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Role of oxidative stress and nitric oxide in regulation of spontaneous tone in aorta of DOCA-salt hypertensive rats

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1 The roles of nitric oxide (NO), superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2) in the modulation of spontaneous tone were investigated in isolated aorta from deoxycorticosterone acetate (DOCA)-salt hypertensive rats.

2 Increases in preload from 1 to 5 g were accompanied by increases in spontaneous tone in aortic rings from DOCA-salt hypertensive rats but not from SHAM-normotensive rats.

3 Tone was higher in endothelium-denuded aortic rings than in endothelium-intact vessels. Inhibition of nitric oxide synthase (NOS) with 300 μM N^G-nitro-L-arginine methyl ester (L-NAME) increased spontaneous tone.

4 Basal O_2^- generation was higher in aortic rings from DOCA-salt hypertensive rats than in those from SHAM-normotensive rats. Stretch increased O_2^- levels even further in the DOCA-salt group. In rings isolated from DOCA-salt hypertensive rats, administration of the O_2^- scavenger, superoxide dismutase (SOD, 150 U ml^{-1}), or the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase inhibitor, apocynin (100 μM), completely abolished the development of spontaneous tone in endothelium-intact aortic rings but not in endothelium-denuded or in L-NAME-treated rings. SOD and apocynin decreased the generation of O_2^- in endothelium-intact, endothelium-denuded, and L-NAME-treated aortic rings.

5 Oral treatment of DOCA-salt hypertensive rats with the O_2^- scavengers, tempol or tiron, or with apocynin for 3 weeks prevented the development of hypertension and abolished the increases in O_2^- generation and spontaneous tone.

6 Administration of catalase (1000 U ml^{-1}) to aortic rings increased spontaneous tone in vessels from DOCA-salt hypertensive rats.

7 Administration of the cyclooxygenase (COX) inhibitor, valeroyl salicylate, or the thromboxane/prostaglandin antagonist, SQ 29548, to aortic rings abolished tone.

8 The results suggest that NO plays a major role in preventing the generation of spontaneous tone in isolated aortic rings from DOCA-salt hypertensive rats. NADPH-oxidase-derived O_2^- enhanced spontaneous tone by inactivating NO. Endogenous H_2O_2 appears to mitigate the increase in tone. In addition, a COX component may also contribute to spontaneous tone.
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Keywords: DOCA-salt hypertension; spontaneous tone; rat aorta; nitric oxide synthase; superoxide anion; hydrogen peroxide; cyclooxygenase

Abbreviations: ANOVA, analysis of variance; DOCA, deoxycorticosterone acetate; NO, nitric oxide; SNP, sodium nitroprusside; e-NOS, endothelial nitric oxide synthase; BP, blood pressure; L-NAME, N^G-nitro-L-arginine methyl ester; SOD, superoxide dismutase; NADPH, nicotinamide adenine dinucleotide phosphate; ET, endothelin; COX, cyclooxygenase; TP, thromboxane/prostaglandin; sGC, soluble guanylyl cyclase; $[Ca^{2+}]_i$, cytosolic free calcium; K_{Ca}, calcium-activated potassium channel; sGC, soluble guanylyl cyclase; cGMP, cyclic 3′, 5′-guanosine monophosphate; H_2O_2, hydrogen peroxide; $OONO^-$, peroxynitrite; O_2^-, superoxide anion; PGH_2, prostaglandin H_2; VAS, valeroyl salicylate

Introduction

Spontaneous tone observed in isolated blood vessels is defined as the increase of tension in the vessel in response to stretch and in the absence of any exogenous stimulus (Hwa & Bevan, 1986a). This phenomenon was described by W.M. Bayliss in 1902. Spontaneous tone has been reported in several experimental models of hypertension including, the spontaneously hypertensive rat (SHR) (Sekiguchi *et al*., 1998), the angiotensin II-infused model (Di Wang *et al*., 1999), and the deoxycorticosterone acetate (DOCA)-salt model (Rinaldi & Bohr, 1989). In the aorta from DOCA-salt hypertensive rats, spontaneous tone was abolished by a calcium channel blocker or by removal of calcium from the organ bath, suggesting that the generation of spontaneous tone is dependent on calcium influx. Recently, it has been reported that phosphoinositide 3-kinase mediates both enhanced spontaneous and agonist-

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induced contractions in aorta of DOCA-salt hypertensive rats (Northcott *et al*., 2002). However, the contribution of various endogenous modulators to the generation of spontaneous tone in the DOCA-salt hypertensive rat has not been explored.

Reactive oxygen species have been shown to regulate vascular tone. Vascular superoxide anion (O_2^-) formation is enhanced in DOCA-salt hypertensive rats (Somers *et al*., 2000). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase accounts for the increased O_2^- production in these rats (Beswick *et al*., 2001a). Long-term antioxidant administration of the O_2^- scavenger, tempol, or of the NADPH-oxidase inhibitor, apocynin, decreased systolic blood pressure (BP) and reduced O_2^- generation in DOCA-salt hypertensive rats (Beswick *et al*., 2001b). Thus, considerable evidence suggests that reactive oxygen species contribute to the elevation in BP in the DOCA-salt model. Recently, administration of superoxide dismutase (SOD) has been reported to improve the agonist-induced vasorelaxation in aortic rings of DOCA-salt hypertensive rats (Somers *et al*., 2000), suggesting a role for O_2^- in the regulation of vascular tone in this model. However, the possibility that O_2^- may contribute to the spontaneous tone observed in vessels from the DOCA-salt hypertensive rat has not been explored. A potential effect of the increase in O_2^- is inactivation of nitric oxide (NO) (Laight *et al*., 1998), an interaction that could contribute to spontaneous tone. Another reactive oxygen species, hydrogen peroxide (H_2O_2), has been shown to cause both vasoconstriction (Yang *et al*., 1998; Shen *et al*., 2000) and vasorelaxation (Bharadwaj & Prasad, 1995; Yang *et al*., 1999) depending on the tissue. However, a role of H_2O_2 in regulating vascular reactivity in the DOCA-salt hypertensive model has not been reported. We tested the hypothesis that spontaneous tone generated in aortic rings of DOCA-salt hypertensive rats is regulated by a complex interaction involving endothelial-derived NO and reactive oxygen species.

Methods

Animal care and instrumentation

All procedures and protocols were in accordance with guidelines set by the Laboratory Animal Care Committee at the University of Saskatchewan, Saskatoon, Canada. All surgical procedures were performed in anesthetized rats. Anesthesia was induced by inhalation of 4% isoflurane and maintained with 2% isoflurane. An analgesic ($0.03\,mg\,kg^{-1}$ buprenorphine) was administered i.m. postoperatively.

Male Sprague–Dawley rats were purchased from Charles River (Montreal, Canada) and were maintained in our animal quarters under standardized conditions. All rats were maintained on a 12-h light/day cycle and received standard laboratory rat chow and water *ad libitum*. At 8–10 weeks of age, the right kidney was removed through a dorsal flank incision. After 1 week, these rats were assigned randomly into one of two groups: a DOCA-salt-treated or a SHAM-control group. In the DOCA-salt-treated group, a silastic strip impregnated with $100\,mg\,kg^{-1}$ body weight of DOCA (Aldrich Chemical; Milwaukee, WI, U.S.A.) and in the SHAM-control group, a DOCA-free strip was implanted subcutaneously in the midscapular region. A catheter connected to a radiotelemetry capsule (TA11PA-C40, Data Sciences; Minneapolis, MN, U.S.A.) was inserted into the left femoral artery and pushed so that its tip reached the abdominal aorta above the iliac bifurcation for monitoring BP and the capsule containing the transducer was positioned in the left flank region subcutaneously. From this point on, DOCA-salt rats were given a 0.9% NaCl and 0.2% KCl solution and SHAM-control rats were given tap water for drinking *ad libitum* over a 3-week period. In some rats, tempol (10^{-3} M), apocynin (1.5×10^{-3} M), or tiron (10^{-3} M) was added in drinking water during the course of DOCA and SHAM treatment.

BP monitoring

BPs were recorded by a radiotelemetry method in conscious unrestrained rats with a computer-driven data acquisition system (Data Science, Minneapolis, MN, U.S.A.). Details for this method are described elsewhere in this journal (Yu *et al*., 2001a). After 3 weeks of DOCA or SHAM treatment, BP was monitored for 4 h and readings from the last hour were analyzed to establish the baseline BP.

Tension measurements in thoracic aorta rings

After 3 weeks of DOCA or SHAM treatment, rats were killed under pentobarbital anesthesia ($60\,mg\,kg^{-1}$ body weight, i.p.) and the thoracic aorta was cleaned of adherent fat. Rings 7 mm long were cut and mounted on triangular stirrups for isometric tension recording in organ chambers containing 10 ml of Kreb's buffer. The composition of the buffer has been described elsewhere (Ghosh *et al*., 2002). The rings were maintained at 37°C and pH 7.4 with 95% O_2/5% CO_2. During the first hour, the tension (preload) was increased progressively to the optimal tension of 5 g. As a spontaneous tone was evident in rings from DOCA-salt hypertensive rats, sodium nitroprusside (SNP, 10^{-7} M) was introduced for the last 20 min to allow final adjustment of the preload under passive conditions. In this way, the method for setting the preload was standardized and spontaneous tone could not contribute to the preload. On removal of the SNP by washing, aortic rings from DOCA-salt hypertensive rats but not those from SHAM-control-normotensive rats showed an increase in tension.

Various interventions were introduced in a series of separate experiments. Removal of endothelium was achieved by gently rubbing the intimal surface of rings before mounting the tissue in the organ chamber. The loss of vasodilatory responses to acetylcholine (1 μM) was used to confirm endothelium denudation. The role of endothelin (ET-1) was studied by incubating rings with the ET_A antagonist, BQ123 (10 μM), and the ET_B antagonist, BQ788 (1.2 nM, 1.3 and 10 μM), either alone or in combination. Loss of ET-1-induced vasoconstriction was used to confirm the stability of the compounds. The role of NO was determined by incubating rings with N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM). The role of O_2^- was examined by recording responses in the presence and absence of the superoxide scavenger, SOD ($150\,U\,ml^{-1}$), or the NADPH-oxidase inhibitor, apocynin (100 μM). The role of H_2O_2 was determined by recording responses in the presence and absence of catalase ($1000\,U\,ml^{-1}$), an enzyme that reduces H_2O_2 to H_2O and O_2. The role of COX products was determined by incubating aortic rings with one of two COX inhibitors, indomethacin (50 μM) or valeroyl salicylate (VAS) (3 mM), or with the thromboxane/prostaglandin (TP) receptor antagonist,

Table 1 Effect of antioxidants on spontaneous tone and agonist-evoked contraction

	Spontaneous tone	*Phenylephrine (1 μm)*	*KCl (30 mm)*
Groups	*% of maximum contractile response to 120 mm KCl*		
Endothelium intact			
Untreated	38.2±3.1	72.5±2.5	72.0±3.4
SOD	1.80±0.9*	73.4±3.8	77.1±5.2
Apocynin	0.30±0.3*	71.5±1.2	72.6±2.7
Catalase	58.7±6.3*	69.6±0.47	70.6±0.5
Endothelium denuded			
Untreated	54.7±4.9†	74.5±1.7	73.7±4
SOD	59.0±5.2†	70.4±3.4	72.7±3.2
Apocynin	54.5±1.2†	73.5±2.3	73.3±1.5
Catalase	71.0±3.4†*	75.3±6.5	76.1±2.7

Effect of SOD (150 U/ml), apocynin (100 μM), and catalase (1000 U/ml) on spontaneous tone, and on phenylephrine (1 μM)- and KCl (30 mM)-induced contractions in aortic rings from DOCA-salt hypertensive rats in the presence and absence of endothelium. Data are mean±s.e.m. expressed as a percentage of the maximal contractile response to 120 mM of KCl (g) obtained at the end of the tension experiments. In each group, $n=5-8$. *$P<0.05$ compared with untreated rings. †$P<0.05$ compared with endothelium-intact rings.

SQ 29548 (3 μM). These concentrations were selected based on previously reported inhibitory activity (Yang *et al*., 2003). Allopurinol (1 and 10 μM) was used to explore the role of xanthine oxidase.

Each intervention was carried out either acutely after rings developed spontaneous tone or by preincubation in the bath while stretching the tissue to its optimal tension. Following SNP washout, aortic rings were allowed to stabilize for 90 min. For acute treatment, readings were taken after 30–45 min to allow the maximum response to reach a plateau stage. In order to normalize the data from different rings, the contractile response to 120 mM of KCl was determined in each ring at the end of each experiment. The mean peak increase in tension (g) that developed spontaneously following washout of SNP was expressed as a percentage of the contractile response (g) to 120 mM KCl. In separate experiments, the specificity of the antioxidants SOD, apocynin, and catalase on spontaneous tone were tested (Table 1). Each intervention modified spontaneous tone, but did not modify phenylephrine nor 120 mM KCl-evoked responses. Similarly, in other experiments, L-NAME and VAS modified spontaneous tone but not agonist-evoked responses when expressed as a percentage of the maximum response to 120 mM KCl (data not shown). The effects of SQ29458 and furegrelate and of combinations on agonist-evoked contractions were not tested, but they did modify spontaneous tone as described in the results.

Detection of O_2^- by lucigenin chemiluminescence

O_2^- was measured by the lucigenin chemiluminescence method as described elsewhere (Pagano *et al*., 1995). Although a high concentration of lucigenin chemiluminescence has been reported to react with a variety of flavin-containing enzymes to yield O_2^- (Tarpey *et al*., 1999), it has been demonstrated that these phenomena do not exist when a low concentration of lucigenin is used (Li *et al*., 1998). Briefly, at the end of selective organ bath experiments, rings were rinsed with Kreb's buffer and transferred to test tubes containing 500 μl of HEPES buffer (pH 7.4) at 37°C containing lucigenin (5 μM). In some experiments, O_2^- production was measured after isolated aortic rings were incubated in the organ bath for 1 h without a preload (unstretched) and in rings subjected to a preload of 5 g for 1 h (stretched). The tubes were transferred to a luminometer set to read and integrate the arbitrary units of light emitted over a 30 s period. Repeated measurements were collected during 5 min and averaged. A cell permeable nonenzymatic scavenger of O_2^-, tiron (10 mM), was then added to quench the O_2^--dependent chemiluminescence. Repeated measurements were taken again and eight readings over 5 min were averaged. The difference between the initial set of readings and the readings after tiron was taken to calculate O_2^- production.

Protein preparation and Western blot analysis

A portion of the thoracic aorta was cleaned and stored in liquid nitrogen immediately after isolation. The frozen tissue was then homogenized in lysis buffer containing 50 mM Tris, pH 8, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride, and centrifuged at $1000\times g$ at 4°C for 15 min. The supernatant was transferred to a fresh tube and centrifuged at $30{,}000\times g$ at 4°C for 30 min. Protein concentration in the supernatant was determined using the Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.), and 50 μg of total protein was electrophoretically size separated on a 7% SDS–polyacrylmide gel and then transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, U.S.A.) at 4°C overnight. The membrane was blocked with 5% nonfat dry milk in TBS-T (pH 7.6, 20 mM Tris base, 137 mM NaCl, and 0.1% Tween 20) at room temperature for 1 h and then incubated with 1 : 2000 diluted monoclonal mouse anti-endothelial nitric oxide synthase (eNOS) antibody (Transduction Laboratories, Mississauga, ON, Canada) at 4°C overnight. The eNOS protein was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Baie d'Urfe, PQ, Canada). The membrane was then stripped in stripping buffer containing 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 7.6 at 50°C for 30 min and reprobed with 1 : 10,000 monoclonal mouse anti-β-actin antibody (Sigma Aldrich, Oakville, ON, Canada). The densitometric value of the samples was evaluated by densitometry (UN-SCAN-IT, SILK Scientific Corporation, Orem, Utah).

Immunohistochemistry

Sections of the thoracic aorta from DOCA-salt hypertensive rats and SHAM-normtensive rats were fixed in 4% paraformaldehyde in 0.1 M phophate buffer, pH 7.2 overnight at 4°C. After washing with phosphate-buffered saline (PBS), aortic sections were immersed serially in 10, 20, and 30% sucrose in PBS at 4°C until the tissues floated. The aorta was then embedded in OCT compound (VWR, Edmonton, AB, Canada) and frozen in liquid nitrogen, and stored at −80°C until analysis. Cross-sections of the aorta were cut into 6 μm and placed onto poly-L-lysine-coated slides. Before staining, slides were washed with 0.1% BSA in PBS and then were incubated with 0.1% Triton X-100 in PBS for 5 min at room temperature. The sections were blocked in 1 : 30 goat serum in

PBS for 30 min at room temperature. This was followed by incubating with 1 : 50 diluted monoclonal mouse anti-eNOS antibody (Transduction Laboratories, Mississauga, ON, Canada) and 1 : 100 diluted polyclonal rabbit anti-nitrotyrosine antibody (Molecular Probes, Eugene, OR, U.S.A.) at 4°C overnight. After washing with PBS, the sections were incubated at dark with either 1 : 200 diluted fluorescent anti-mouse (Jackson ImmunoResearch, Western Grove, PA, U.S.A.) or 1 : 200 diluted fluorescent anti-rabbit secondary antibody (Sigma, Saint Louis, MI, U.S.A.) at room temperature for 1 h.

Drugs

Lucigenin, L-NAME), SNP, catalase, acetylcholine, phenylephrine, tiron, tempol, SOD, apocynin, and indomethacin were purchased from Sigma Chemical Co, Oakville, ON, Canada. VAS and SQ 29,548 were obtained from Cyaman Chemical Company, Ann Arbor, MI, U.S.A. Peptides were purchased from American Peptide Company, Sunnyvale, CA, U.S.A. Kreb's solution salts were of analytical grade and obtained from BDH, Toronto, ON, Canada. All drug solutions and buffer were prepared fresh every day just before the experiment. Drugs added to the organ baths were in aliquots of $<1\%$ of the bath solution volume. All experiments were carried out with an appropriate vehicle control.

Data analysis

All values are expressed as means $\pm$ s.e.m. Comparisons between groups were based on analysis of variance (ANOVA). If ANOVA indicated significance, simultaneous multiple comparisons were based on Scheffe's multiple comparison procedure. When there were only two groups, statistical comparisons were carried out by Student's *t*-test. Significance was accepted when P was <0.05.

Results

Effect of DOCA treatment on BP

At the end of 3 weeks of the DOCA-salt treatment, the mean arterial pressure of conscious unrestrained DOCA-salt hypertensive rats was significantly higher than that recorded in SHAM-control rats (Table 2).

Preload and spontaneous tone

The development of spontaneous tone with increasing preload is shown in Figure 1. In all experiments, the preload was increased in 1 g increments to an optimal tension of 5 g. Tone developed spontaneously in rings from DOCA-salt hypertensive rats (Figure 1b, but not in rings from SHAM-control rats (Figure 1a). The increase in tone was often accompanied by oscillations in tension. The relationship between preload and spontaneous tone is shown in Figure 1c. In these experiments, a preload of 1, 3, or 5 g was set in the presence of SNP and the peak increase in tension following washout of SNP was plotted as a function of the preload. Tone increased as a function of the preload in DOCA-salt hypertensive rats, suggesting a direct relationship between stretch and contraction.

Table 2 Effect oral administration of antioxidant on mean arterial pressure, superoxide production, and spontaneous tone generation

Groups	*Mean arterial pressure (mmHg)*	*Superoxide anion production (mU mg−1 min−1)*	*Spontaneous tone (% of maximum contractile response to 120 mm KCl)*
SHAM control	107 $\pm$ 4 (16)	875 $\pm$ 135	1.25 $\pm$ 0.6
+ Tempol	103 $\pm$ 6 (6)	758 $\pm$ 199	1.00 $\pm$ 0.5
+ Tiron	96 $\pm$ 4 (6)	715 $\pm$ 73	0.50 $\pm$ 0.25
DOCA-salt	161 $\pm$ 10 (21)*	3166 $\pm$ 232*	39.2 $\pm$ 2.3*
+ Tempol	108 $\pm$ 5 (6)†	824 $\pm$ 265†	4.80 $\pm$ 2†
+ Tiron	99 $\pm$ 3 (6)†	702 $\pm$ 97†	0.00†
+ Apocynin	122 $\pm$ 0.9 (4)†	1041 $\pm$ 314†	9.30 $\pm$ 6.1†

Effect of 3 weeks of tempol (10^{-3} M) or tiron (10^{-2} M) on mean arterial pressure, superoxide anion generation, and spontaneous tone in SHAM-normotensive rats and DOCA-salt hypertensive rats, and of apocynin (1.5×10^{-3} M) in DOCA-salt hypertensive rats. The numbers in parentheses indicate number of animals for each group. *$P<0.05$ compared with the SHAM-normotensive control animals; $^{\dagger}P<0.05$ compared with DOCA control animals.

Role of extracellular calcium

Spontaneous tone generated in rings from DOCA-salt hypertensive rats was abolished in the presence of a calcium-free buffer. Replacing the buffer with calcium (2.6 mM) restored spontaneous tone (data not shown). In the presence of 2.6 mM calcium, the L-type calcium channel blocker, nifedipine (10^{-7} M) blocked ($n=5$, $P<0.001$) spontaneous tone (data not shown).

Role of endothelium and NO

In endothelium-denuded aortic rings from DOCA-salt hypertensive rats at 5 g resting tension, the developed peak tension was 2.9 $\pm$ 0.4 g, which represented 54.78 $\pm$ 4.9% of the maximum contraction to 120 mM KCl (5.36 $\pm$ 0.38 g). In endothelium-intact aortic rings, spontaneous tone was lower for any given preload compared to endothelium-denuded rings (Figure 1c). At a preload of 5 g, spontaneous tone reached 39.2 $\pm$ 3.1% of the maximal response evoked by 120 mM KCl in endothelium-intact rings. Maximal contractile responses to 120 mM of KCl were significantly lower in aortic rings from SHAM-normotensive rats (4.1 $\pm$ 0.4 g). Aortic rings from normotensive SHAM-control rats failed to develop tone after washing out SNP (1.1 $\pm$0.57% of KCl contraction).

Pretreatment or acute treatment of aortic rings from DOCA-salt hypertensive rats with L-NAME (300 μM) increased spontaneous tone. Spontaneous tone was 61.38 $\pm$ 8.9% of the maximal response evoked by 120 mM KCl in rings pretreated with L-NAME ($n=8$). In contrast, spontaneous tone was 40.22 $\pm$ 2.1% in untreated aortic rings with a functional NO system. The response in L-NAME-pretreated rings was virtually identical ($P=1$) to that observed in endothelium-denuded preparations. L-NAME did not increase spontaneous tone any further in endothelium-denuded aortic rings from DOCA-salt hypertensive rats.

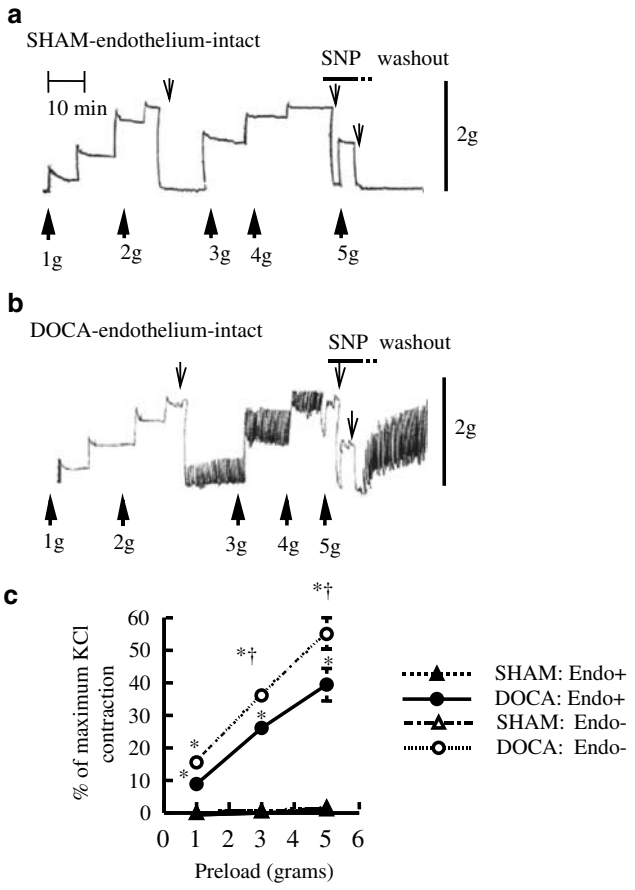

Figure 1 Relationship of preload to spontaneous tone in aortic rings isolated from SHAM-control rats (a) and DOCA-salt hypertensive rats (b) in the presence of the endothelium. The rings were stretched over a period of 1 h in 1 g increments (solid arrows) to an optimum tension of 5 g for development of smooth muscle tone. SNP (10^{-7} M) was added to the bath for the last 20 min to allow adjustment of preload under passive conditions. Arrows pointing down indicate readjustment of the baseline. Pooled data in the presence (Endo+) and absence (Endo−) of endothelium is shown in (c). In these experiments, a preload of 1, 3, or 5 g was set in the presence of SNP and the peak increase in tension following washout of SNP was plotted as a function of the preload. Values are mean ± s.e.m. expressed as a percentage of the maximal contractile response evoked by 120 mM of KCl. If not shown, error bars are within the range of the symbol. $^{*}P<0.05$ compared with SHAM-control group. $^{\dagger}P<0.05$ compared with the rings with intact endothelium.

Removal of the endothelium or pretreatment of rings with L-NAME failed to evoke spontaneous tone in aortic rings from SHAM-control rats.

Effect of DOCA treatment on e-NOS protein expression

e-NOS protein (Western blot analysis and immunohistochemistry) was present in the aorta of both DOCA-salt and SHAM rats. There was no significant difference in e-NOS protein expression in the aorta of DOCA-salt and SHAM rats (data not shown).

Effect of BQ-123 and BQ-788 on spontaneous tone

Pretreatment or the acute administration with the ET_A receptor antagonist, BQ123 (10 μM), and the ET_B receptor antagonist, BQ788 (1.2 nM, 1.3 and 10 μM), had no significant effect on the development of spontaneous tone in aortic rings from DOCA-salt hypertensive rats (data not shown). These antagonists together did attenuate contractions evoked by ET-1.

Effect of DOCA treatment on superoxide production

O_2^- production in isolated endothelium-denuded and endothelium-intact aortic rings incubated in the organ bath for 1 h without a preload (unstretched) and in rings subjected to a preload of 5 g for 1 h (stretched) is shown in Figure 2a. The basal superoxide production (unstretched rings) was much higher in rings isolated from the DOCA-salt hypertensive rat than in those isolated from SHAM rats. Exposure of the rings to a preload of 5 g for 1 h increased O_2^- production in the DOCA-salt group both in the presence and absence of endothelium. In the SHAM group, the tendency of increased superoxide generation in response to stretch was not statistically significant ($P=0.72$).

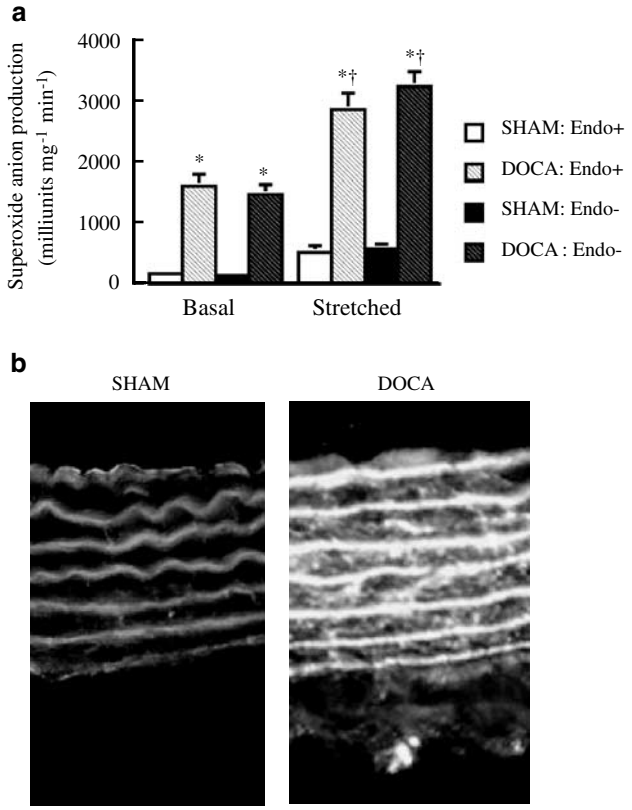

Figure 2 Superoxide production determined by the lucigenin chemiluminescence method in either unstretched (Basal) or stretched aortic rings of DOCA-salt hypertensive rats and SHAM-control rats in the presence (Endo+) and absence (Endo−) of endothelium (a). Values are mean ± s.e.m. expressed in arbitrary units (mg^{-1} min^{-1}). $^{*}P<0.001$ compared with superoxide level in normotensive SHAM rats. $^{\dagger}P<0.001$ compared with the basal superoxide level in unstretched rings form DOCA-salt hypertensive rats. (b) Immunohistochemical localization of 3-nitrotyrosine in aorta of SHAM-normotensive and DOCA-salt hypertensive rats. 3-Nitrotyrosine staining was increased in all layers of the aortic wall in DOCA-salt hypertensive rats. Original magnification ×40. The images shown are representative of similar results observed in preparations from six to eight rats.

O_2^- production was unaffected by either L-NAME pretreatment or endothelium denudation in aortic rings from DOCA-salt hypertensive rats compared with untreated rings (Figure 5). Pretreatment with nifedipine did not affect superoxide production in either endothelium-intact and endothelium-denuded aortic rings from DOCA-salt hypertensive rats or SHAM-normotensive rats.

Localization of 3-nitrotyrosine by immunohistochemistry

3-Nitrotyrosine protein moieties were assessed as a marker of oxidative stress to confirm the chemiluminescence findings. Immunohistochemistry performed with a polyclonal antibody raised against 3-nitrotyrosine to localize 3-nitrotyrosine revealed greater staining in endothelium, smooth muscle layers, and adventitia, in aorta from DOCA-salt hypertensive rats compared with SHAM-normotensive rats (Figure 2b). Immunoreactivity was not observed when the anti-3-nitrotyrosine antibody was preincubated with 3-nitrotyrosine antibody (10 mM) or when the primary antibody was omitted, indicating that the staining was specific.

Effect of chronic oral administration of tempol, apocynin, and tiron

A 3-week administration of tempol, apocynin, and tiron into the drinking water prevented the increase in the mean BP in DOCA-salt hypertensive rats (Table 2). O_2^- generation was increased dramatically in aorta isolated from DOCA-salt hypertensive rats. Treatment of DOCA-salt hypertensive rats with tempol, tiron, or apocynin reduced the generation of O_2^- in aorta from DOCA-treated rats to rates that were not significantly different from those observed in untreated SHAM-normotensive rats. Aortic rings from these tempol-, apocynin-, and tiron-treated DOCA-salt hypertensive rats did not generate any spontaneous tone in tension studies even with endothelium removed. Tempol and tiron did not have a significant effect on BP, O_2^- generation, or spontaneous tone in SHAM-normotensive rats. Apocynin was not tested in SHAM-control rats.

Effect of administration of SOD, tempol, and apocynin to organ baths

SOD (150 U ml^{-1}) did not prevent the generation of spontaneous tone in endothelium-denuded (Figure 3c) aortic rings, or in L-NAME-treated aortic rings (data not shown), from DOCA-salt hypertensive rats when added directly to the organ bath. In contrast, SOD abolished spontaneous tone when endothelial function remained intact (Figure 3b and c). In other rings, SOD (150 U ml^{-1}) administered acutely after spontaneous tone had developed, resulted in dramatic decreases in tone. SOD had no effect in aortic rings from SHAM-control rats.

Another cell permeable scavenger of O_2^-, tempol (100 μM) also inhibited spontaneous tone in endothelium-intact aortic rings from DOCA-salt hypertensive rats and also had no effect on the tone in endothelium-denuded and L-NAME-treated aortic rings (data not shown).

Pretreatment of aortic rings from DOCA-salt hypertensive rats with the NADPH-oxidase inhibitor, apocynin (100 μM), had no effect on the generation of spontaneous tone in the absence of the endothelium (Figure 4c) or in L-NAME-treated aortic rings (data not shown). In contrast, apocynin abolished spontaneous tone when endothelial function remained intact (Figure 4b and c). Apocynin had no effect in the aortic rings from SHAM-control rats.

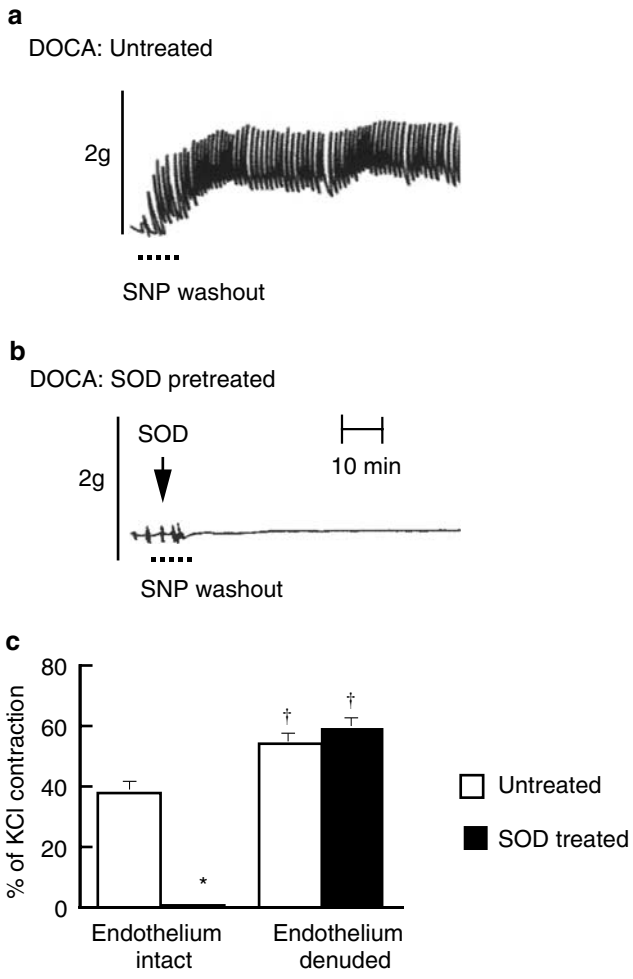

Figure 3 Spontaneous tone in aortic rings isolated from DOCA-salt hypertensive rats when a ring was untreated (a) and when a ring was pretreated with 150 U ml^{-1} of SOD (b). Pooled values expressed as a percentage of the maximal contractile response to 120 mM KCl are shown in (c). Values are mean$\pm$s.e.m., $n=7$ in each group. If not shown, error bars are within the height of the symbol. $^*P<0.01$ compared with SOD-untreated rings. $^\dagger P<0.05$ compared with endothelium-intact-untreated rings. SNP – sodium nitroprusside.

O_2^- generation was decreased dramatically in aortic rings from DOCA-salt hypertensive rats that were treated with SOD or apocynin (Figure 5). Indeed, there was no significant difference in O_2^- generation among aortic rings from SHAM-control rats (data not shown) and SOD- or apocynin-treated aortic rings from DOCA-salt hypertensive rats. In endothelium-denuded and L-NAME-treated rings from DOCA-salt hypertensive rats, SOD, and apocynin also decreased the generation of O_2^- dramatically (Figure 5) even though they did not affect the spontaneous tone (Figure 4c).

Effect of xanthine oxidase inhibition

Acute administration or pretreatment of aortic rings from DOCA-salt hypertensive rats and SHAM-normotensive rats

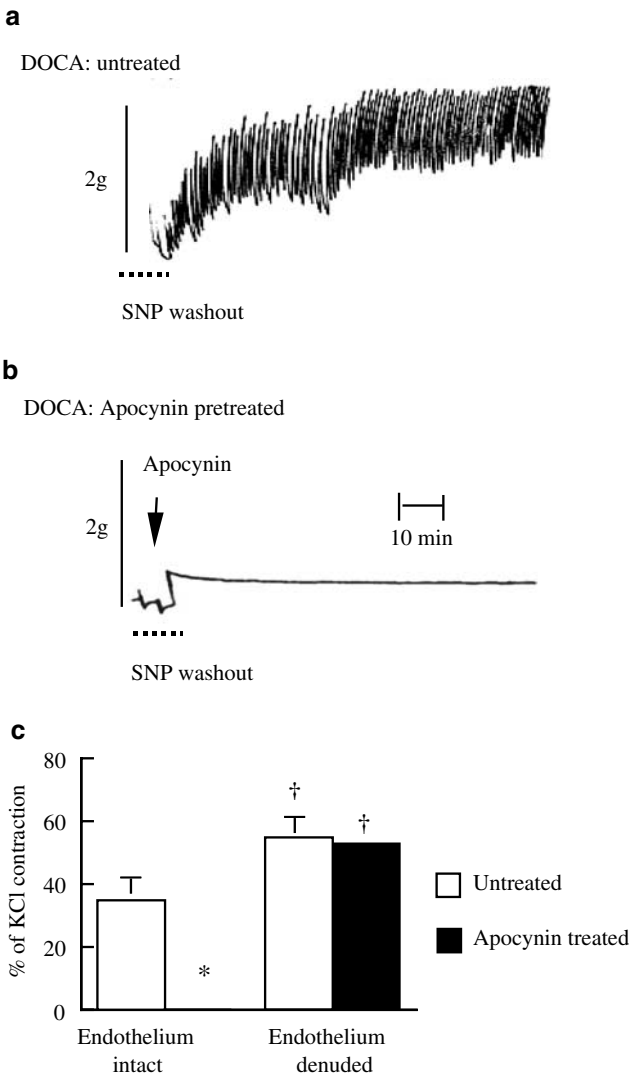

Figure 4 Spontaneous tone in aortic rings isolated from DOCA-salt hypertensive rats when a ring was untreated (a), and when a ring was pretreated with 100 μM of apocynin (b). Pooled values are expressed as a percentage of maximal contractile response to 120 mM KCl (c). Values are mean $\pm$ s.e.m., $n = 5$ in each group. If not shown, error bars are within the height of the symbol. $^{*}P < 0.01$ compared with apocynin-untreated rings. $^{\dagger}P < 0.05$ compared with endothelium-intact-untreated rings. SNP – sodium nitroprusside.

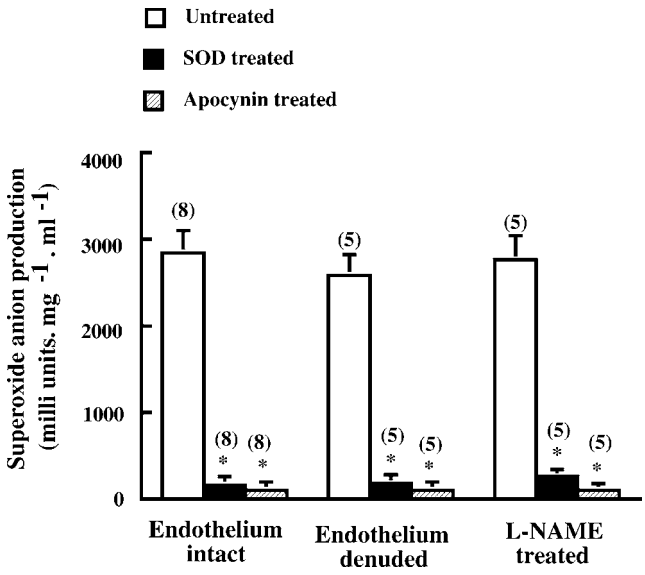

Figure 5 Effect of 150 U ml^{-1} of SOD and 100 μM of apocynin on the superoxide production determined by the lucigenin chemiluminescence method in endothelium-intact, endothelium-denuded, and L-NAME-treated aortic rings from DOCA-salt hypertensive rats. Values are expressed as mean $\pm$ s.e.m. The number on the top of each column indicates number of animals for that group. $^{*}P < 0.001$ compared with superoxide level in SOD/apocynin-untreated rings.

with allopurinol (1 and 10 μM), an inhibitor of xanthine oxidase, did not have any significant effect on spontaneous tone either in the presence or absence of endothelium (data not shown). Allopurinol also had no significant effect on the O_2^- generation in both groups of rats (data not shown).

Effect of catalase on spontaneous tone

The role of endogenous H_2O_2 in the modulation of spontaneous tone and O_2^- levels was tested in rings treated with catalase. In aortic rings from DOCA-salt hypertensive rats, catalase (1000 U ml^{-1}) increased spontaneous tone significantly (Figure 6b). Catalase also increased tone significantly in endothelium-denuded, SOD-treated and L-NAME-treated aortic rings from DOCA-salt hypertensive rats, but did not increase tone in apocynin-treated rings (Figure 6c). In aortic rings from SHAM-normotensive rats, catalase evoked a significant but a small increase in tone (10.35 $\pm$ 2.98% of KCl contraction).

Effect of cyclooxygenase (COX) and TP receptor antagonist

Acute administration or pretreatment of the aortic rings with the COX inhibitor, VAS (3 mM), or the TP receptor antagonist, SQ 29548, had only a modest or no effect on the generation of spontaneous tone in endothelium-denuded aortic rings from DOCA-salt hypertensive rats (Figure 7d). In contrast, both VAS and SQ29548 abolished spontaneous tone in aortic rings with an intact endothelium (Figure 7b–d). A TXA_2 synthase inhibitor, furegrelate (50 μM), had no effect on the spontaneous tone in either endothelium-intact or endothelium-denuded aortic rings from DOCA-salt hypertensive rats. In aortic rings from SHAM-control rats, these agents had no significant effect.

Importantly, O_2^- generation was not significantly different in aortic rings treated with VAS or SQ 29548 compared with the untreated rings (data not shown).

Discussion

The tension that developed spontaneously in aortic rings isolated from the DOCA-salt hypertensive rats was directly proportional to the preload. Tension increased dramatically as the preload was increased from 1 to 5 g. The final adjustment of the preload was determined in the presence of SNP to ensure that the preload was set in the relaxed passive state, a method described previously by others (Di Wang *et al*., 1999).

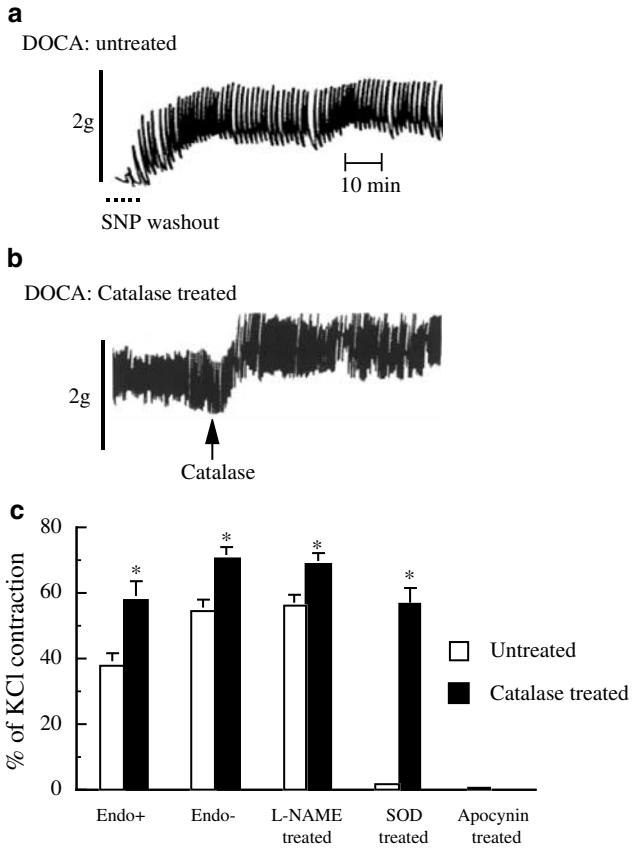

Figure 6 Spontaneous tone in aortic rings isolated from DOCA-salt hypertensive rats when the ring was untreated (a) or when the ring was treated acutely with 1000 U ml^{-1} of catalase (b). (c) The pooled data expressed as percentages of the maximal contractile response to 120 mM KCl in rings untreated or treated with catalase. If not shown, error bars are within the height of the symbol. Values are mean ± s.e.m., $n=6$ in each group. $^{*}P<0.05$ compared with untreated rings. SNP – sodium nitroprusside.

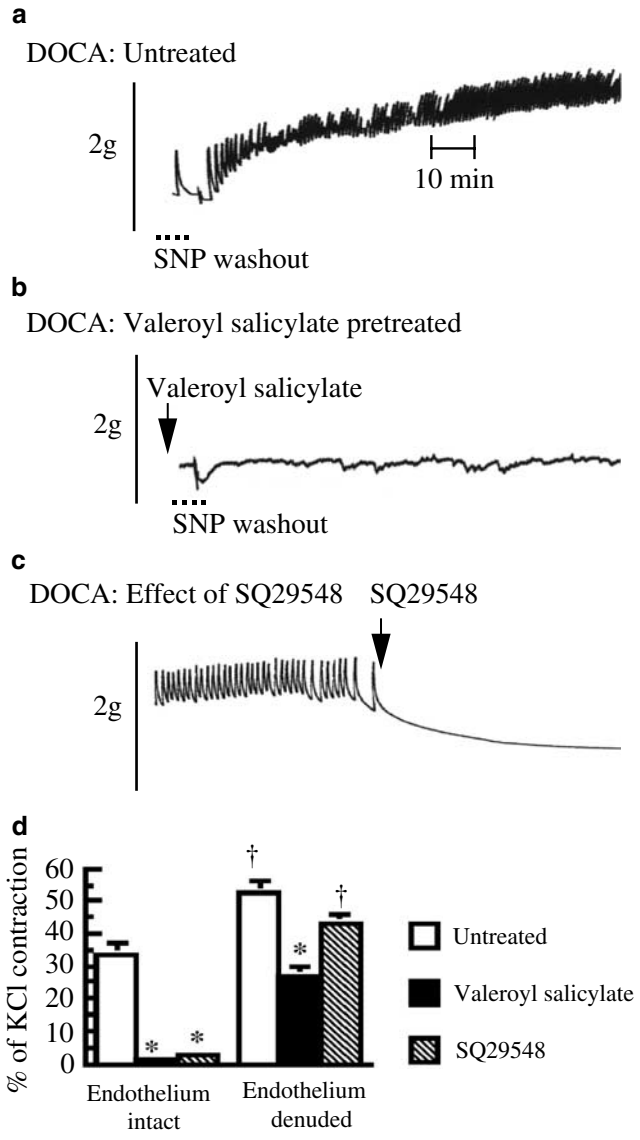

Figure 7 Spontaneous tone in aortic rings isolated from DOCA-salt hypertensive rats when a ring was untreated (a), when a ring was pretreated with 3 mM of VAS (b), and in a ring treated acutely with 3 μM of SQ 29548 (c). (d) Pooled values expressed as a percentage of maximal contractile response to 120 mM KCl in the presence and absence of endothelium. If not shown, error bars are within the height of the symbol. Values are mean ± s.e.m., $n=4$ in each group. $^{*}P<0.05$ compared with untreated rings. $^{\dagger}P<0.05$ compared with endothelium intact rings. SNP – sodium nitroprusside.

Washout of the SNP resulted in reproducible increases in active tension. The development of active tension in response to stretch appears to be *the vitro* correlate of myogenic tone. In small resistance arteries, the resistance of the vessel changes in response to changes in transmural pressure (Scotland *et al.*, 2001). When the transmural pressure increases, these vessels respond to the elevated pressure by constricting, a phenomenon termed myogenic tone (Bayliss, 1902). It has been suggested that there is a calcium transient in smooth muscle of pressurized resistance arteries during myogenic tone (Miriel *et al.*, 1999). A similar phenomenon has been described in larger arteries of hypertensive animals (Sunano *et al.*, 1992; 1996). In our study, removal of extracellular calcium from the buffer inhibited the tone completely and addition of calcium restored the tone. This confirms the finding of other investigators that described spontaneous tone as a calcium-dependent phenomenon (Hwa & Bevan, 1986b; Rinaldi & Bohr, 1989; Di Wang *et al.*, 1999). Since nifedipine prevented the tone, influx of extracellular calcium through L-type calcium channels appears to be critical for generation of spontaneous tone. However, nifedipine did not interfere with superoxide generation in either SHAM-control rats or DOCA-salt hypertensive rats. Thus, a significant role of L-type calcium channel in the increased generation of O_2^- observed in DOCA-salt hypertensive rats can be excluded.

Whether spontaneous tone is a consequence of, or a cause of, the hypertensive state is uncertain. The increase in tension in response to stretch appears to be specific to the hypertensive state, since spontaneous tone failed to develop in aortic rings isolated from SHAM-control rats even at the highest preload of 5 g. Importantly, rats treated with DOCA and salt remained normotensive when treated chronically with tempol or apocynin, and aortic rings from these rats failed to develop spontaneous tone whether the endothelium was intact or removed. In contrast, when hypertension was allowed to develop, acute treatment of aortic rings with tempol or

apocynin abolished spontaneous tone when the endothelium remained intact, but failed to abolish tone when the endothelium had been removed. These findings are more consistent with the interpretation that spontaneous tone is a consequence of hypertension and not the DOCA-salt regimen *per se*. It follows that spontaneous tone should be present in all models of hypertension, an assertion that appears consistent with those hypertensive models that have been tested. Spontaneous tone in aorta from the SHR stroke-prone strain has been reported: active tone was 40% of potassium-induced contractions (Sunano *et al*., 1996). Spontaneous tone has also been reported in the Ang II-infused model of hypertension, where spontaneous tone was $52 \pm 5.6\%$ of KCl-induced contractions (Di Wang *et al*., 1999). Chronic treatment of rats with the alpha and beta blocking agent, carvedilol, prevented the development of hypertension in SHRSP and the associated development of spontaneous tone (Sunano *et al*., 1997). Finally, the ET_A/ET_B antagonist, bosentan, has been reported to reduce total peripheral resistance and BP dramatically in conscious unrestrained DOCA-salt hypertensive rats (Yu *et al*., 2001b), yet ET_A- and ET_B receptor antagonism had no effect on spontaneous tone when administered acutely to isolated aortic rings in our experiments. In the final analysis, the possibility that spontaneous tone may contribute to the hypertensive state cannot be excluded, but the evidence appears more consistent with the interpretation that spontaneous tone is a consequence of hypertension.

The development of spontaneous tone appears to be independent of the endothelium because spontaneous tone was present both in the presence and absence of the endothelium. Nevertheless, the possibility that endothelial-derived vasoconstrictor or vasodilator factors might contribute to or modulate the tone cannot not be excluded. It has been suggested that ET can increase the generation of O_2^- in DOCA-salt hypertensive rats by stimulation of NADPH oxidase (Li *et al*., 2003). However,ET-1 does not appear to contribute to spontaneous tone directly because preincubation or acute administration of the ET_A receptor antagonist, BQ123, or the ET_B receptor antagonist, BQ788, did not have any effect on spontaneous tone. Moreover, endothelial denudation increased the magnitude of spontaneous tone. We conclude that ET-1 *per se* does not contribute to spontaneous tone in DOCA-salt hypertensive rats.

In contrast, endothelial-derived NO plays an important role in limiting the development of spontaneous tone (Figure 8). Several observations are consistent with this conclusion. Firstly, removal of the endothelium increased spontaneous tone. Secondly, inhibition of NOS by L-NAME increased tone in the endothelium-intact preparation. Thirdly, in endothelium-denuded preparations L-NAME did not increase the tone any further. Thus, NO appears to be an important modulator of spontaneous tone. Initially, it was tempting to postulate that a deficiency in the NO system in the DOCA-salt hypertensive rat accounted for the development of spontaneous tone. However, e-NOS protein expression was similar in DOCA-salt and SHAM rats. Moreover, endothelium-denudation and L-NAME did not generate spontaneous tone in rings from normotensive SHAM-control rats. These findings indicate that a deficiency of the NO system *per se* cannot account for the development of spontaneous tone. Nevertheless, NO does act to suppress tone in the DOCA-salt model.

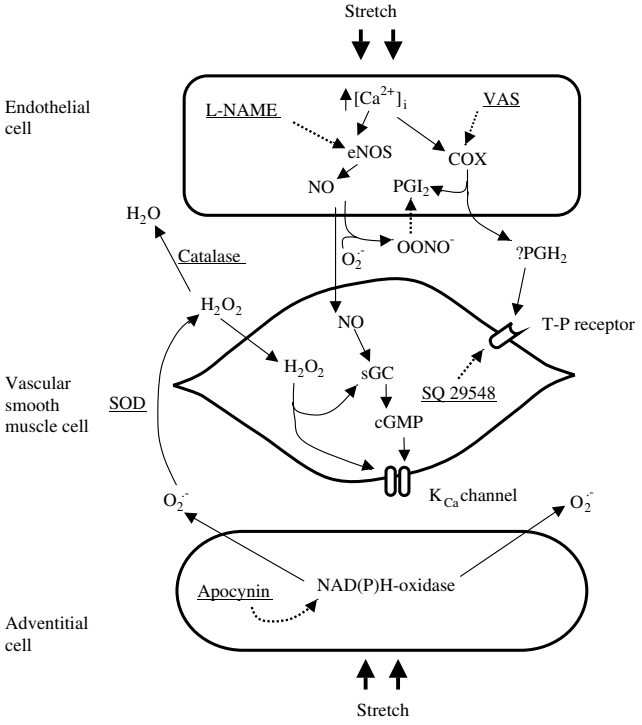

Figure 8 Proposed schematic suggesting the mechanism for spontaneous tone and interaction of various modulators in the generation of spontaneous tone in the aorta of DOCA-salt hypertensive rats. $[Ca^{2+}]_i$ – cytosolic-free calcium; K_{Ca} – calcium-activated potassium channel; sGC – soluble guanylyl cyclase; cGMP – cyclic 3′; 5′-guanosine monophosphate; eNOS – endothelial nitric oxide synthase; L-NAME – N^G-nitro-L-arginine methyl ester; H_2O_2 – hydrogen peroxide; $OONO^-$ –peroxynitrite; NO – nitric oxide; T/P – thromboxane-prostaglandin; O_2^- – superoxide anion; PGH_2 – prostaglandin H_2; VAS – VAS (see text for details).

We suggest that spontaneous tone in aortic rings of DOCA salt hypertensive rat depends on increased O_2^- generation (Figure 8). This conclusion is based on the following observations. Firstly, O_2^- production was significantly increased in aortic rings of DOCA-salt hypertensive rats compared to SHAM-control rats. The increased O_2^- production agrees with previous work where it had been reported that vascular superoxide generation is high in DOCA-salt hypertensive rats (Somers *et al*., 2000; Wu *et al*., 2001). Secondly, preincubation with the O_2^- scavenger, SOD, completely prevented spontaneous tone in aortic rings in the presence of endothelium. We also confirmed that SOD treatment resulted in decreased O_2^- generation. Thirdly, stretch increased the generation of O_2^- in rings from DOCA-salt hypertensive rats even above the elevated basal levels observed in these rats. Thus, stretch was associated with parallel increases in O_2^- generation and spontaneous tone in the DOCA-salt animals. In contrast, stretch failed both to generate O_2^- and to evoke spontaneous tone in SHAM rats.

Potential mechanisms contributing to the enhanced generation of O_2^- generation include eNOS, xanthine oxidase, and NADPH oxidase. The production of O_2^- has been ascribed to uncoupled eNOS in SHRSP (Kerr *et al*., 1999). However, in our experiments, inhibition of e-NOS with L-NAME did not interfere with the generation of O_2^-. Moreover, L-NAME reduces NO production, and consequently, the formation of

peroxynitrite ($OONO^-$). Since spontaneous tone was not reduced in the aortic rings treated with L-NAME, it is also unlikely that $OONO^-$ accounts for spontaneous tone in DOCA-salt hypertensive rats. Recently, it has been shown that $OONO^-$ can inactivate prostacyclin synthase (Zou *et al*., 2002). Since, COX inhibitors decreased the tone, we cannot rule out the possibility that $OONO^-$ had an indirect effect on the generation of spontaneous tone. Inhibition of COX and xanthine oxidase also had no effect on the production of O_2^- in both stretched and unstretched rings from DOCA-salt hypertensive rats. Therefore, our data indicate that it is unlikely that these enzymes contribute in a significant way to the increased generation of O_2^- in aorta of DOCA-salt hypertensive rats. On the other hand, it has been reported that NADPH oxidase activity is increased in the aorta of DOCA-salt hypertensive rats and that this increase is associated with elevated superoxide production (Beswick *et al*., 2001a). In our study, the NADPH-oxidase inhibitor, apocynin, prevented the generation of spontaneous tone in aortic rings with intact endothelium, and it inhibited the generation of O_2^- in both stretched and unstretched aortic rings from DOCA-salt hypertensive rats. In view of these considerations, we conclude that NADPH-oxidase derived O_2^- generation is a major contributor to the development of spontaneous tone in the DOCA-salt hypertensive rat.

The site accounting for O_2^- generation by NADPH oxidase is unclear. NADPH oxidase is expressed in endothelial cells (Zafari *et al*., 1998), vascular smooth muscle cells (Griendling *et al*., 1994; Zalba *et al*., 2000), and cells in the adventitial layer (Di Wang *et al*., 1999). NADPH oxidase can also be expressed by infiltrating neutrophils (Clozel *et al*., 1991) and macrophages/monocytes (Dinauer *et al*., 1987). In addition, an increased inflammatory response and macrophage/monocyte activity has been reported in the DOCA-salt model of hypertension (Beswick *et al*., 2001b). In our immunohistochemistry study, we found that 3-nitrotyrosine was significantly higher in aortic walls of DOCA-salt hypertensive rats compared to that of SHAM-normotensive rats, and it was distributed in all layers of the wall of aorta. We also found that the removal of endothelium did not interfere with the increase O_2^- production in aortic rings from DOCA-salt hypertensive rats. In endothelium-denuded rings, SOD and apocynin did not have any effect on the spontaneous tone, but decreased the generation of O_2^- significantly. These two observations suggest that the endothelial layer is not the major source for O_2^- in this rat model of hypertension. Although the exact location of NADPH oxidase was not determined in the present study, various observations suggested that either adventitial fibroblasts or vascular smooth muscle cells or both contribute to the increase in O_2^- production in DOCA-salt hypertensive rats.

The mechanisms by which O_2^- contributes to spontaneous tone may be related, at least in part, to its interaction with NO. The production of NO appears unimpaired in DOCA-salt hypertensive rats because e-NOS protein expression is similar in both groups of rats, and because L-NAME and endothelial denudation increased the magnitude of tone in these rats. We also observed that SOD failed to decrease spontaneous tone in the rings that were pretreated with L-NAME or in endothelium-denuded rings, although it was able to decrease the level of O_2^- in those rings. Therefore, we conclude that SOD decreased spontaneous tone in endothelium-intact rings by scavenging O_2^- and thereby freeing endothelial-derived NO to relax the tissue. It is also possible that in DOCA-salt hypertensive rats, increased O_2^- is responsible for increased calcium influx in vascular smooth muscle cells because the maximum contractile response induced by 120 mM KCl is significantly higher in aortic rings of DOCA-salt hypertensive rats compared to that in SHAM-normotensive rats.

The possibility that H_2O_2 might play a role in modulating spontaneous tone merits consideration (Figure 8). SOD is known to catalyze the formation of H_2O_2 from O_2^- and H_2O. Opinion on the vascular effects of H_2O_2 is controversial. It has been reported to contract isolated rat aortic rings (Yang *et al*., 1998; Shen *et al*., 2000). On the contrary, H_2O_2 has also been suggested to be a vasodilator in rat aorta (Yang *et al*., 1999), rabbit aorta (Bharadwaj & Prasad, 1995), and porcine coronary arteries (Hayabuchi *et al*., 1998). In our functional study, catalase, a scavenger of H_2O_2 evoked an increase in the intensity of spontaneous tone in aortic rings from DOCA-salt hypertensive rats when endothelium was removed or in rings pretreated with L-NAME or SOD. Importantly, catalase did not evoke any increase in tone when rings were pretreated with the NADPH-oxidase inhibitor, apocynin, a situation characterized by the lack of production of O_2^-. Since O_2^- is an important precursor in the formation of H_2O_2, the lack of effect of catalase in apocynin-treated vessels is not surprising. Accordingly, we suggest that in the stretched aortic rings of DOCA-salt hypertensive rats, any endogenous H_2O_2 formed from the interaction of O_2^- and H_2O acts as a vasodilator to protect the tissue against generation of spontaneous tone.

In addition to oxidative stress, it is apparent that a COX product contributes to the development of spontaneous tone, since both indomethacin and VAS reduced the tone as did the TP antagonist. The effects of these products are mostly endothelium dependent, although in the endothelial denuded rings VAS had a small but significant inhibitory effect on the tone. While detailed studies on the nature of this COX product is beyond the scope of this study, it is clear that, in addition to O_2^-, a COX product also contributes to the development of spontaneous tone in the DOCA-salt hypertensive rat.

In summary, aortic rings from DOCA-salt hypertensive rats but not those from SHAM-normotensive rats develop spontaneous tone in response to stretch and in the absence of any agonist. Figure 8 is a proposed scheme to summarize the complex interaction of various factors modulating spontaneous tone in aortic rings from DOCA-salt hypertensive rats. The endothelium, and in particular endothelial-derived NO, plays a protective role limiting the development of tone. However, the activity of the NO system *per se* is insufficent to prevent spontaneous tone. Strikingly, an increase in NADPH-derived O_2^- appears to be a major factor in the generation of spontaneous tone. At least part of the effect of O_2^- appears to be related to its interaction with NO. The generation of H_2O_2 from O_2^- appears to be a mechanism that mitigates the development of spontaneous tone by O_2^-. Finally, a COX component also appears to contribute to the development of spontaneous tone in the DOCA-salt hypertensive rat. These events may contribute to the pathogenesis and impaired vascular reactivity in mineralocorticoid hypertension.

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