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Tempol, an antioxidant, restores endothelium-derived hyperpolarizing factor-mediated vasodilation during hypertension

Ayotunde S.O. Adeagbo[a,*], Irving G. Joshua[a], Cameron Falkner[b], Paul J. Matheson[a,c]

[a] *Department of Physiology and Biophysics, Health Sciences Center, A-1214, University of Louisville, Louisville, KY 40292, USA*
[b] *Department of Biochemistry and Molecular Biology, University of Louisville, Louisville, KY 40292, USA*
[c] *Louisville Veterans Affairs Medical Center, Louisville, KY 40206, USA*

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Abstract

Acetylcholine releases a non-prostanoid endothelium-derived hyperpolarizing factor (EDHF) and nitric oxide from physiological salt solution perfused rat mesenteric arteries. This study reports an impairment in EDHF-mediated vasodilation in deoxycorticosterone acetate (DOCA)-salt hypertensive versus control normotensive rats. Nitric oxide-mediated vasodilation to acetylcholine was not altered in the animals. We hypothesize that free radical species generated as by-products of arachidonic acid metabolism contribute to impaired EDHF-mediated dilation in DOCA-salt hypertension. With or without reduced nicotinamide adenine dinucleotide phosphate (NADPH) as co-factor, arterial microsomes generate free radical species upon incubation with arachidonic acid. The production of free radicals was significantly higher in DOCA-salt versus control rat microsomes, and was totally eliminated by addition of cyclooxygenase-2 inhibitors NS-398 or celecoxib at 30 μM. Treatment of DOCA-salt rats with tempol (an antioxidant; 15 mg/kg, i.p., 21 days) alleviates hypertension; improves acetylcholine-induced EDHF-mediated vasodilation in DOCA-salt rats, and decreases arachidonic acid-driven microsomal free radical production. Serum level of 8-isoprostanes is elevated in DOCA-salt hypertension versus control or sham-salt rats, and the increase was reversed by tempol treatment. These results show that EDHF-mediated dilation of rat mesenteric arteries is impaired in DOCA-salt induced hypertension. Our data also suggest that cyclooxygenase-2 mediates free radical production, and that free radicals modulate the EDHF-mediated vascular response in DOCA-salt induced hypertension.

Keywords: Endothelium-derived hyperpolarizing factor; DOCA-salt hypertension; Tempol; Free radical species; Mesenteric vascular bed

1. Introduction

Endothelium-dependent vasodilation is impaired in human (Miyoshi et al., 1997; Shimokawa, 1998; Lind et al., 2000) and animal forms of hypertension (Fujii et al., 1992; Kimura and Nishio, 1999), including deoxycorticosterone acetate (DOCA)-salt-induced hypertension (Millette et al., 2000). The mechanisms proposed include: altered nitric oxide synthase activity (Sullivan et al., 2002); increased production of endothelium-derived prostanoid contracting factors and endothelins (Luscher, 1990); increased generation/availability of superoxide anions (Somers et al., 2000); and decreased influence of endothelium-derived hyperpolarizing factor (EDHF) (Fujii et al., 1992). These mechanisms vary in importance with different types of blood vessels and with different forms of hypertension. In the spontaneously hypertensive rats (SHR), for example, blunted endothelium-dependent relaxation found in the aorta is due to increased formation of contractile endoperoxide products (Luscher, 1990; Cordellini, 1999), while in rat mesenteric arteries, blunted hyperpolarization (Fujii et al., 1992) and/or altered nitric oxide synthase 3 distribution (Sullivan et al., 2002) accounts for impaired endothelium-dependent vasodilation.

Acetylcholine elicits dilation of perfused rat mesenteric arterial bed by releasing two distinct, non-prostanoid relaxing factors (Adeagbo and Triggle, 1993). The first, described as endothelium-derived hyperpolarizing factor (EDHF), is insensitive to nitric oxide synthase inhibitors, and vasodilates by opening K_{Ca} channels. The second can be inhibited by nitric oxide synthase inhibitors when extracellular K^+concentration is raised to about 25 mM and is characterized as endothelium-derived nitric oxide (EDNO).

* Corresponding author. Tel.: +1-502-852-7561; fax: +1-502-852-6239.
E-mail address: asadea01@louisville.edu (A.S.O. Adeagbo).

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In the present study, we first determined the influence of DOCA-salt induced hypertension on EDHF- and EDNO-mediated components of acetylcholine-induced vasodilation.

Elevated production of free radical species contributes to the onset or progression of spontaneous (Kerr et al., 1999), Dahl salt-sensitive (Swei et al., 1999) and mineralocorticoid-salt-induced (Somers et al., 2000; Beswick et al., 2001a,b) hypertension in rats, as well as development of hypertension in humans (Kumar and Das, 1993; Lacy et al., 2000). Free radical species are generated as by-products during the oxidative metabolism of either arachidonic acid by cyclo-oxygenases, lipoxygenases and cytochrome *P*450 enzymes (Puntarulo and Cederbaum, 1998), or L-arginine by nitric oxide synthases (Porasuphatana et al., 2003), and during activation of NADH/NADPH-dependent oxidases by hypertension (Beswick et al., 2001a; Li et al., 2003). Since endothelium-dependent vasodilation of perfused rat mesenteric bed is accompanied by the release of arachidonic acid (Adeagbo et al., 2001), which we presume as the precursor for EDHF synthesis, we hypothesize that free radical by-products of cytochrome *P*450-mediated metabolism of arachidonic acid contribute to endothelium dysfunction during DOCA-salt-induced hypertension. We tested this hypothesis by investigating the effects of a broad spectrum antioxidant, tempol (Mitchell et al., 1990; Laight et al., 1997; Zhang et al., 1998), on blood pressure and on EDHF-mediated vasodilation of DOCA-salt treated rats, and by measuring microsomal generation of free radical species from arachidonic acid under different experimental paradigms.

2. Methods

2.1. General animal care

Our experimental procedures conform to "Guiding Principles for Research Involving Animals and Human Beings" of the American Physiological Society (2002). Male Sprague–Dawley rats (Harlan, Indianapolis) were maintained on standard rat diet and water ad libitum for at least 1 week in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved facilities before use for experiments. The project was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Louisville. Acute experiments were started in the morning on rats (225–305 g) that had been fasted overnight.

2.2. Experimental hypertension

Experiments were performed on four groups of age-matched, male Sprague–Dawley rats. Group I (control): rats with two kidneys; fed normal rat chow and water drink. Groups II, III and IV rats were uninephrectomized surgically under pentobarbital (50 mg/kg; i.p) anesthesia. Group II (sham-salt): implanted s.c with placebo pellets, and fed high NaCl + K diet. Group III (DOCA-salt): implanted s.c with DOCA (100 mg) 21-day-release pellets and fed with high NaCl + K diet. Group IV (DOCA-salt/Tempol): implanted s.c with DOCA (100 mg) 21-day-release pellets and fed with high NaCl + K diet plus daily intra-peritoneal administration of tempol (15 mg/kg) for 21 days prior to use in experimental protocols. Blood pressures of all groups of rats were measured indirectly by a digital tail cuff plethysmography (Letica; model 5001).

2.3. Vascular perfusion studies

Mesenteric vascular beds of rats were excised under pentobarbitone (60 mg/kg, i.p.) anesthesia and perfused (5 ml/min; Masterflex peristaltic pump) in vitro with physiological salt solution at 37 °C. The physiological salt solution is composed of the following (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 12.5 and glucose, 11.1. Excess (25 mM) K^+ depolarizing physiological salt solution was prepared by substituting an equimolar amount of K^+ for Na^+; the pH of all physiological salt solution after bubbling with 95% O_2, 5% CO_2 gas mixture was 7.4. Vascular beds were routinely perfused with physiological salt solution, which in EDHF protocols contained nitro-L-arginine methyl ester (L-NAME; 100 μM) plus indomethacin (5 μM) to block endogenous syntheses of nitric oxide and prostanoid, respectively. For experimental protocols involving endothelium-derived nitric oxide (EDNO) investigations, high (25 mM) K^+ physiological salt solution was used to perfuse the vascular beds. Perfusion pressures were recorded with Statham pressure transducers coupled to a Grass polygraph (model 7H).

In all relaxation studies, we generated vascular tone by infusion of an α_1-adrenoceptor selective agonist, cirazoline (0.2–0.5 μM). Acetylcholine, 1-ethyl-2-benzimidazolinone (1-EBIO) or 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl) phenyl]-2*H*-benzimidazol-2-one (NS-1619) was injected into the perfused vascular beds in bolus doses and the dilator responses (expressed as % of prevailing tonus) were compared in the groups of rats used. The effects of the dilator agonists were also examined in the absence or during infusion of apamin (0.5 μM) plus charybdotoxin (0.1 μM) in order to ascertain the dependence or otherwise of the responses on the opening of K_{Ca} channels.

2.4. Preparation of arterial microsomes

Mesenteric vessels were minced with a sharp pair of scissors in a beaker containing 20-ml 0.1 M phosphate plus 0.15% KCl buffer (homogenization buffer; pH 7.4). The buffer contained phenylmethylsulfonyl fluoride and a cocktail of six other protease inhibitors with broad specificity for the inhibition of aspartic, cysteine, and serine proteases as well as aminopeptidases. The homogenates were centrifuged for 15 min at 20,000 × *g* (4 °C); the supernatant layer was retained while the tissue debris (bottom) and

floating fat (top) layers were discarded. The supernatant was centrifuged further for 1 h at 100,000 × *g* (4 °C). The cytosolic (top) layer was discarded while the microsomal pellets (bottom portion) were reconstituted in 0.5-ml homogenization buffer containing 15% glycerol (to preserve their functional integrity). This was eventually dispersed evenly with a glass pestle and aliquots made according to the desired experiments, or stored at B80 °C. The total protein content of the microsomal fractions was estimated (Bradford, 1976). Microsomes were prepared from arteries of control, sham-salt, and DOCA-salt rats in most cases, but in some experiments, we also used microsomes obtained from rats treated with DOCA-salt plus tempol (15 mg/kg, i.p.) for 21 days. In the latter cases, microsomes were isolated as described above.

2.5. Microsomal production and spectrofluorimetric determination of free radicals

Arachidonic acid-dependent free radical production was investigated with microsomes obtained from control, sham-salt or DOCA-salt arteries. Reaction cuvettes contained 2 mg of microsomal protein in 2-ml 40 mM potassium phosphate buffer (pH 7.4) plus 1 mM sodium azide. The reaction mixtures were set up as follows: (1) 2-mg boiled microsomal protein plus or minus 0.5 mM NADPH plus 0.1 mM arachidonic acid [negative control]; (2) 2-mg microsomal protein plus or minus 0.5 mM NADPH plus 0.1 mM arachidonic acid [control microsomes], and (3) 2-mg microsomal protein plus or minus 0.5 mM NADPH plus 0.1 mM arachidonic acid [DOCA-salt microsomes]. We evaluated the potential role of cytochrome *P*450 and cyclooxygenase enzyme systems in mediating arachidonic acid-dependent free radical production in arterial microsomes. In such cases, we studied the influence of a *P*450 inhibitor miconazole (100 μM), and cyclooxygenase inhibitors mefenamic acid (nonselective; 100 μM) or NS-398 and celecoxib (cyclooxygenase-2 selective; 30 μM) on free radical production in DOCA-salt arterial microsomes. The desired concentration of a particular inhibitor under study was added to the reaction cuvettes, and allowed to equilibrate with the microsomes prior to addition of arachidonic acid.

Generally, reaction cuvettes were incubated for 10 min at 37 °C in a final volume of 2 ml and in the presence of 2′,7′-dichlorofluorescein diacetate (DCFDA; 5 μM). Following 2–3-min incubation, arachidonic acid (0.1 mM) was added to each cuvette, and the fluorescence generated by the reaction mixture was monitored in a Perkin Elmer Luminescence Spectrometer (LS50B) at excitation and emission wavelengths of 488 and 525 nm, respectively. Fluorescence was quantified as the changes in formation rate of arachidonic acid-dependent fluorescent product(s). Corrections for autofluorescence were made by inclusion of parallel blanks (i.e., assay mixture without microsomes, or without arachidonic acid) in each experiment.

2.6. Measurement of serum 8-isoprostane levels

8-Isoprostane (8-*epi*-prostaglandin $F_{2\alpha}$) is produced non-enzymatically via superoxide anion radical-mediated tissue oxidation of membrane phospholipids (Banerjee et al., 1992). The levels of this lipid derivative in sera harvested from control, DOCA-salt and DOCA-salt plus tempol groups of rats were measured with a commercially available enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). The assays were performed on freshly harvested sera. Samples and standards were assayed in triplicate and blanked according to the manufacturer's instructions, and as previously reported (Krysztopik et al., 2000). Polynomial regession of standard curves demonstrated a strong correlation ($r^2 = 0.988$) between concentration (ng/ml) and measured absorbance (405 nm).

2.7. Drugs

Acetylcholine hydrobromide, arachidonic acid, reduced nicotinamide adenine dinucleotide phosphate (NADPH), N^{ω}-nitro-L-arginine methyl ester, indomethacin, 1-ethyl-2-benzimidazolinone (1-EBIO), 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl) phenyl]-2*H*-benzimidazol-2-one (NS-1619), charybdotoxin, apamin, NS-398 [*N*-(2-cyclohexyl-4-nitrophenyl)-methane sulfonamide] and mefenamic acid were purchased from Sigma (St. Louis, MO, USA). Cirazoline hydrochloride was purchased from Research Biochem. (Natick, MA, USA). The Protease Inhibitor Cocktail Set III and phenylmethylsulfonyl fluoride were purchased from Calbiochem-Novabiochem (La Jolla, CA, USA). Tempol-4-hydroxy-[2,2,6,6-tetramethylpiperidine-1-oxyl] was purchased from Aldrich-Sigma, Milwaukee, WI, USA. The stock solutions of compounds were made in distilled water except indomethacin, NS-398 and celecoxib, which were dissolved in dimethyl sulfoxide (DMSO).

2.8. Data analysis

Changes in perfusion pressure were expressed as a percentage of the arterial perfusion pressure before the administration of a vasodilator agent. Values are expressed as mean ± S.E.M., and differences between the mean values were compared using either Student's *t* test for paired observations, or the Repeated Measures Analysis of Variance (ANOVA; multiple comparisons). The Dunnett's multiple comparison test was used to determine differences between the means of individual groups. The difference between means was considered significant when *P* was less than 0.05.

3. Results

The systolic as well as diastolic blood pressures were significantly ($P < 0.05$) higher in uninephrectomized rats that received subcutaneous implants of DOCA (100 mg; 21

Table 1
Blood pressure, heart mass, body mass and heart-to-body mass ratio in 3-week DOCA-salt-treated rats and age-matched salt-treated sham and control untreated rats

Parameters	Control	Sham-salt	DOCA-salt
Systolic/diastolic blood pressure (mm Hg)	125 ± 7/108 ± 3 (n = 37)	137 ± 3/106 ± 6 (n = 30)	194 ± 5/166 ± 4[a] (n = 32)
Heart mass (mg)	1398 ± 28 (n = 37)	1440 ± 40 (n = 30)	1860 ± 32[b] (n = 32)
Body mass (g)	350 ± 6 (n = 37)	358 ± 10 (n = 30)	301 ± 9[c] (n = 32)
Heart/body mass ratio (mg/g)	3.99 ± 0.50 (n = 37)	4.02 ± 0.48 (n = 30)	6.18 ± 0.22[d] (n = 32)

Data represent the mean ± S.E.M.; n in parentheses. [a, b, c] and [d] denote statistical difference ($p < 0.05$) from corresponding control and sham-salt values.

day-release) pellets and fed with potassium-supplemented high sodium chloride diet, and 1% saline drink. The age-matched control and sham-salt treated rats did not develop high blood pressure (Table 1). The body mass of DOCA-salt rats was significantly less than those of control and sham-salt rats; the heart mass and the heart mass-to-body mass ratios were significantly ($P < 0.05$) higher in DOCA-salt treated rats versus control and sham-salt rats (see Table 1).

3.1. Vasodilation in control versus DOCA-salt hypertensive rat vessels

The basal perfusion pressures of physiological salt solution-perfused mesenteric vascular beds were statistically similar for all groups of rats: 20.8 ± 0.5 mm Hg (control), 22.0 ± 0.8 mm Hg (sham-salt), and 21.6 ± 0.4 mm Hg (DOCA-salt). We characterized EDHF-mediated responses in vascular beds perfused with physiological salt solution containing L-NAME (100 μM) plus indomethacin (5 μM) to block nitric oxide synthase and cyclooxygenases, respectively. Conversely, perfusion of vascular beds with 25 mM K^+ physiological salt solution minimizes EDHF-related mechanisms and such tissues were used to characterize nitric oxide-dependent mechanisms. The continuous infusion of cirazoline (0.5–1.0 μM and 0.1–0.3 μM) produced sustained, statistically similar increases in perfusion pressure for control/sham-salt, and DOCA-salt vessels, respectively. Cirazoline-induced tone under EDHF- or nitric oxide-dependent conditions was sustained for a period of over 5 h. Bolus applications of acetylcholine (1×10^{-9}–1×10^{-5} mol) elicited dose-dependent decreases in perfusion pressure (index of vasodilation). Acetylcholine-induced EDHF responses were abolished by a combination of apamin (0.5 μM) with charybdotoxin (0.1 μM) while EDNO-mediated vasodilation during high K^+ physiological salt solution perfusion was markedly blocked by L-NAME (100 μM) (Fig. 1). EDHF-, but not EDNO-mediated dilations elicited by acetylcholine were significantly blunted in DOCA-salt-induced hypertensive, compared to sham-salt and control vascular beds (Fig. 2). However, acetylcholine pD_2 values (index of tissue sensitivity) were statistically similar for vessels of all groups: 9.87 ± 0.13 (control), 9.86 ± 0.13 (sham-salt) and 9.56 ± 0.15 (DOCA-salt) rats, respectively.

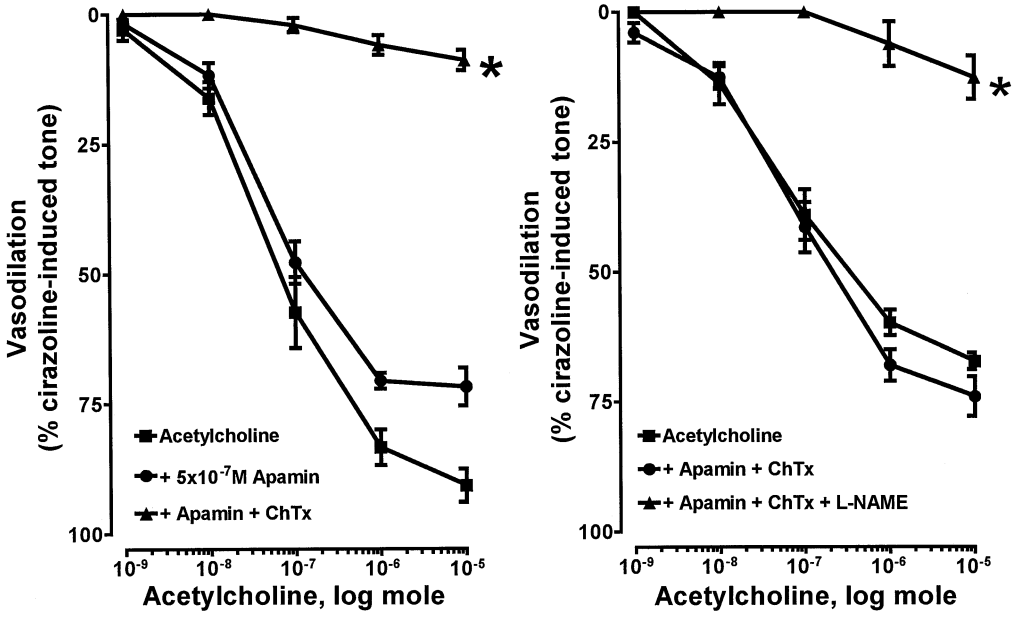

Fig. 1. EDHF- (left panel) and nitric oxide- (right panel) mediated dilator responses elicited by acetylcholine in PSS-perfused rat mesenteric vascular beds. EDHF responses were recorded during combined blockade of nitric oxide synthase and cyclooxygenases with L-NAME (100 μM) plus indomethacin (5 μM), respectively, while nitric oxide-mediated responses (EDNO) were recorded in vascular beds perfused with 25 mM K^+PSS. Each data point on the graphs represents the mean ± S.E.M. (n = 8). The asterisks (*) denote statistically significant differences ($p < 0.05$).

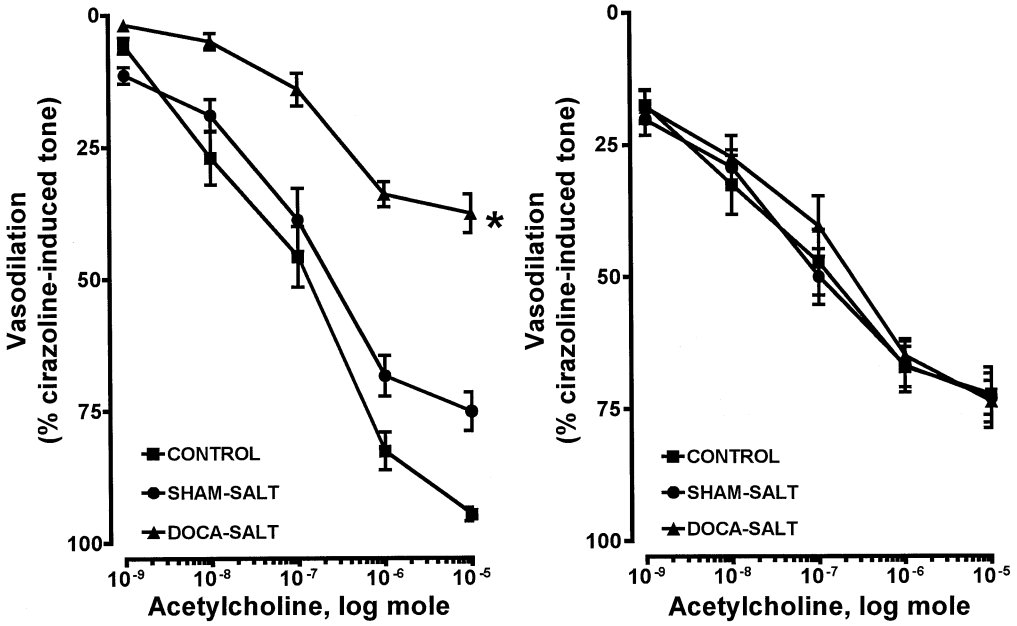

Fig. 2. Acetylcholine-induced EDHF- (left panel) and nitric oxide- (right panel) mediated responses in perfused mesenteric beds of control (kidneys; normal feed and water drink), sham-salt (high-salt diet plus 1% salt drink) and DOCA-salt (high salt diet, 1% salt drink plus DOCA implants) rats. Each data point on the graphs represents the mean ± S.E.M. ($n=9$). The asterisk (*) denotes statistically significant difference ($p<0.05$) from control or sham-salt curves.

1-EBIO (1×10^{-6}–1×10^{-3} mol) and NS-1619 (1×10^{-5} mol), endothelial and vascular smooth muscle K_{Ca} channel activators, respectively, also elicited dilator responses of mesenteric arteries from all three groups of rats, but the responses were not significantly different (Fig. 3).

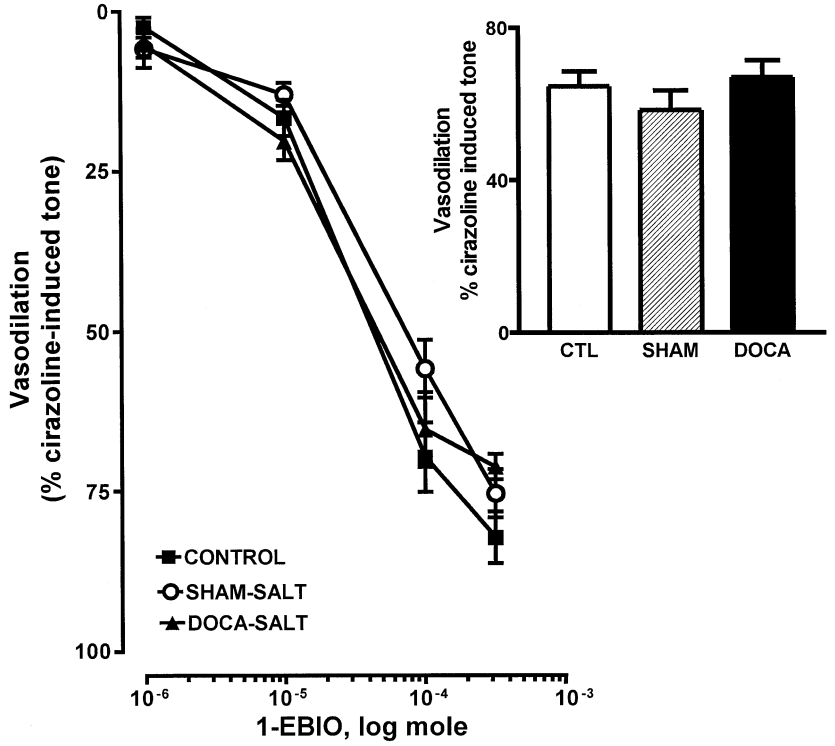

Fig. 3. Dose-responses to 1-EBIO in control (CTL), sham-salt (Sham) and DOCA-salt rat vascular beds. The bar graph (insert) indicates vasodilator responses to NS-1619 (1×10^{-4} mol) in vascular beds isolated from the three groups of rats. Each data point on the graph represents the mean ± S.E.M. ($n=5$); each bar column represents the mean ± S.E.M. ($n=4$).

Table 2
Influence of tempol (15 mg/kg, i.p., 21 days) treatment on blood pressure, and serum isoprostanes levels in age-matched control, sham-salt and DOCA-salt rats

Parameters	Control	Sham-salt	DOCA-salt
Systolic/diastolic blood pressure	122 ± 4/104 ± 6 (n = 8)	140 ± 12/108 ± 8 (n = 8)	188 ± 9/152 ± 6[a] (n = 8)
Serum isoprostane (ng/ml)	0.15 ± 0.09 (n = 9)	0.20 ± 0.08 (n = 5)	0.77 ± 0.025[b] (n = 8)
	Control/tempol	Sham-salt/tempol	DOCA-salt/tempo1
Systolic/diastolic blood pressure	119 ± 6/101 ± 8 (n = 5)	136 ± 8/110 ± 6 (n = 5)	146 ± 10/112 ± 8[c] (n = 6)
Serum isoprostane (ng/ml)	0.19 ± 0.02 (n = 5)	0.26 ± 0.06 (n = 5)	0.36 ± 0.14[d] (n = 6)

Data represent the means ± S.E.M.; n in parentheses.
[a] Statistical difference (P < 0.05) from corresponding control and sham-salt values.
[b] Statistical difference (P < 0.05) from corresponding control and sham-salt values.
[c] Statistical difference (P < 0.05) from [a], i.e., DOCA-salt values.
[d] Statistical difference (P < 0.05) from [b], i.e., DOCA-salt values.

3.2. Modulation of EDHF-mediated vasodilation by tempol

Daily treatment of DOCA-salt rats with tempol (15 mg/kg, i.p) for 21 days significantly lowered elevated blood pressure of DOCA-salt rats (Table 2), and also restored EDHF-mediated vasodilation (Fig. 4). Tempol treatment did not significantly alter blood pressure or EDHF-mediated vasodilation of control or sham-salt rats (see Table 2).

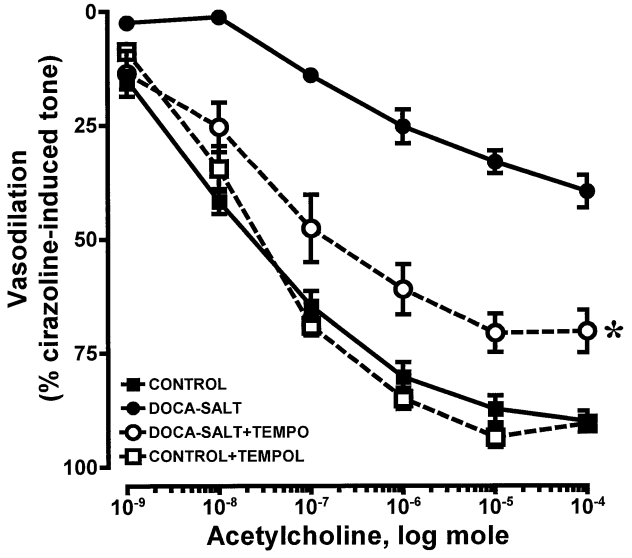

Fig. 4. Effects of treatment with an antioxidant, tempol (15 mg/kg, i.p., 21 days), on EDHF-mediated dilation of mesenteric arterial bed elicited by acetylcholine. Each data point on the graph represents the mean ± S.E.M. (n = 7). The asterisk (*) denotes statistically significant difference (p < 0.05) between DOCA-salt and DOCA-salt + tempol curve.

3.3. Microsomal production of free radical species

The dye DCFDA enters the cells or microsomes and becomes de-esterified by intramicrosomal esterases to dichlorofluorescein (DCF), which oxidize and fluoresce in the presence of free radicals (LeBel and Bondy, 1990; Bondy and Naderi, 1994). Our initial experiments indicate that addition of arachidonic acid to microsomes caused an increase in the rate of DCF oxidation and fluorescence, which is an index of arachidonic acid-dependent increases in free radical production. There were no significant increases in DCF oxidation in the absence of arachidonic acid, or microsomes, and in reaction mixtures containing only DCFDA plus phosphate buffer over many hours. It took

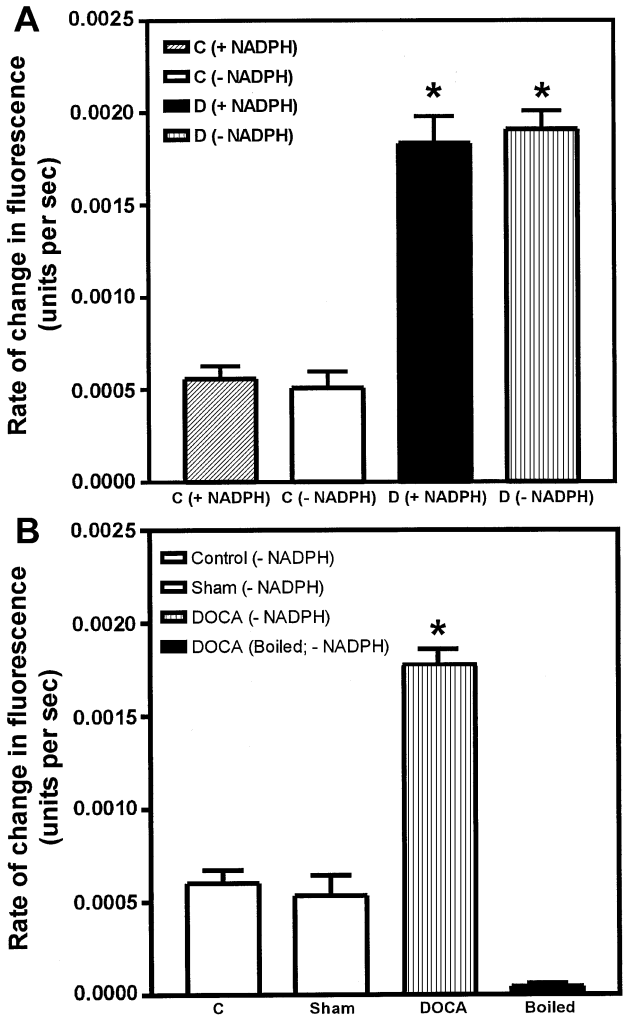

Fig. 5. Arachidonic acid-dependent free radical production in arterial microsomes of rats. A—Control (C; + NADPH), Control (C; − NADPH), DOCA-salt (D; + NADPH) and DOCA-salt (D; − NADPH) microsomes, respectively. B—Control (C), sham-salt (Sham), DOCA-salt (D), and boiled DOCA-salt microsomes. All experiments in panel B were performed without NADPH. Each bar column represents the mean ± S.E.M. (n = 6 rats). The asterisks (*) denote statistically significant differences (P < 0.05): DOCA plus or minus NADPH versus Controls plus or minus NADPH (A); DOCA versus Control, Sham or boiled microsomes (B).

the presence of viable microsomes of control, sham-salt or DOCA-salt arteries, and arachidonic acid to emit fluorescence from the addition of DCFDA to reaction cuvettes. Denaturing arterial microsomes (control or DOCA-salt) by boiling for 5 min completely prevented arachidonic acid-dependent free radical production (Fig. 5). Mesenteric arterial microsomes of DOCA-salt rats produced significantly stronger fluorescence compared to those of control and sham-salt rats (see Fig. 5). Microsomal free radical production decreased by 62.7 ± 5.0% in DOCA-salt/tempol, but remained unchanged in control/tempol or sham-salt/tempol rats.

Incubation of DOCA-salt arterial microsomes with either NS-398 or celecoxib at 30 μM completely prevented arachidonic acid dependent free radical production (Fig. 6). Mefenamic acid (100 μM), a nonselective cyclooxygenase inhibitor, significantly reduced the rate of free radical production while miconazole (100–300 μM) was totally ineffective (see Fig. 6).

3.4. Serum levels of 8-isoprostanes

Serum 8-isoprostane (8-*epi*-prostaglandin $F_{2\alpha}$) level is significantly (threefold) higher in DOCA-salt versus control or sham-salt rats (see Table 2). As with systolic and diastolic blood pressures, 8-isoprostanes levels also significantly decreased in tempol (15 mg/kg; i.p, 21 days)-treated DOCA-salt rats. Tempol treatment did not alter isoprostane levels in control or sham-salt rats.

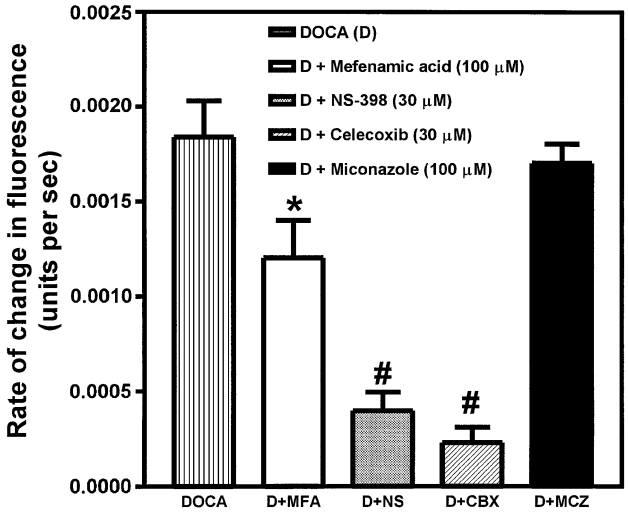

Fig. 6. The effects of inhibitors of cyclooxygenases and cytochrome *P*450 on arachidonic acid-dependent free radical production. Microsomes from DOCA-salt mesenteric arteries were incubated with buffer plus mefenamic acid (D + MFA; 100 μM), NS-398 (D + NS; 30 μM), celecoxib (D + CBX; 30 μM) and miconazole (MCZ; 100 μM), respectively, before the addition of arachidonic acid. NADPH was not added to reaction mixtures. Each bar column represents the mean ± S.E.M. ($n = 6$ rats). The asterisk (*) denotes statistically significant difference ($P < 0.05$): D + MFA versus DOCA; # denotes statistical difference ($P < 0.05$): DOCA versus D + NS, or D + CBX values.

4. Discussion

The principal findings of the present study are threefold: (1) EDHF- but not EDNO-mediated dilation in response to acetylcholine is attenuated in vascular beds of DOCA-salt hypertensive rats compared to control and sham-salt rat vessels. Vasodilation elicited by K_{Ca} channel activators, 1-EBIO and NS-1619, is not altered. (2) Arterial microsomes incubated with arachidonic acid produce free radical species in the presence or absence of a co-factor, NADPH; microsomes of DOCA-salt hypertensive vessels produce significantly more free radicals versus those from control, normotensive vessels. Serum isoprostane levels also increased significantly in DOCA-salt versus control rats. (3) Treatment of DOCA-salt rats with an antioxidant, tempol, alleviates hypertension, improves acetylcholine-induced EDHF-mediated vasodilation, and lowers serum isoprostane levels.

Endothelium-dependent relaxation is impaired in many forms of experimental (Mombouli and Vanhoutte, 1999) as well as in human hypertension (Panza et al., 1990; Treasure et al., 1992; Miyoshi et al., 1997; Houghton et al., 1998). Endothelium dysfunction in hypertension has been attributed to decreased endothelium-derived nitric oxide (EDNO) levels in Dahl salt-sensitive rats exposed to high salt intake, due to a deficiency in substrate for nitric oxide synthesis (Chen and Sanders, 1991). However, using vasoconstrictor responses to intra-arterial infusion of N^G-monomethyl-L-arginine as an index of endothelial function does not reveal an impairment of EDNO in salt-sensitive and salt-resistant patients (Miyoshi et al., 1997). The present study reveals an impairment of EDHF-, but not EDNO-mediated component of vasodilation elicited by acetylcholine during DOCA-salt induced hypertension. This finding agrees with those reported for mesenteric arteries of the spontaneously hypertensive rat (Fujii et al., 1992) and in salt-sensitive hypertensive patients (Miyoshi et al., 1997).

The mechanisms for endothelium dysfunction vary with the type of blood vessel/vascular bed, and with forms of hypertension. Reduction in EDHF-mediated vasodilation can occur if agonist receptor function, or the transmembrane K^+ channel that EDHF opens, is altered. Such alterations are not evident in the present study. Acetylcholine pD_2 values, index of tissue sensitivity, were statistically similar for vascular beds of control, sham-salt and DOCA-salt groups of rats. Also, vasodilation resulting from either the opening of endothelial K_{Ca} by 1-EBIO (Adeagbo, 1999) or smooth muscle K_{Ca} by NS-1619 (Devor et al., 1996) was statistically similar in vascular beds of DOCA-salt, compared to sham-salt and control rats. Thus, it is unlikely that the dysfunction in EDHF-mediated vasodilation occurred at the muscarinic receptors, or at the vascular K_{Ca} channel levels.

We tested an alternative hypothesis that the reduction in an agonist-induced, EDHF-mediated vasodilation in DOCA-salt hypertension is due to enhanced free radical

status, which instigates a diversion of precursor arachidonic acid toward the formation of vasoconstrictors, e.g., isoprostanes. This hypothesis is founded on our observations that treatment of DOCA-salt rats with tempol, a hydrophilic nitroxide antioxidant (Mitchell et al., 1990), alleviates hypertension, restores acetylcholine-induced EDHF vasodilation, and reduces serum isoprostanes. These observations are intriguing and suggest that free radical species modulate EDHF-mediated dilation of rat mesenteric arterial bed.

The alleviation by tempol of DOCA-salt induced hypertension, as observed in this study, agrees conceptually with a role for free radical species in the pathogenesis of the disease. Tempol can influence vascular reactivity by modulating the direct or indirect actions of free radical species on the vasculature. In the present study, tempol did not alter the vasodilator response elicited by 1-EBIO, thus negating a direct influence of free radical species on K_{Ca} channels. 1-EBIO elicits arterial smooth muscle hyperpolarization by opening endothelial K_{Ca} (Adeagbo, 1999), notably, the intermediate-conductance or IK_{Ca} channels (Bychkov et al., 2002). Treatment with tempol has been demonstrated to normalize blood pressure and renal vascular resistance in the spontaneously hypertensive rat (SHR; Schnackenberg and Wilcox, 1999; Schnackenberg et al., 1998), and also to attenuate elevated systolic blood pressure, as well as suppress renal NF-κB-binding activity and aortic superoxide accumulation in mineralocorticoid induced hypertension (Beswick et al., 2001b). More recently, chronic treatment with tempol was shown to prevent vascular remodelling and progression of hypertension in salt-loaded stroke prone SHRs (Park et al., 2002). The latter authors attributed the blood-pressure-lowering effects of tempol to decreased generation of superoxide anions (O_2^-).

Endothelium-dependent vasodilation of rat mesenteric arteries is accompanied by the release of arachidonic acid (Adeagbo et al., 2001), and enzymatic metabolism of this fatty acid by cyclooxygenases, lipoxygenases or cytochrome *P*450s generates free radical species including lipid (L^-), lipid alkoxyl (LOO^-), lipid peroxides and superoxide anions (Fleming et al., 2001; O'Donnell, 2003). These radical species are also all produced during lipid peroxidation. We established arachidonic acid as the source of free radical species by measuring the fluorescence emitted by dichlorofluorescein or DCF, the oxidation product of a non-fluorescent dye, dichlorofluoresceine diacetate or DCFDA. This dye, DCFDA, enter cells or microsomes, becomes de-esterified by intramicrosomal esterases to DCF, which is then oxidized by free radical species to give measurable fluorescence (LeBel and Bondy, 1990; Bondy and Naderi, 1994). Viable microsomes and arachidonic acid are obligatory for measurable fluorescence to occur. No fluorescence was observed with the dye DCFDA in phosphate buffer without microsomes, or with boiled microsomes. Thus, the source of free radical species that is modulated by the antioxidant tempol to bring about alleviation of high blood pressure and restoration of EDHF vasodilation is arachidonate metabolism.

Cyclooxygenases and CYPs are relevant to the present because they, unlike lipoxygenases, are microsomal enzymes. However, CYPs but not cyclooxygenases require NADPH as co-factor for activity. Since arachidonic acid-dependent free radical production (1) occurs with/without NADPH, and (2) is not affected by miconazole, a nonselective CYP inhibitor, it is unlikely that CYPs mediate their production. However, in DOCA-salt induced hypertension, there is an up-regulation of inflammatory mediators (Ammarguellat et al., 2002) as well as increased expression of cyclooxygenase-2 (Fitzgerald and Patrono, 2001), and products of this pathway contribute to endothelial dysfunction in hypertension (Widlansky et al., 2003). Data from human studies showing improved endothelium-dependent dilation after treatment with nonselective cyclooxygenase inhibitors (Taddei et al., 1997) or with a selective inhibitor of cyclooxygenase-2, celecoxib (Widlansky et al., 2003) support the putative role of cyclooxygenase(s) in endothelium dysfunction. In the present study, we found that NS-398 and celecoxib, two structurally distinct cyclooxygenase-2 selective inhibitors, depressed free radical production in DOCA-salt microsomes. Thus, our data strongly implicate cyclooxygenase-2-mediated catabolism of arachidonic acid as the probable source of free radicals in microsomes of mesenteric arteries of rats.

An elevated production of free radical species such as evident from the present study contributes to the onset or progression of mineralocorticoid-salt induced hypertension in rats (Somers et al., 2000; Beswick et al., 2001a,b) as well as development of hypertension in humans (Kumar and Das, 1993; Lacy et al., 2000). Free radicals can initiate the peroxidation of arachidonic acid resulting in the production of vasoconstrictors such as isoprostanes (Banerjee et al., 1992; Takahashi et al., 1992) and/or attack the heme moiety of cytochrome *P*450s to induce a suicide inactivation of the hemoprotein (Serbinova et al., 1992) to decrease the formation of vasodilator epoxyeicosatrienoic acids (or the putative EDHF). Our study shows a significant increase in serum isoprostane levels during DOCA-salt hypertension, which was significantly lowered by tempol treatment. Therefore, the cellular mechanisms underlying the actions of tempol in lowering serum isoprostanes/improving EDHF-mediated vasodilation and in attenuating DOCA-salt hypertension may be due to inhibition of peroxidation processes. Tempol can react with these radicals to terminate the chain reactions associated with lipid peroxidation (Zhang et al., 1998).

We conclude that EDHF-mediated dilation of rat mesenteric arteries is impaired in DOCA-salt induced hypertension. The impairment is apparently unrelated to receptor or K_{Ca} channel function. Second, treatment with an antioxidant, tempol, alleviates DOCA-salt induced hypertension and significantly improves EDHF-mediated vasodilation. Both of these effects appear to be related to lowering of

the free radical induced formation of isoprostanes. These data suggest that free radical species, probably emanating from cyclooxygenase-2-mediated lipid metabolism and/or lipid peroxidation, modulates EDHF-mediated vascular regulation in DOCA-salt induced hypertension.

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References

Adeagbo, A.S.O., 1999. 1-Ethyl-2-benzimidazolinone stimulates endothelial K_{Ca} channels and nitric oxide formation in rat mesenteric vessels. Eur. J. Pharmacol. 379, 151–159.

Adeagbo, A.S.O., Triggle, C.R., 1993. Varying extracellular [K^+]: a functional approach to separating EDHF- and EDNO-related mechanisms in perfused rat mesenteric arterial bed. J. Cardiovasc. Pharmacol. 21, 423–429.

Adeagbo, A.S.O., Oyekan, A.O., Joshua, I.G., Falkner, K.C., Prough, R.A., Pierce, W.M., 2001. Epoxyeicosatrienoic acid (EET) release mediates nitric oxide-independent dilation of rat mesenteric vessels. In: Vanhoutte, P.M. (Ed.), EDHF 2000. Taylor and Francis, London, pp. 195–206. Chapter 22.

American Physiological Society, 2002. Guiding principles for research involving animals and human beings. Am. J. Physiol., Regul. Integr. Comp. Physiol. 283, 281–283.

Ammarguellat, F.Z., Gannon, P.O., Amiri, F., Schiffrin, E.L., 2002. Fibrosis, matrix metalloproteinases, and inflammation in the heart of DOCA-salt hypertensive rats: role of ET_A receptors. Hypertension 39, 679–684.

Banerjee, M., Kang, K.H., Morrow, J.D., Roberts, L.J., Newman, J.H., 1992. Effects of a novel prostaglandin, 8-epiPGF_{2a}, in rabbit lung in situ. Am. J. Physiol. 263, H660–H663.

Beswick, R.A., Dorrance, A.M., Leite, R., Webb, R.C., 2001a. NADH/NADPH oxidase and enhanced superoxide production in the mineralocorticoid hypertensive rat. Hypertension 38, 1107–1111.

Beswick, R.A., Zhang, H., Marable, D., Catravas, J.D., Hill, W.D., Webb, R.C., 2001b. Long-term antioxidant administration attenuates mineralocorticoid hypertension and renal inflammatory response. Hypertension 37, 781–786.

Bondy, S.C., Naderi, S., 1994. Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species. Biochem. Pharmacol. 48, 155–159.

Bradford, M.M., 1976. A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bychkov, R., Burnham, M.P., Richards, G.R., Edwards, G., Weston, A.H., Feletou, M., Vanhoutte, P.M., 2002. Characterization of a charybdoto xin-sensitive intermediate conductance Ca^{2+}-activated K^+ channel in porcine coronary endothelium: relevance to EDHF. Br. J. Pharmacol. 137, 1346–1354.

Chen, P.Y., Sanders, P.W., 1991. L-arginine abrogates salt-sensitive hypertension in Dahl/Rapp rats. J. Clin. Invest. 88, 1559–1567.

Cordellini, S., 1999. Endothelium dysfunction in DOCA-salt hypertension: possible involvement of prostaglandin endoperoxides. Gen. Pharmacol. 32, 315–320.

Devor, D.C., Singh, A.K., Frizell, R.A., Bridges, R.A., 1996. Modulation of Cl^- secretion by benzimidazolones: I. Direct activation of a Ca^{2+}-dependent K^+ channels. Am. J. Physiol. 271, L775–L784.

Fitzgerald, G.A., Patrono, C., 2001. The coxibs, selective inhibitors of cyclooxygenase-2. N. Engl. J. Med. 345, 433–442.

Fleming, I., Michaelis, U.R., Bredenkotter, D., Fisslthaler, B., Dehghani, F., Brande, R.P., Busse, R., 2001. Endothelium-derived hyperpolarizing factor synthase (Cytochrome *P*450 2C9) is a functionally significant source of reactive oxygen species in coronary arteries. Circ. Res. 88, 44–51.

Fujii, K., Tominaga, M., Ohmori, S., Kobayashi, K., Koga, T., Takata, Y., Fujishima, M., 1992. Decreased endothelium-dependent hyperpolarization to acetylcholine in smooth muscle of the mesenteric artery of spontaneously hypertensive rats. Circ. Res. 70, 660–669.

Houghton, J.L., Davidson, C.A., Kuhner, P.A., Torossov, M.T., Strogatz, D.S., Carr, A.A., 1998. Heterogenous vasomotor responses of coronary conduit and resistance vessels in hypertension. J. Am. Coll. Cardiol. 31, 374–382.

Kerr, S., Brosnan, M.J., McIntyre, M., Reid, J.L., Doniniczak, A.F., Hamilton, C.A., 1999. Superoxide anion production is increased in a model of genetic hypertension. Role of endothelium. Hypertension 33, 1353–1358.

Kimura, K., Nishio, I., 1999. Impaired endothelium-dependent relaxation in mesenteric arteries of reduced renal mass hypertensive rats. Scand. J. Clin. Lab. Invest. 59, 199–204.

Krysztopik, R.J., Matheson, P.J., Spain, D.A., Garrison, R.N., Wilson, M.A., 2000. Lazaroid and pentoxifylline suppress sepsis-induced increases in renal vascular resistance via altered arachidonic acid metabolism. J. Surg. Res. 93 (1), 75–81.

Kumar, K.V., Das, U.N., 1993. Are free radicals involved in the pathobiology of human essential hypertension? Free Radic. Res. Commun. 19, 59–66.

Lacy, F., Kailasam, M.T., O'Connor, D.T., Schmid-Schnbein, G.W., Parmer, R.J., 2000. Plasma hydrogen peroxide production in human essential hypertension: role of heredity, gender, and ethnicity. Hypertension 36, 878–884.

Laight, D.W., Bethelon, J.J., Andrews, T.J., Carrier, M.J., Anggard, E.E., 1997. Assessment of superoxide anion scavenging activity of some common spin traps at physiological temperature and pH by microassay. Br. J. Pharmacol. 122, P130.

LeBel, C.P., Bondy, S.C., 1990. Sensitive and rapid quantification of oxygen reactive species formation in rat synaptosomes. Neurochem. Int. 17, 435–440.

Li, L., Fink, G.D., Watts, S.W., Northcott, C.A., Galligan, J.J., Pagano, P.J., Chen, A.F., 2003. Endothelin-1 increases vascular superoxide via endothelin A-NADPH oxidase pathway in low-renin hypertension. Circulation 107, 1053–1058.

Lind, L., Granstam, S.-O., Millgard, J., 2000. Endothelium-dependent vasodilation in hypertension: a review. Blood Pressure 9, 4–15.

Luscher, T., 1990. Imbalance of endothelium-derived relaxing and contracting factors: a new concept in hypertension. Am. J. Hypertens. 3, 317–330.

Millette, E., de Champlain, J., Lamontagne, D., 2000. Altered coronary dilation in deoxycorticosterone acetate-salt hypertension. J. Hypertens. 18, 1783–1793.

Mitchell, J.B., Samuni, A., Krishna, M.C., DeGraff, W.G., Ahn, M.S., Samuni, U., Russo, A., 1990. Biologically active metal-independent superoxide dismutase mimetics. Biochemistry 29, 2802–2807.

Miyoshi, A., Suzuki, M., Masai, M., Iwasaki, T., 1997. Impairment of endothelial function in salt-sensitive hypertension in humans. Am. J. Hypertens. 10, 1083–1090.

Mombouli, J.-V., Vanhoutte, P.M., 1999. Endothelial dysfunction: from physiology to therapy. J. Mol. Cell. Cardiol. 31, 61–74.

O'Donnell, V.B., 2003. Free radicals and lipid signaling in endothelial cells. Antioxid. Redox Signal. 5, 195–203.

Panza, J.A., Quyyumi, A.A., Brush Jr., J.E., Epstein, S.E., 1990. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. N. Engl. J. Med. 323, 22–27.

Park, J.B., Touyz, R.M., Chen, X., Schiffrin, E.L., 2002. Chronic treatment with a superoxide dismutase mimetic prevents vascular remodelling and progression of hypertension in salt-loaded stroke-prone spontaneously hypertensive rats. Am. J. Hypertens. 15, 78–84.

Porasuphatana, S., Tsai, P., Rosen, G.M., 2003. The generation of free radicals by nitric oxide synthase. Comp. Biochem. Physiol. 134 (Part C), 281–289.

Puntarulo, S., Cederbaum, A.I., 1998. Production of reactive oxygen species by microsomes enriched in specific human cytochrome *P*450 enzymes. Free Radic. Biol. Med. 24, 1324–1330.

Schnackenberg, C.G., Wilcox, C.S., 1999. Two-week administration of tempol attenuates both hypertension and renal excretion of 8-iso prostaglandin F_{2a}. Hypertension 33, 424–428.

Schnackenberg, C.G., Welch, W.J., Wilcox, C.S., 1998. Normalization of blood pressure and renal vascular resistance in SHR with a membrane-permeable superoxide dismutase mimetic: role of nitric oxide. Hypertension 32, 59–64.

Serbinova, E., Ivanova, S., Kirova, A., Kitanova, S., Packer, L., Kagan, V., 1992. Cytochrome *P*-450 under conditions of oxidative stress: role of antioxidant recycling in the protection mechanisms. Adv. Exp. Med. Biol. 316, 223–230.

Shimokawa, H., 1998. Endothelial dysfunction in hypertension. J. Atheroscler. Thromb. 4, 118–127.

Somers, M.J., Mavromatis, K., Galis, Z.S., Harrison, D.G., 2000. Vascular superoxide production and vasomotor function in hypertension induced by deoxycorticosterone acetate-salt. Circulation 101, 1722–1728.

Sullivan, J.C., Pollock, D.M., Pollock, J.S., 2002. Altered nitric oxide synthase 3 distribution in mesenteric arteries of hypertensive rats. Hypertension 39, 597–602.

Swei, A., Lacy, F., Delano, F.A., Parks, D.A., Schmid-Schonbein, G.W., 1999. A mechanism of oxygen free radical production in the Dahl hypertensive rat. Microcirculation 6, 179–187.

Taddei, S., Virdis, A., Ghiadoni, L., Magagna, A., Salvetti, A., 1997. Cyclooxygenase inhibition restores nitric oxide activity in essential hypertension. Hypertension 29, 274–279.

Takahashi, K., Nammour, T.M., Fukunaga, M., Ebert, J., Morrow, J.D., Roberts II, L.J., Hoover, R.L., Badr, K.F., 1992. Glomerular actions of a free radical-generated novel prostaglandin 8-epi-prostaglandin F_{2a}, in the rat: evidence for interaction with thromboxane A2 receptors. J. Clin. Invest. 90, 136–141.

Treasure, C.B., Manoukian, S.V., Klein, J.L., Vita, J.A., Nabel, E.G., Renwick, G.H., Selwyn, A.P., Alexander, R.W., Ganz, P., 1992. Epicardial coronary artery responses to acetylcholine are impaired in hypertensive patients. Circ. Res. 71, 776–781.

Widlansky, M.E., Price, D.T., Gokce, N., Eberhardt, R.T., Duffy, S.J., Holbrook, M., Maxwell, C., Palmisano, J., Keaney, J.F., Morrow, J.D., Vita, J.A., 2003. Short- and long-term COX-2 inhibition reverses endothelial dysfunction in patients with hypertension. Hypertension 42, 1–6.

Zhang, R., Pinson, A., Samuni, A., 1998. Both hydroxylamine and nitroxide protect cardiomyocytes from oxidative stress. Free Radic. Biol. Med. 24, 66–75.