam.es
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conditions. However, at the vascular level it is well established that nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidase, present in all three vessel layers (Griendling *et al*., 1994; Mohazzab *et al*., 1994; Chamseddine and Miller, 2003), is the main source of $O_2^{\bullet-}$ (Paravicini and Touyz, 2006; Vaziri and Rodríguez-Iturbe, 2006). An increase of $O_2^{\bullet-}$ production has been observed in human and different experimental models of hypertension, including spontaneously hypertensive rats (SHR) (Bouloumie *et al*., 1997; McIntyre *et al*., 1999; Cai and Harrison, 2000; Griendling *et al*., 2000; Lassegue and Griendling, 2004). More specifically, the enhanced $O_2^{\bullet-}$ generation in hypertension is a known result of the activation of vascular NAD(P)H oxidase (Zalba *et al*., 2001; Cruzado *et al*., 2005).

The mechanisms whereby increased $O_2^{\bullet-}$ production might contribute to high blood pressure are currently under active investigation. However, it is also well known that by interacting with NO, $O_2^{\bullet-}$ forms peroxynitrite, thus decreasing NO availability for smooth muscle relaxation. Hypertension is associated with changes in vascular responses, such as impairment of endothelium-dependent vasodilator responses or enhancement of vasoconstrictor response to different agonists (Marín, 1993; Marín and Rodríguez-Martínez, 1997; Mattei *et al*., 1997). Several studies have analysed the relationship between increased $O_2^{\bullet-}$ production and the impairment of endothelium-dependent relaxation in hypertension (for review see Touyz, 2004; Feletou and Vanhoutte, 2006). However, the $O_2^{\bullet-}$ contribution to the altered vasoconstrictor responses in hypertension as well as its relationship with the iNOS-derived NO is less studied. The present study was performed to analyse how hypertension might alter the role of $O_2^{\bullet-}$ in the vasoconstrictor responses to phenylephrine, the sources of this $O_2^{\bullet-}$ and its relationship with iNOS-derived NO.

Methods

Animals

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications no. 85-23, revised 1996) and complies with the current Spanish and European laws (RD 223/88 MAPA and 609/86). Six-month-old male normotensive Wistar Kyoto (WKY) and SHR rats were used. Systolic BP was recorded with an automatic sphygmomanometer using a tail-cuff method device placed on the tail of pretrained rats, which had spent 1 h in a warm chamber at 37 °C, and were restrained. Measurements of BP were repeated at least three times, and the average systolic BP was calculated: 131.1 ± 2.8 for WKY, $n = 13$, and 189.7 ± 1.1 mm Hg for SHR, $n = 6$ ($P < 0.05$). Rats were killed by decapitation and the thoracic aorta was removed and placed in Krebs–Henseleit solution (in mM: 115 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4.7H_2O$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 11.1 glucose and 0.01 Na_2EDTA) at 4 °C.

Vascular reactivity experiments

Vascular reactivity was studied in aortic segments by isometric tension recording, as described previously (Álvarez *et al*., 2005). Briefly, two parallel stainless steel pins were introduced through the lumen of the segments: one was fixed to the organ bath wall and the other one was connected to a force transducer (Grass FT03C, Quincy, MA, USA), which in turn was connected to a Grass model TD polygraph. Segments were incubated in an organ bath containing 5 ml of Krebs–Henseleit solution at 37 °C, continuously bubbled with a 95% O_2–5% CO_2 mixture (pH 7.4). An optimal resting tension of 1.5 g was applied to all aortic segments. This tension was adjusted every 15 min during a 60-min equilibration period before adding drugs. Aortic segments were initially exposed to 75 mM KCl to test their functional integrity. The presence of endothelium was confirmed by the effect of 10 μM ACh (Sigma Chemical Co., St Louis, MO, USA) on segments contracted with phenylephrine (Sigma Chemical) at a concentration that produces close to 50% of the contraction induced by KCl. After 60 min of washout, concentration–response curves to phenylephrine or 5-hydroxytryptamine (Sigma Chemical) were performed. A single concentration-dependent curve was performed in each segment. The effects of superoxide dismutase (SOD; Sigma Chemical), catalase (Sigma Chemical), the NAD(P)H oxidase inhibitor apocynin (Sigma Chemical), the xanthine oxidase inhibitor allopurinol (Research Biochemicals Incorporated, Natick, MA, USA) and/or the selective iNOS inhibitor *N*-(3-(aminomethyl)benzyl) acetamidine hydrochloride (1400W; Calbiochem, San Diego, CA, USA) were investigated by their addition 30 min before phenylephrine or 5-hydroxytryptamine. Some experiments were performed in the presence of the protein synthesis inhibitor dexamethasone (Sigma Chemical), which was added at the time the aorta was first placed in the Krebs–Henseleit solution. In another set of experiments, endothelium was mechanically removed by rubbing the intimal surface.

Western blot analysis

iNOS expression was analysed in aortic segments from WKY and SHR in either basal conditions (immediately after dissection of the artery from the animal) or in segments under similar conditions of reactivity experiments. Segments were frozen in liquid nitrogen and stored at −70 °C until analysis.

Segments were homogenized in ice-cold Tris-EDTA buffer (in mM: Tris-50, EDTA-1.0, pH 7.4). Homogenates (30 μg protein per lane) and prestained molecular SDS-PAGE standards (Bio-Rad Laboratories, Hercules, CA, USA) were electrophoretically separated on a 7.5% SDS-PAGE and then transferred to polyvinyl difluoride membranes overnight at 4 °C, using a Mini Trans-Blot Cell System (Bio-Rad) containing 25 mM Tris, 190 mM glycine, 20% methanol and 0.05% SDS. Mouse macrophages were used for iNOS positive controls. Then, the membrane was blocked for 60 min at room temperature in Tris-buffered solution (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) with 5% powdered non-fat milk. Next, the membrane was incubated for 1 h at room temperature with mouse monoclonal antibody for iNOS (1:10 000; Transduction Laboratories, Lexington, UK). After washing, the membrane was incubated with anti-mouse IgG

antibody (1:2000) conjugated to horseradish peroxidase (Transduction Laboratories). The membrane was thoroughly washed and the immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminescence system (ECL Plus, GE Healthcare, Little Chalfont, UK) and subjected to autoradiography (Hyperfilm ECL, GE Healthcare). Signals on the immunoblot were quantified with an NIH Image V1.56 computer program. The same membrane was used to determine α-actin expression, and the content of the latter was used to correct iNOS expression in each sample by means of a monoclonal antibody anti-α-actin (1:3 000 000; Sigma Chemical).

Results are expressed as the ratio between signals on the immunoblot corresponding to iNOS and to α-actin. To compare the results of protein expression within the same experiment and with other experiments, we assigned a value of 1 to the ratio in arteries from WKY and used that value to calculate the relative density of the other bands in the same gel.

RT-PCR real-time assay

iNOS mRNA was determined in aortic segments in similar conditions to those used for protein expression determination. Total RNA was obtained using TRIzol (Invitrogen Life Technologies, Philadelphia, PA, USA). A total of 1 μg of DNAse I-treated RNA was reverse transcribed into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) in a 50 μl reaction. PCR was performed in duplicate for each sample using 0.5 μl of cDNA as a template for iNOS, 1 × TaqMan Universal PCR Master Mix (Applied Biosystems) and 10 × of Taqman Gene Expression Assays (Applied Biosystems, Rn00561646_m1) in a 20 μl reaction. Quantitative RT-PCR was carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the following conditions: 2 min 50 °C, 10 min 95 °C followed by 40 cycles of 15 s 95 °C and 1 min 60 °C. As a normalizing internal control we amplified β2 microglobulin (Rn00560865_m1). To calculate the relative index of gene expression, we employed the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) using WKY samples as calibrator.

In situ *detection of vascular* $O_2^{\bullet-}$ *production*

The oxidative fluorescent dye dihydroethidium was used to evaluate $O_2^{\bullet-}$ production *in situ*, as described previously (Jiménez-Altayó *et al*., 2006). Hydroethidine freely permeates cells and, in the presence of $O_2^{\bullet-}$, is oxidized to ethidium bromide, which is trapped by intercalation with DNA. Ethidium bromide is excited at 546 nm and has an emission spectrum of 610 nm. Frozen tissue segments from control and apocynin (0.3 mM, 30 min)-incubated arteries were cut into 10-μm thick sections and placed on a glass slide. Serial sections were equilibrated under identical conditions for 30 min at 37 °C in Krebs-HEPES buffer (in mM: NaCl 130, KCl 5.6, $CaCl_2$ 2, $MgCl_2$ 0.24, HEPES 8.3, glucose 11, pH 7.4). Fresh buffer containing dihydroethidium (2 μM) was applied topically onto each tissue section, cover-slipped and incubated for 30 min in a light-protected humidified chamber at 37 °C, and then viewed in a fluorescent laser scanning confocal microscope (Leica TCS SP2 equipped with a krypton/argon laser, × 40 objective), using the same imaging

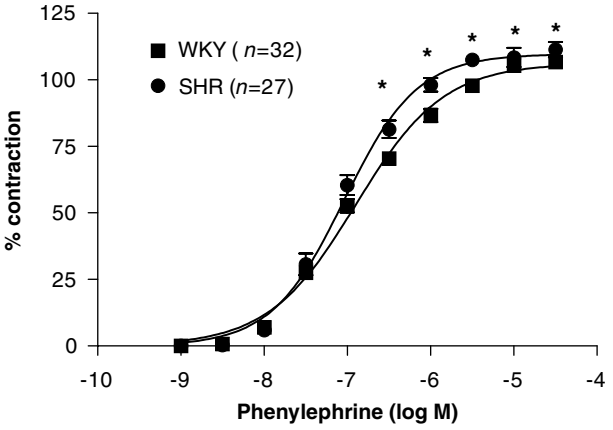

Figure 1 Concentration–response curve to phenylephrine in intact aortic segments from normotensive (WKY) and hypertensive (SHR). Results are expressed as a percentage of the response to 75 mM KCl for the number of animals indicated in parentheses. ANOVA (two-way): $*P<0.05$ vs control. WKY, Wistar Kyoto rat; SHR, spontaneously hypertensive rat.

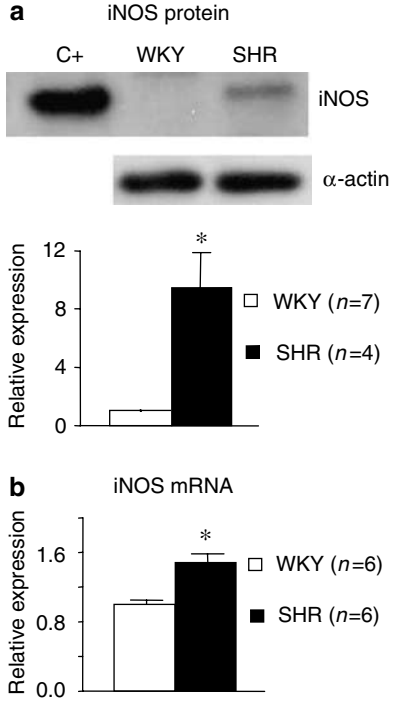

Figure 2 (**a**) Representative western blot and densitometric analysis for the inducible isoform of nitric oxide synthase (iNOS) in aortic segments from WKY and SHR; mouse macrophages were used for iNOS positive controls (C +). (**b**) Quantitative RT-PCR assessment of iNOS mRNA expression in the same segments. Results are expressed as the relative expression of protein or mRNA in SHR compared to WKY. ANOVA (one-way): $*P<0.05$. The number of animals are indicated in parentheses. WKY, Wistar Kyoto rat; SHR, spontaneously hypertensive rat.

settings in each case. Fluorescence was detected with a 568 nm longpass filter. Elastin autofluorescence was used to delimit the different layers of the vascular wall and was visualized by excitation at 488 nm and detection at 535 nm. For fluorescence quantification, four rings per animal were sampled for each experimental condition and averaged. The mean fluorescence densities in the target region were calculated.

Data analysis and statistics

Vasoconstrictor responses induced by phenylephrine or 5-hydroxytryptamine were expressed as a percentage of the tone generated by 75 mM KCl. The maximum response (E_{max}) and EC_{50} values were calculated by a non-linear regression analysis of each individual concentration–response curve using GraphPad Prism Software (San Diego, CA, USA).

Results are expressed as mean ± s.e.m. of the number of rats indicated; differences were analysed using Student's *t*-test, one-way or two-way ANOVA followed by a Bonferroni test. A *P*-value below 0.05 was considered significant.

Results

Phenylephrine (1 nM–30 μM) induced concentration-dependent contraction in aortic segments that was slightly greater in SHR than in WKY (Figure 1), as reported previously (Álvarez *et al*., 2005, 2007). 5-hydroxytryptamine (0.1–100 μM) also induced concentration-dependent contractions greater ($P<0.05$) in SHR (E_{max}: 153.8 ± 4.9% of the tone generated by 75 mM KCl; EC_{50}: 2.26 ± 0.31 μM; $n=14$) than in WKY (E_{max}: 123.8 ± 6.9%; EC_{50}: 4.23 ± 0.54 μM; $n=9$). The mean values for KCl contraction in aorta were 3100 ± 39 and

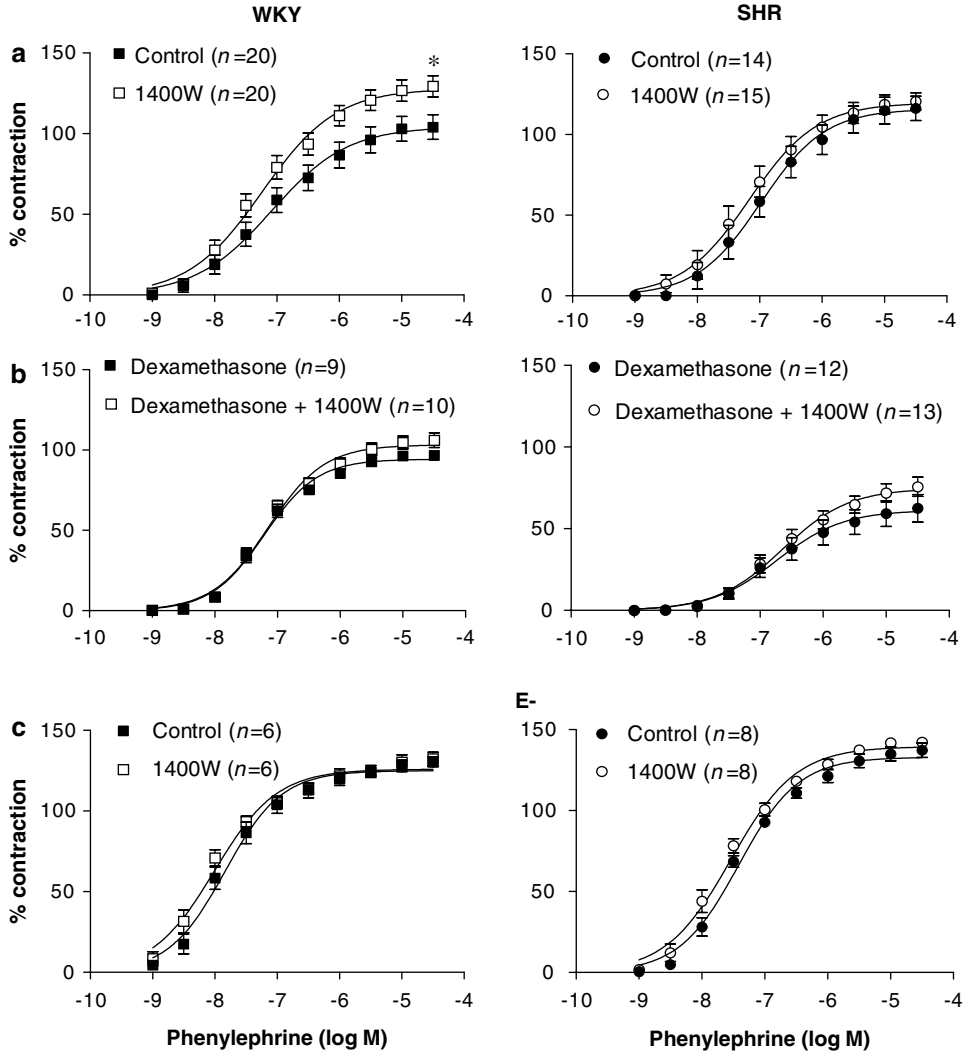

Figure 3 Effect of 1400W (10 μM) on the concentration–response curve to phenylephrine in: (**a**) intact aortic segments from WKY and SHR, (**b**) segments incubated with dexamethasone (1 μM) and (**c**) endothelium-denuded segments (E−). Results are expressed as a percentage of the response to 75 mM KCl for the number of animals indicated in parentheses. ANOVA (two-way): *$P<0.05$ vs control. WKY, Wistar Kyoto rat; SHR, spontaneously hypertensive rat.

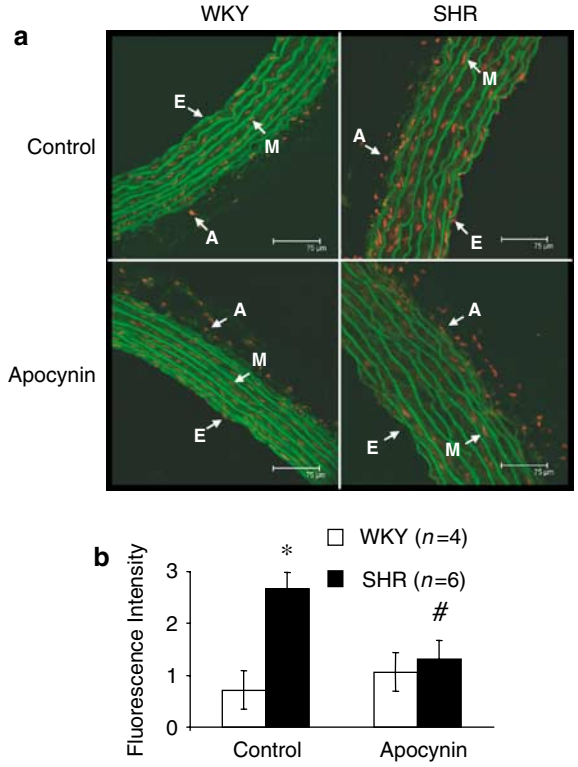

Figure 4 (**a**) Representative fluorescent photomicrographs and (**b**) quantitative analysis of confocal microscopic sections of aortic segments from WKY and SHR incubated without (control) or with apocynin (0.3 mM). Vessels were labelled with the oxidative dye dihydroethidium, which produces a red fluorescence when oxidized to ethidium bromide by superoxide anion ($O_2^{\bullet-}$). Natural autofluorescence of elastin was used to delimit the different layers of the vascular wall (appears green). Image size 375 × 375 μm. Images were captured with a fluorescence confocal microscope (× 40 oil immersion objective, zoom 1). E = endothelial layer; M = media layer; A = adventitial layer. $^*P<0.05$ vs WKY, $^{\#}P<0.05$ vs control by Student's *t*-test. WKY, Wistar Kyoto rat; SHR, spontaneously hypertensive rat.

2913 ± 49 mg for WKY ($n=42$) and SHR ($n=42$), respectively ($P<0.05$).

Participation of iNOS-derived NO in vasoconstrictor responses

In basal conditions, neither iNOS protein nor mRNA expression could be detected in aortic segments from either WKY or SHR (results not shown). In aortic segments from both strains under similar conditions of reactivity experiments, iNOS protein and mRNA expressions were detected, and they were greater in arteries from hypertensive than from normotensive animals (Figure 2). iNOS protein expression was abolished in arteries from both strains when the endothelial layer was removed or when the arteries had been incubated with dexamethasone (1 μM) (results not shown).

The selective iNOS inhibitor 1400W (10 μM) increased the vasoconstrictor response induced by phenylephrine in aorta from WKY, but did not modify it in aorta from SHR (Figure 3a). The effect of 1400W was abolished by co-incubation with dexamethasone (4 μM) (Figure 3b) as well as by endothelium removal (Figure 3c). 1400W did not affect 5-hydroxytryptamine responses in segments from both strains (results not shown).

Participation of reactive oxygen species on vasoconstrictor responses

Production of $O_2^{\bullet-}$ was greater in aortic segments from hypertensive than normotensive animals (Figure 4). Pre-incubation of vessels with apocynin (0.3 mM) decreased $O_2^{\bullet-}$ production in segments from hypertensive animals (Figure 4), confirming the participation of NAD(P)H oxidase on $O_2^{\bullet-}$ production in this strain.

To study the contribution of $O_2^{\bullet-}$ in the response to phenylephrine, experiments were performed in the presence of SOD, which dismutates $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2). SOD (150 U ml^{-1}) reduced the phenylephrine contraction only in aorta from SHR (Figure 5a). The participation of H_2O_2 in response to phenylephrine was analysed in experiments performed in the presence of catalase, which transforms two molecules of H_2O_2 to water and oxygen. Catalase (1000 U ml^{-1}) did not modify the response to phenylephrine in either strain (Figure 5b). To exclude the possibility that the inhibitory effect observed with SOD could be due to the H_2O_2 produced by the added SOD, segments were pre-incubated with SOD (150 U ml^{-1}) plus catalase (1000 U ml^{-1}). Co-incubation of SOD with catalase did not modify the observed inhibitory effect of SOD in segments from hypertensive animals (data not shown).

The decreased contractile response to phenylephrine in the presence of SOD observed in SHR could be to due to the increased bioavailability of iNOS-derived NO. To test this hypothesis, we analysed the effect of 1400W on phenylephrine responses in arteries incubated with SOD. In these conditions, the contractile response to phenylephrine was potentiated and this potentiation was greater in SHR than in WKY (Figure 5c).

To study if NAD(P)H oxidase and/or xanthine oxidase were the sources for the $O_2^{\bullet-}$ involved in the phenylephrine responses, the arteries were preincubated with apocynin or allopurinol, respective inhibitors of these enzymes. Apocynin (0.3 mM), but not allopurinol (0.3 mM), inhibited the contractile response to phenylephrine in both strains, and this effect was greater in aortic segments from SHR than in WKY (Figure 6).

To assess if the greater participation of $O_2^{\bullet-}$ on vasoconstrictor responses observed in aorta from hypertensive rats was specific for phenylephrine or other agonists were similarly affected, we analysed the effect of SOD and apocynin on vasoconstrictor response to 5-hydroxytryptamine. Figure 7a shows that apocynin inhibited 5-hydroxytryptamine response in aorta from both strains. This inhibitory effect was greater in SHR than in WKY, as shown by the modification of both E_{max} (control: 153.8 ± 4.9%; apocynin: 127.4 ± 6.9%, $P<0.05$) and EC_{50} (control: 2.26 ± 0.31; apocynin: 3.43 ± 0.35 μM, $P<0.05$) observed in this strain; in contrast, in WKY, apocynin only diminished E_{max} (control: 123.8 ± 6.9%; apocynin: 96.7 ± 5.0%, $P<0.05$), without changes in EC_{50} (control: 4.23 ± 0.54; apocynin: 5.20 ± 0.77 μM, $P<0.05$). Moreover, SOD reduced 5-hydroxytryptamine response only in aorta from SHR (Figure 7b). The decrease of the contractile response to 5-hydroxytryptamine in the presence of SOD observed in SHR could be also due to the increased bioavailability of iNOS-derived NO because, in these conditions, the contractile response to 5-hydroxytryp-

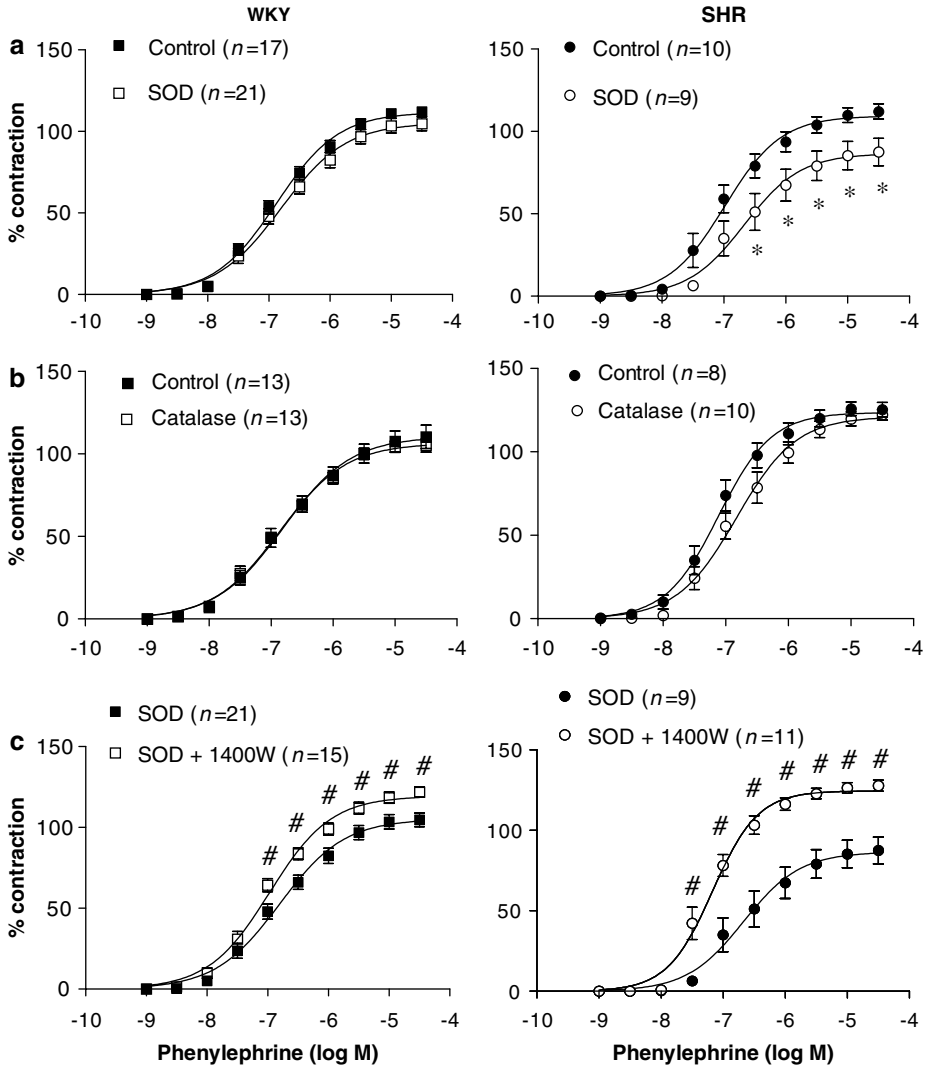

Figure 5 Effect of (**a**) superoxide dismutase (SOD; 150 U ml^{-1}) and (**b**) catalase (1000 U ml^{-1}) on the concentration–response curve to phenylephrine in aortic segments from SHR and WKY. (**c**) Effect of 1400W (10 μM) on the concentration–response curve to phenylephrine in aortic segments from WKY and SHR treated with SOD. Results are expressed as a percentage of the response to 75 mM KCl for the number of animals indicated in parentheses. ANOVA (two-way): *$P<0.05$ vs control; $^{\#}P<0.05$ vs SOD. SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

tamine was potentiated by 1400W only in this strain (Figure 7c).

Discussion

Hypertension is associated with increased activity and/or expression of iNOS (Wu *et al.*, 1996; Chou *et al.*, 1998; Vaziri *et al.*, 1998; Briones *et al.*, 2000, 2002a) as well as increased production of $O_2^{\bullet-}$ (Kerr *et al.*, 1999; Zalba *et al.*, 2001) in different vascular beds; these changes might contribute to the alterations in vascular tone occurring in this pathology. The main results of the present study suggest that the increased production of $O_2^{\bullet-}$ derived from NAD(P)H oxidase, observed in aorta from hypertensive rats, counteracts the enhanced production of NO derived from iNOS, occurring in hypertension, and the modulation exerted by NO of vasoconstrictor responses.

Expression of iNOS protein and mRNA was detected in aortic segments after incubation in the organ bath but not under basal conditions, and these expressions were greater in segments from SHR than WKY, as already described (Wu *et al.*, 1996; Chou *et al.*, 1998; Vaziri *et al.*, 1998; Briones *et al.*, 2002a). The observed iNOS expression might be due, at least partially, to low levels of endotoxin in the incubation medium (Rees *et al.*, 1990; Alonso *et al.*, 1998). The fact that dexamethasone prevented this expression supports this possibility. However, we cannot discount the possibility that iNOS was present in freshly excised aorta but at levels too low to detect and that incubation in the organ bath merely increased iNOS levels. In any case, our results suggest that aorta from SHR might be more susceptible to upregulation of iNOS. Hypertension has been considered a chronic inflammatory disease with elevated vascular and plasma levels of proinflammatory cytokines (Savoia and Schiffrin, 2006; Vaziri and Rodríguez-Iturbe, 2006). It is then possible that

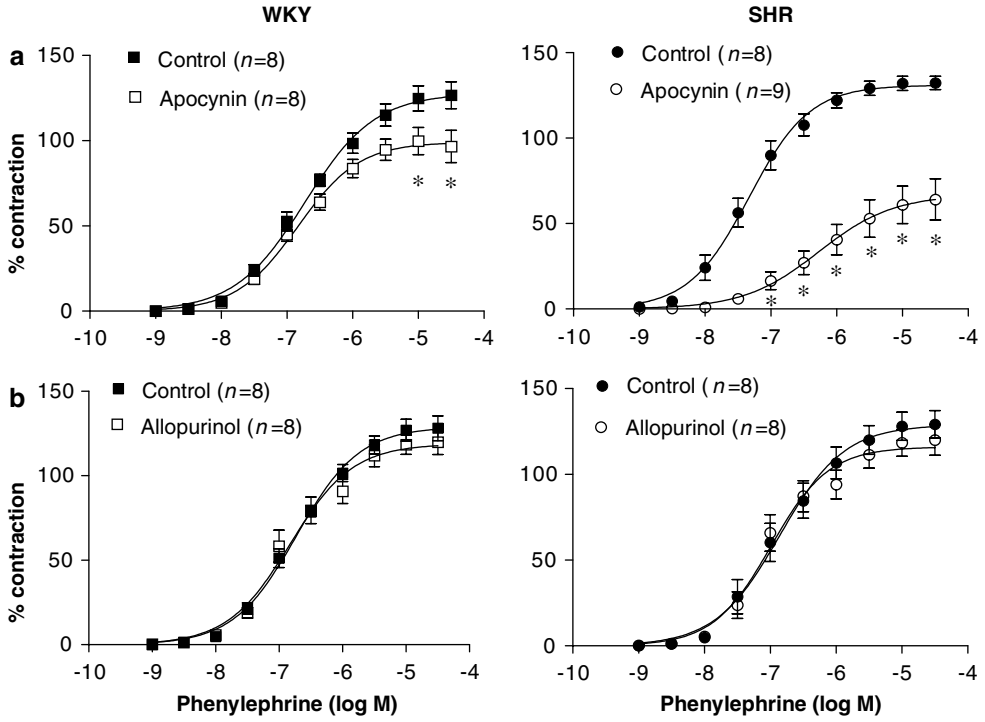

Figure 6 Effect of (**a**) apocynin (0.3 mM) and (**b**) allopurinol (0.3 mM) on the concentration–response curve to phenylephrine in aortic segments from WKY and SHR. Results are expressed as a percentage of the response to 75 mM KCl for the number of animals indicated in parentheses. ANOVA (two-way): $*P<0.05$ vs control. WKY, Wistar Kyoto rat; SHR, spontaneously hypertensive rat.

the increased proinflammatory cytokines would increase the *in vivo* expression of inducible enzymes, such as iNOS. The physiological relevance of the increased iNOS expression is unknown. Conflicting results regarding the role of iNOS in the control of blood pressure are reported. Thus, iNOS does not seem to play a significant role in preventing DOCA-salt-induced hypertension (Sun *et al*., 2005). However, Hong *et al*. (2000) demonstrated that the iNOS inhibitor aminoguanidine decreased blood pressure in SHR. Furthermore, overexpression of iNOS in the rostral ventrolateral medulla increases urinary noradrenaline excretion and blood pressure via activation of the sympathetic nervous system, which is mediated by an increase in oxidative stress (Kimura *et al*., 2005).

The modulation induced by iNOS-derived NO of the phenylephrine contraction, but not of 5-hydroxytryptamine, in aorta from normotensive rats was demonstrated by the increased phenylephrine response observed in the presence of the selective iNOS inhibitor 1400W. In addition, this effect was abolished by dexamethasone, demonstrating that the effect of 1400W was selective for iNOS. Other investigators have also described modulation by iNOS-derived NO of vasoconstrictor responses to adrenergic agonists in different vascular beds after incubation with lipopolysaccharide (Briones *et al*., 2000; O'Brien *et al*., 2001). Surprisingly, 1400W had no effect on segments from SHR despite the greater iNOS expression we observed in this strain. iNOS is expressed in the three layers of the vascular wall (Zhang *et al*., 1999; Hernanz *et al*., 2003, 2004; Briones *et al*., 2005). The fact that endothelium removal abolished the effect of 1400W suggests that endothelial iNOS is responsible for the NO production that modulates the phenylephrine responses in aortic segments.

Reactive oxygen species, such as $O_2^{\bullet-}$ and H_2O_2, modulate vascular tone and their increase has been involved in the vascular alterations associated to hypertension. Both oxidative stress and hypertension seem to constitute a self-perpetuating cycle in which the initiating factor of this vicious cycle varies in different forms of hypertension (Vaziri and Rodríguez-Iturbe, 2006). $O_2^{\bullet-}$ induces vasoconstriction through different mechanisms; among them, it is well established that $O_2^{\bullet-}$ reacts with and inactivates NO, producing the peroxynitrite anion, $ONOO^-$. Also, the addition of $O_2^{\bullet-}$-generating systems to organ baths produces contraction in aorta and cerebral arteries (Shen *et al*., 2000; Hernanz *et al*., 2003), confirming that $O_2^{\bullet-}$ acts as a vasoconstrictor factor. Several authors have demonstrated the involvement of $O_2^{\bullet-}$ in vasoconstrictor responses to 5-HT (Srivastava *et al*., 2002) or to adrenoceptor agonists (Srivastava *et al*., 1998; Briones *et al*., 2002b). However, no direct modulation of contractile responses by $O_2^{\bullet-}$ has been reported by others (Girouard and de Champlain, 2004). The fact that SOD diminished the contractile response to phenylephrine and 5-hydroxytryptamine only in aortic segments from hypertensive rats indicates the involvement of $O_2^{\bullet-}$ in this strain and agrees with the increased $O_2^{\bullet-}$ production widely reported in hypertension (Kerr *et al*., 1999; Zalba *et al*., 2001; Paravicini and Touyz, 2006; Vaziri and Rodríguez-Iturbe, 2006; present results). In agreement, major modulation of contractile responses by $O_2^{\bullet-}$ in vessels from hypertensive subjects has been described (Püntmann *et al*., 2005). On the other hand, H_2O_2 has dual effects at

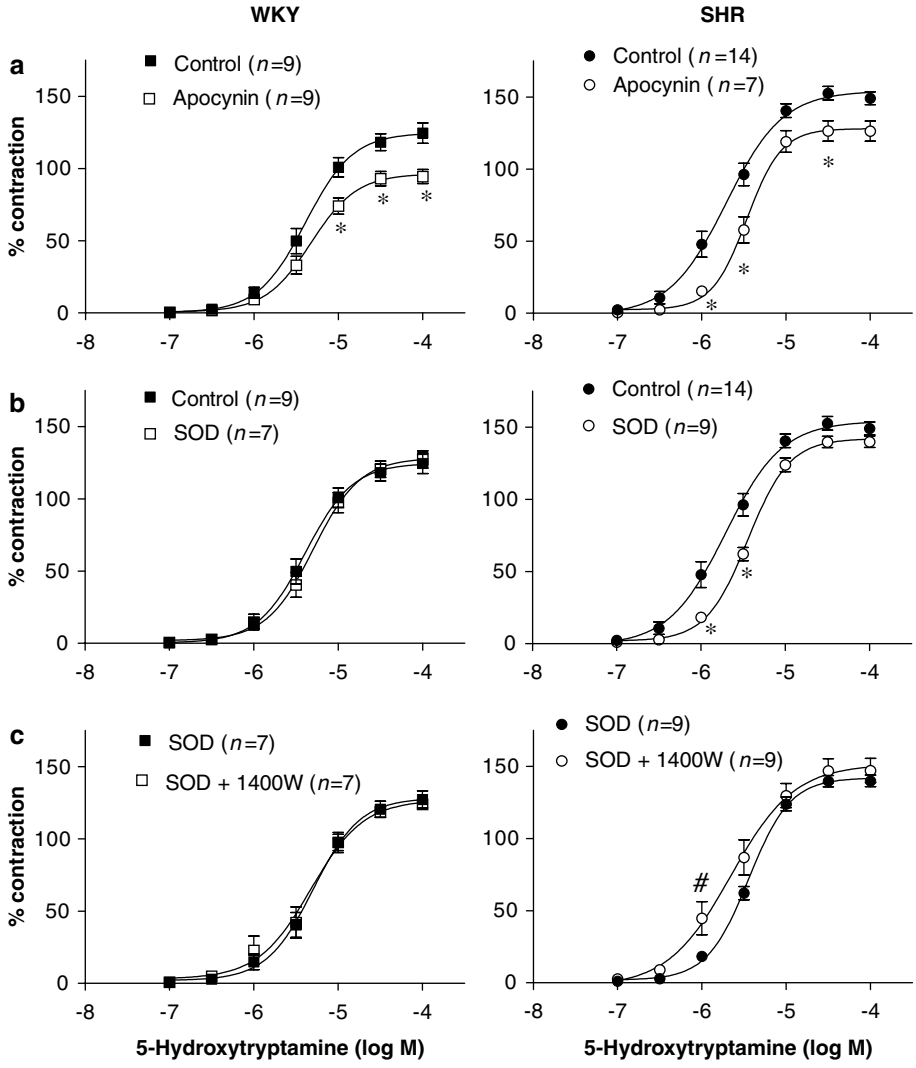

Figure 7 Effect of (**a**) apocynin (0.3 mM) and (**b**) superoxide dismutase (SOD; 150 U ml^{-1}) on the concentration–response curve to 5-hydroxytryptamine in aortic segments from SHR and WKY. (**c**) Effect of 1400W (10 μM) on the concentration–response curve to 5-hydroxytryptamine in aortic segments from WKY and SHR treated with SOD. Results are expressed as a percentage of the response to 75 mM KCl for the number of animals indicated in parentheses. ANOVA (two-way): *$P<0.05$ vs control; $^{\#}P<0.05$ vs SOD. SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

vascular level, since it can produce both vasoconstriction and vasodilation (Ardanaz and Pagano, 2006). Nevertheless, its participation in the responses to vasoconstrictor agents is contradictory. Thus, Girouard and de Champlain (2004) and Thakali *et al*. (2005) have proposed that endogenous H_2O_2 is not involved in the contractions, respectively, induced by phenylephrine and endothelin-1. However, other studies have shown that catalase reduces the contractile response to noradrenaline (Srivastava *et al*., 1998). In our study, catalase affected neither phenylephrine responses nor the effect of SOD, thus excluding H_2O_2 involvement from this response.

As mentioned above, 1400W increased the contractile response to phenylephrine only in aorta from WKY, suggesting a greater modulation of contractile responses by iNOS-derived NO in segments from normotensive rats despite the smaller iNOS expression found in this strain. It is possible that the increased $O_2^{\bullet-}$ observed in SHR might react with iNOS-derived NO, thereby decreasing NO bioavailability, and thus explaining the lack of effect of 1400W in this strain. The fact that the combination of SOD plus 1400W increased the phenylephrine response more in SHR than WKY confirms this hypothesis and correlates with the higher iNOS expression observed in SHR vessels.

It is well established that NAD(P)H oxidase is the main source of $O_2^{\bullet-}$ at vascular level (Paravicini and Touyz, 2006; Vaziri and Rodríguez-Iturbe, 2006). Thus, the suggestion that NAD(P)H oxidase plays an important role in the control of the vascular tone is now evident (Souza *et al*., 2001). Apocynin, an inhibitor of NAD(P)H oxidase, diminished the vasoconstrictor responses to phenylephrine more in aorta from SHR than in WKY, whereas allopurinol, a xanthine oxidase inhibitor, did not modify the phenylephrine response in either strain. In agreement, Miyagawa *et al*. (2007) recently found that apocynin, but not allopurinol, reduced noradrenaline responses in rat femoral arteries from SHR but not in WKY. Moreover, apocynin reduced $O_2^{\bullet-}$ production in aorta from hypertensive rats. The vasoconstrictor responses induced by 5-hydroxytryptamine

were also decreased by apocynin only in SHR, although the magnitude of inhibition was greater for the α-adrenoceptor agonist. This suggests different degree of modulation of the contractile responses by $O_2^{\bullet-}$ depending on the vasoconstrictor used, but confirms the greater role of $O_2^{\bullet-}$ in vessels from hypertensive rats. Taken together, these results point to NAD(P)H oxidase as responsible for the increased $O_2^{\bullet-}$ production in vessels from hypertensive animals. In this sense, increased activity and expression of NAD(P)H oxidase with hypertension has been reported (Zalba *et al.*, 2001; Cruzado *et al.*, 2005; Vaziri and Ni, 2005). NAD(P)H oxidase activation is also involved in the contraction induced by angiotensin II in radial arteries from hypertensive patients (Püntmann *et al.*, 2005) as well as in the spontaneous tone observed in aorta from DOCA-salt hypertensive rats (Ghosh *et al.*, 2004) and SHR (Lodi *et al.*, 2006). However, we cannot exclude the possible involvement of other sources of $O_2^{\bullet-}$. For instance, $O_2^{\bullet-}$ production in aorta from SHR was reduced by N^G-nitro-L-arginine methyl ester, indicating $O_2^{\bullet-}$ generation from uncoupled endothelial NOS in hypertension (Li *et al.*, 2006).

In conclusion, our results demonstrate that hypertension increases iNOS expression but decreases the bioavailability and the modulation elicited by iNOS-derived NO of contractile responses in aorta as a result of the increased $O_2^{\bullet-}$ production from NAD(P)H oxidase. Although similar results were not found in mesenteric resistance vessels (unpublished results) and the results obtained in aorta might not to be relevant to blood pressure regulation, the findings found here would contribute to the explanation of the altered vasoconstrictor responses observed in different vessels, associated with hypertension.

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Conflict of interest

The authors state no conflict of interest.

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