

Endothelin-1-induced oxidative stress in DOCA-salt hypertension involves NADPH-oxidase-independent mechanisms

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A B S T R A C T

We have demonstrated recently [Callera, Touyz, Teixeira, Muscara, Carvalho, Fortes, Schiffrin and Tostes (2003) *Hypertension* **42**, 811–817] that increased vascular oxidative stress in DOCA (deoxycorticosterone acetate)-salt rats is associated with activation of the ET (endothelin) system via ET_A receptors. The exact source of ET-1-mediated oxidative stress remains unclear. The aim of the present study was to investigate whether ET-1 increases generation of ROS (reactive oxygen species) in DOCA-salt hypertension through NADPH-oxidase-dependent mechanisms. Xanthine oxidase, eNOS (endothelial nitric oxide synthase) and COX-2 (cyclo-oxygenase-2) were also examined as potential ET-1 sources of ROS as well as mitochondrial respiration. DOCA-salt and control UniNX (uninephrectomized) rats were treated with the ET_A antagonist BMS182874 (40 mg · day⁻¹ · kg⁻¹ of body weight) or vehicle. Plasma TBARS (thiobarbituric acid-reacting substances) were increased in DOCA-salt compared with UniNX rats. Activity of NADPH and xanthine oxidases in aorta, mesenteric arteries and heart was increased in DOCA-salt rats. BMS182874 decreased plasma TBARS levels without influencing NADPH and xanthine oxidase activities in DOCA-salt rats. Increased p22^{phox} protein expression and increased p47^{phox} membrane translocation in arteries from DOCA-salt by rats were not affected by BMS182874 treatment. Increased eNOS and COX-2 expression, also observed in aortas from DOCA-salt rats, was unaltered by BMS182874. Increased mitochondrial generation of ROS in DOCA-salt rats was normalized by BMS182874. ET_A antagonism also increased the expression of mitochondrial MnSOD (manganese superoxide dismutase) in DOCA-salt rats. In conclusion, activation of NADPH oxidase does not seem to be the major source of oxidative stress induced by ET-1/ET_A in DOCA-salt hypertension, which also appears to be independent of increased activation of xanthine oxidase or eNOS/COX-2 overexpression. Mitochondria may play a role in ET-1-driven oxidative stress, as evidenced by increased mitochondrial-derived ROS in this model of hypertension.

Key words: DOCA (deoxycorticosterone acetate)-salt hypertension, endothelin-1, ET_A receptor, NADPH oxidase, oxidative stress.

Abbreviations: Ang II, angiotensin II; BH₄, tetrahydrobiopterin; COX, cyclo-oxygenase; DOCA, deoxycorticosterone acetate; eNOS, endothelial nitric oxide synthase; ET, endothelin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; MDA, malondialdehyde; ROS, reactive oxygen species; RT, reverse transcription; SBP, systolic blood pressure; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reacting substance; UniNX, uninephrectomized; VSMC, vascular smooth muscle cell.

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INTRODUCTION

Oxidative stress, characterized by increased bioavailability of ROS (reactive oxygen species), plays an important role in the development and progression of cardiovascular dysfunction associated with hypertensive disease [1]. There are a number of sources of ROS, including neutrophil-like membrane-associated NADPH oxidase, xanthine oxidase, myeloperoxidase, uncoupled eNOS (endothelial nitric oxide synthase) and spillover from mitochondrial respiratory chain [2]. Of the numerous ROS generated in the vasculature, $O_2^{\bullet-}$ (superoxide anion) and H_2O_2 appear to be particularly important. Emerging evidence indicates NADPH oxidase as a major source for vascular $O_2^{\bullet-}$ production [1,2]. Under physiological conditions, ROS produced in the course of normal metabolism are fully inactivated by an elaborate cellular antioxidant system comprising several enzymes [3]. The first line of defence against ROS production comprises SOD (superoxide dismutase), catalase and the glutathione system. In hypertension, an imbalance between ROS generation and antioxidant capacity depletion results in enhanced ROS bioavailability and consequent oxidative stress [4,5].

Among the humoral factors implicated in systemic cardiovascular changes occurring in hypertension, ET-1 (endothelin-1) has been highlighted to increase vascular ROS generation [6–8]. It has been reported that both ET-1 and vascular $O_2^{\bullet-}$ levels are increased in DOCA (deoxycorticosterone acetate)-salt hypertension [9–15], a model known for decreased plasma renin activity [16]. Consistent with these observations, we reported previously the ET-1-dependent ROS accumulation in DOCA-salt rats [17]. It has been proposed that NADPH oxidase contributes to ET-1-induced oxidative stress via an ET_A pathway in DOCA-salt hypertension [9,18–20]; however, evidence for a direct role of ET-1 in NADPH-oxidase-driven oxidative stress in DOCA-salt hypertension is still lacking.

Because mechanisms whereby ET-1 mediates oxidative stress have not been fully characterized in hypertension, the present study investigated whether NADPH oxidase is involved in ET-1-induced oxidative stress in DOCA-salt rats. Xanthine oxidase, eNOS and COX (cyclooxygenase)-2 were also examined as potential ET-1 sources of vascular ROS, as well as mitochondrial formation of ROS and antioxidant defences in this model of hypertension. To address these issues, we determined the activity of NADPH and xanthine oxidases, expression of NADPH oxidase subunits, translocation of cytoplasmic $p47^{phox}$ to the membrane and eNOS/COX-2 expression in cardiovascular tissues from DOCA-salt rats treated with BMS172874, an antagonist of ET_A receptors. The detection of $O_2^{\bullet-}$ production by isolated intact mitochondria and the expression of mitochondrial MnSOD (manganese SOD) were also evaluated in these

experimental groups. Secondly, we evaluated whether depletion of the glutathione antioxidant system was implicated in enhanced ROS activity in this model of hypertension.

METHODS

Animal experiments

The study protocol was approved by the Animal Care Committees of the University of Sao Paulo and the Clinical Research Institute of Montreal. Rats were housed under conditions of constant humidity and temperature and subjected to 12 h light/dark cycles. Male Wistar rats, aged 8 weeks, were studied. DOCA-salt hypertension was induced as described previously [21] and, simultaneously, rats received either the ET_A antagonist BMS182874 ($40 \text{ mg} \cdot \text{day}^{-1} \cdot \text{kg}^{-1}$ of body weight, p.o. per gavage) or vehicle. SBP (systolic blood pressure) was measured weekly in unanaesthetized animals by an indirect tail-cuff method (PowerLab 4/S; ADInstruments). At the end of 5 weeks of treatment, rats were submitted to the experimental procedures described below.

Measurement of NADPH oxidase activity in tissue homogenates

Aortic, mesenteric arteries and heart were analysed for NADPH and xanthine oxidase activities using 10% (w/v) homogenates prepared in Krebs solution [120 mmol/l NaCl, 25 mmol/l NaHCO_3 , 4.7 mmol/l KCl, 1.18 mmol/l KH_2PO_4 , 1.18 mmol/l MgSO_4 , 2.5 mmol/l CaCl_2 , 0.026 mmol/l EDTA and 5.5 mmol/l glucose (pH 7.4)] with a glass-to-glass homogenizer. Activity of NADPH and xanthine oxidases was measured by a luminescence assay with $5 \mu\text{mol/l}$ lucigenin as the electron acceptor and $100 \mu\text{mol/l}$ NADPH as the substrate. The reaction was started by the addition of NADPH to the homogenate. Luminescence was measured every 1.8 s for 3 min in a luminometer (Lumistar Galaxy; BMG Labtechnologies). Buffer blank was subtracted from each reading. Activity was expressed as arbitrary units/mg of protein. Protein concentrations were determined with protein assay reagent (Bio-Rad Laboratories).

Measurement of lucigenin-derived luminescence in isolated heart mitochondria

Mitochondria were isolated from frozen hearts. Tissues were homogenized in 5 vol. of sucrose buffer [10 mmol/l Hepes (pH 7.6), 0.25 mol/l sucrose, 1 mmol/l EGTA and 0.5% BSA]. The homogenate was then centrifuged at 1500 g for 10 min at 4°C . The supernatant was collected and centrifuged at 10000 g for 10 min at 4°C . The resulting mitochondrial pellet was washed once and then

resuspended in 0.2 ml of sucrose buffer. The reaction mixture contained 0.5 mg of protein/ml of mitochondria in the absence or presence of 6 mmol/l succinate in respiration buffer [70 mmol/l sucrose, 220 mmol/l mannitol, 2 mmol/l Hepes, 2.5 mmol/l KH_2PO_4 , 2.5 mmol/l MgCl_2 , 0.5 mmol/l EDTA and 0.1 % BSA (pH 7.4)]. The lucigenin-derived luminescence response was initiated by adding 5 $\mu\text{mol/l}$ lucigenin to the reaction mixture. Luminescence was measured every 1.8 s for 3 min before or 30 min after substrate addition.

Plasma measurement of TBARS (thiobarbituric acid-reacting substances)

Blood was collected in EDTA, centrifuged and plasma levels of TBARS were measured by a colorimetric method. Briefly, plasma was mixed with 2 % butylated hydroxytoluene and quintanilla reagent (26 mmol/l thiobarbituric acid and 15 % trichloroacetic acid). The mixture reaction was boiled for 15 min. Thereafter, the reaction mixture was cooled and centrifuged at 3000 g for 10 min. The soluble phase was measured with a spectrophotometer at a wavelength of 535 nm. In parallel, MDA (malondialdehyde) standards were diluted in the range of 0–4 $\mu\text{mol/l}$. TBARS values were expressed in nmol/ml MDA equivalents.

Glutathione system analysis

Blood was collected in heparin and total glutathione was measured in erythrocyte lysates by a colorimetric assay (GSH-420, 21023 kit; OxisResearch). For glutathione peroxidase and reductase activities in heart and mesenteric arteries homogenates, rats were perfused with isotonic saline prior to tissue extraction and a colorimetric assay was performed (GPx-340, 21017 and GR-340, 21018D kits; OxisResearch).

Western blotting experiments

Mesenteric arteries were used for total fraction protein analysis. Arteries were homogenized in lysis buffer A [50 mmol/l Tris/HCl (pH 7.4), 1 % Nonidet P40, 0.5 % sodium deoxycholate, 150 mmol/l NaCl, 1 mmol/l EDTA, 0.1 % SDS, 1 mmol/l PMSF, 1 $\mu\text{mol/l}$ pepstatin A, 1 $\mu\text{mol/l}$ leupeptin and 1 $\mu\text{mol/l}$ aprotinin] and the protein supernatant was separated by centrifugation. Aortas were used for cell fraction separation as follows. Frozen aortas were homogenized in lysis buffer B [50 mmol/l Tris/HCl (pH 7.4), 5 mmol/l EGTA and 2 mmol/l EDTA, 0.1 mmol/l PMSF, 1 $\mu\text{mol/l}$ pepstatin A, 1 $\mu\text{mol/l}$ leupeptin and 1 $\mu\text{mol/l}$ aprotinin]. Half of the homogenate was used for total fraction protein analysis. The remaining portion was used for membrane and cytosolic separation. Homogenates were centrifuged at 50 000 g for 30 min at 4 °C, thereby isolating the cytosolic fraction in the supernatant. The particulate fraction

was incubated under constant shaking for 30 min at 4 °C in lysis buffer B containing 1 % Triton X-100 and re-centrifuged at 50 000 g for 30 min at 4 °C. Heart mitochondria were isolated as described above and resuspended in lysis buffer C [0.1 mol/l Tris/HCl (pH 7.4), 0.25 mol/l sucrose, 5 mmol/l EDTA and 2 % (w/v) SDS].

Western blotting was performed as described in detail previously [22]. Membranes were incubated with antibodies characterized previously [23] that specifically recognize NADPH oxidase subunits, p22^{phox}, the membrane-associated subunit (clone 44.1; diluted 1:1000), and p47^{phox}, the cytoplasmic-associated subunit (clone 43.27; diluted 1:750), monoclonal antibodies to eNOS and iNOS (inducible nitric oxide synthase; diluted 1:1000; BD Transduction Laboratories) or polyclonal antibodies to COX-2 (diluted 1:750; Upstate) and to MnSOD (diluted 1:5000; Stressgen Bioreagents). A monoclonal antibody to α -actin (Sigma–Aldrich) was used as loading control and was carried out on the same membranes for total protein and cytosolic fraction. For membrane fractions and mitochondrial preparations, Ponceau Red staining was used as a loading control. VSMCs (vascular smooth muscle cells) stimulated with 1 $\mu\text{g/ml}$ LPS (lipopolysaccharide; from *Escherichia coli* 055:B5; Sigma–Aldrich) were used as a positive control for iNOS and COX-2 immunoblots, and brain homogenate was used as a positive control for MnSOD.

RT (reverse transcription)-PCR analysis

RT-PCR, used to evaluate gene expression of NADPH subunits, was performed as described previously [21]. Total cellular RNA was isolated from arteries and left ventricles. After DNA contaminant digestion, first-strand cDNA was synthesized using 3 μg of total RNA. cDNA was amplified using the following set of primers (5'–3'): p22^{phox} (200 bp amplified), sense CACGCAGTGGTACTTTGGTG, antisense CACGGACAGCAGTAAGTGGGA; p47^{phox} (200 bp amplified) sense GGCCAAAGATGGCAAGAATA, antisense TGTC AAGGGGCTCCAAATAG; and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; 400 bp amplified), sense GGTGCTGAGTATGTCGTGGA, antisense TTCAGCTCTGGGATGACCTT. For duplex PCR, GAPDH was used as an internal control for the co-amplification. The best annealing temperature was chosen for each set of primers, and experiments were also performed to confirm that the number of cycles used in the duplex PCRs was within the linear amplification range. The following conditions were used: final volume of 25 μl , 2.5 units *Taq* Platinum polymerase (Life Technologies), an initial denaturing cycle at 94 °C for 3 min, and subsequent cycles with denaturation at 94 °C for 30 s, annealing at 65 °C (p22^{phox}) or 60 °C (p47^{phox}) for 45 s and extension at 72 °C for 45 s. Numbers of amplification cycles were 30 for p22^{phox} and 28 for p47^{phox}. PCR products underwent electrophoresis on a 1 % (w/v) agarose gel containing ethidium bromide

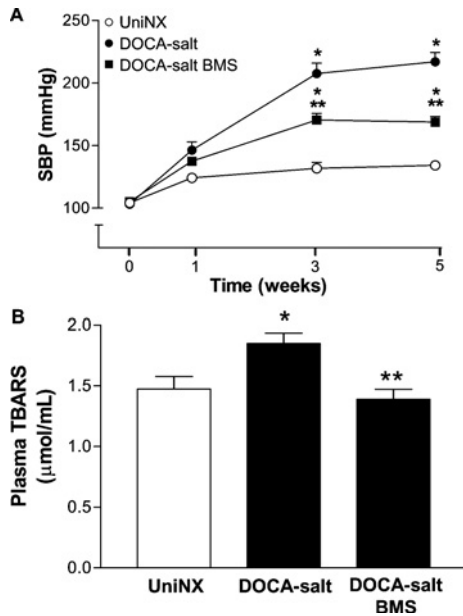


Figure 1 Evolution of SBP (A), and plasma concentration of TBARS (B), a marker of oxidative stress, in UniNX rats ($n = 6$), DOCA-salt rats ($n = 8$) and DOCA-salt rats treated with BMS182874 ($n = 8$)

Values are means \pm S.E.M. * $P < 0.05$ compared with UniNX rats; ** $P < 0.05$ compared with DOCA-salt rats.

(0.5 mg/ml) and band intensities were measured using a software (Kodak Digital Science). Signals were expressed relatively to the intensity of GAPDH in each co-amplified sample.

Data analysis

Results are expressed as means \pm S.E.M.; n indicates the number of animals. Statistical significance was evaluated by ANOVA or Student's t test, as appropriate; $P < 0.05$ was considered to be significant.

RESULTS

Blood pressure measurement

SBP increased progressively after DOCA treatment and salt loading (Figure 1A). At 5 weeks of treatment, SBP in DOCA-salt rats ($n = 8$) was significantly higher ($P < 0.01$) than in UniNX rats ($n = 6$). Treatment with the ET_A antagonist BMS182874 significantly lowered ($P < 0.01$) SBP in DOCA-salt rats ($n = 8$).

Plasma TBARS

Systemic oxidative stress was evaluated by levels of TBARS. As shown in Figure 1(B), plasma TBARS levels were significantly increased ($P < 0.01$) in DOCA-salt rats compared with UniNX rats. BMS182874 treatment significantly decreased ($P < 0.001$) levels of TBARS in DOCA-salt rats.

Measurement of glutathione and antioxidant enzymes activity

Levels of total glutathione and glutathione peroxidase and reductase activities are shown in Table 1. Glutathione content in erythrocytes was similar in DOCA-salt and UniNX rats. Glutathione peroxidase activity, as well as glutathione reductase activity, measured in erythrocytes, heart and mesenteric arteries were not different between DOCA-salt and UniNX rats.

ROS generation

NADPH- and xanthine-dependent generation of $O_2^{\bullet-}$ were measured in vascular and non-vascular tissue homogenates from BMS182874-treated and untreated DOCA-salt rats and compared with those from UniNX rats. Figures 2(A) and 2(B) show that lucigenin-derived luminescence was significantly higher ($P < 0.05$) in aortic and mesenteric arteries for DOCA-salt rats exposed to NADPH than in vessels from UniNX rats. In hearts from DOCA-salt rats, significantly increased ($P < 0.05$) NADPH-dependent generation of $O_2^{\bullet-}$, compared with

Table 1 Glutathione system in DOCA-salt rats

Values are means \pm S.E.M. The number of animals is indicated in parentheses. Hb, haemoglobin.

	UniNX rats	DOCA-salt rats
Total glutathione		
Erythrocytes (μ g)	259.3 \pm 25.8 ($n = 6$)	245.9 \pm 13.0 ($n = 6$)
Glutathione peroxidase		
Erythrocytes (m-unit/g of Hb)	0.33 \pm 0.1 ($n = 5$)	0.31 \pm 0.1 ($n = 5$)
Mesenteric arteries (m-units/mg of protein)	96.5 \pm 11.3 ($n = 6$)	83.7 \pm 8.5 ($n = 6$)
Heart (m-units/mg of protein)	256.4 \pm 17.6 ($n = 6$)	231.7 \pm 17.7 ($n = 6$)
Glutathione reductase		
Erythrocytes (m-units/g of Hb)	1.3 \pm 0.4 ($n = 6$)	1.5 \pm 0.5 ($n = 8$)
Mesenteric arteries (m-units/mg of protein)	5.1 \pm 0.2 ($n = 6$)	6.2 \pm 0.9 ($n = 5$)
Heart (m-units/mg of protein)	4.2 \pm 0.4 ($n = 5$)	4.8 \pm 0.7 ($n = 6$)

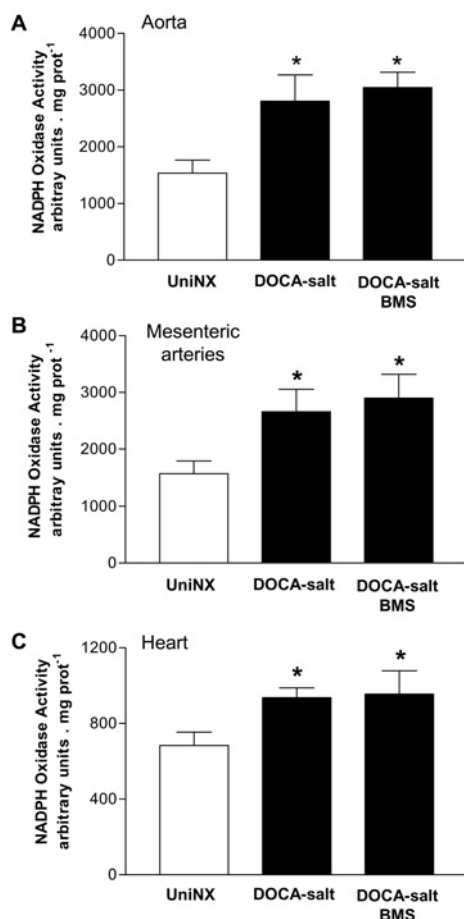


Figure 2 NADPH-dependent $O_2^{\bullet-}$ production in aortas (A), mesenteric arteries (B) and hearts (C) from UniNX rats, DOCA-salt rats and DOCA-salt rats treated with BMS182874. Values are means \pm S.E.M. ($n = 5-8$). * $P < 0.05$ compared with UniNX rats.

those from UniNX rats, was also observed (Figure 2C). Xanthine-dependent $O_2^{\bullet-}$ production was significantly increased ($P < 0.05$) in mesenteric arteries and heart from DOCA-salt rats compared with UniNX rats (Figure 3). Figures 2 and 3 also show that BMS182874 had no effect on increased $O_2^{\bullet-}$ generation in both arteries and hearts from DOCA-salt rats.

Subunits gene and protein expression of NADPH oxidase

Figure 4 shows NADPH oxidase subunit gene expression in mesenteric arteries from DOCA-salt and UniNX rats. Significant ($P < 0.05$) p22^{phox} gene overexpression was observed in mesenteric arteries of DOCA-salt compared with UniNX rats (Figure 4A). Vascular p47^{phox} gene expression was not different between DOCA-salt and UniNX rats. As shown in Figure 5, significantly increased vascular p22^{phox} protein expression ($P < 0.03$) was also observed in DOCA-salt compared with UniNX rats, whereas p47^{phox} protein expression was similar in these

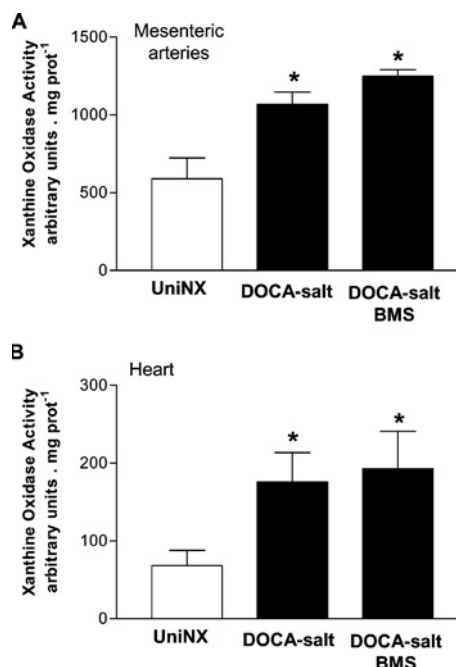


Figure 3 Xanthine-oxidase-dependent $O_2^{\bullet-}$ production in mesenteric arteries (A) and hearts (B) from UniNX rats, DOCA-salt rats and DOCA-salt rats treated with BMS182874. Values are means \pm S.E.M. ($n = 5-6$). * $P < 0.05$ compared with UniNX rats.

two groups. BMS182874 had no effect on NADPH oxidase subunit protein expression in vascular tissues from DOCA-salt and UniNX rats.

p47^{phox} translocation

To evaluate further whether ET-1 induces NADPH oxidase activation, we assessed the effects of BMS182874 on NADPH oxidase subunit protein expression in cell fractions (membrane and cytosol) of aorta. As shown in Figure 6, both total and membrane fractions of p22^{phox} were significantly increased ($P < 0.05$) in aortas from DOCA-salt rats compared with UniNX rats. BMS182874 had no effect on aortic p22^{phox} protein content in DOCA-salt rats. Total p47^{phox} content was similar in aortas from DOCA-salt and UniNX rats (Figure 7A). In aortas from DOCA-salt rats, the expression of p47^{phox} was significantly decreased ($P < 0.05$) in the cytosolic fraction (Figure 7B), and significantly increased ($P < 0.05$) in the membrane fraction (Figure 7C), indicating increased translocation, compared with those from UniNX rats. BMS182874 had no effect on p47^{phox} protein expression in cell fractions from aortas from DOCA-salt rats (Figure 7).

COX-2, eNOS and iNOS protein expression

Expression of COX-2 (Figure 8A) and eNOS (Figure 8B) was significantly greater ($P < 0.01$ and $P < 0.05$

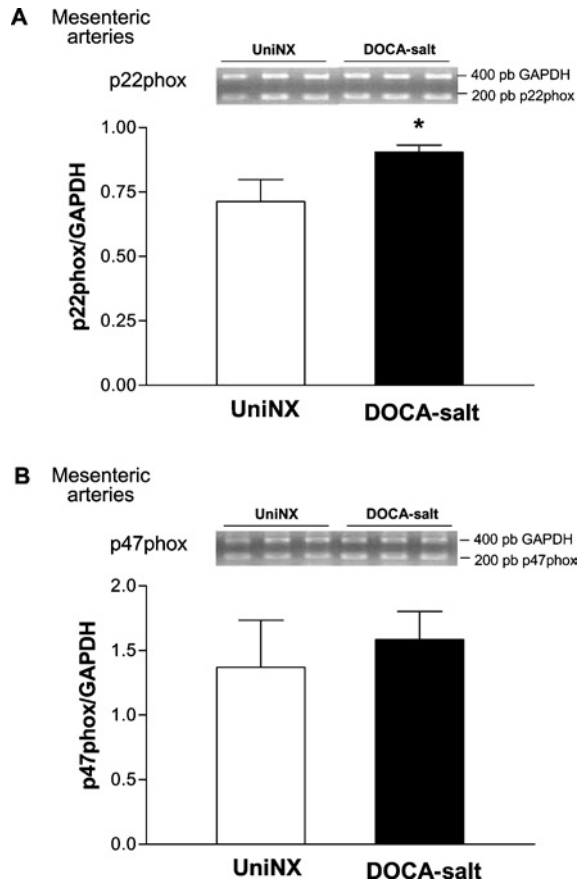


Figure 4 Gene expression of p22^{phox} (A) and p47^{phox} (B) from mesenteric arteries of DOCA-salt and UniNX rats determined by RT-PCT

Upper panels, representative RT-PCR products of 3 μ g of total RNA extracted. Lower panels, densitometric values normalized to the corresponding RT-PCR products of GAPDH ($n = 4-6$). * $P < 0.05$ compared with UniNX rats.

respectively) in aortas from DOCA-salt rats compared with UniNX rats. BMS182874 had no effect on eNOS protein expression in aortas from DOCA-salt rats. iNOS protein expression was similar in DOCA-salt and UniNX rats; however, BMS182874 significantly decreased ($P < 0.03$) the basal levels of iNOS in DOCA-salt aortas (Figure 8C).

Mitochondrial ROS generation and antioxidant defence

Succinate-dependent generation of $O_2^{\bullet-}$ was measured in heart mitochondrial preparations from BMS182874-treated and -untreated DOCA-salt rats and compared with those from UniNX rats. Figure 9(A) shows that lucigenin-derived luminescence was significantly higher ($P < 0.05$) in mitochondria from DOCA-salt rats than UniNX rats. BMS182874 treatment significantly decreased ($P < 0.05$) the $O_2^{\bullet-}$ generation in intact mitochondria from hearts of DOCA-salt rats.

Figure 9(B) shows that MnSOD protein expression was similar in heart mitochondrial preparations from DOCA-salt and UniNX rats. BMS182874 significantly increased ($P < 0.05$) the basal levels of MnSOD in the DOCA-salt group.

DISCUSSION

It is now clearly established that the enzyme NADPH oxidase plays a major role as an important source of $O_2^{\bullet-}$ in vascular cells and contributes significantly to the functional and structural alterations present in hypertension. This is particularly evident in Ang II (angiotensin II)-dependent forms of hypertension. However, sources other than NADPH oxidase, such as xanthine oxidase, COX, mitochondrial enzymes and uncoupled eNOS, may also contribute to oxidative damage in pathological conditions. The relative contribution of different ROS-generating enzymes probably depends on the underlying stimulus driving ROS formation. We have reported previously [24] that, in VSMCs, Ang II potentially activates NADPH oxidase to generate ROS, whereas ET-1 stimulates ROS formation through mitochondrial enzyme-dependent processes.

ET-1 plays an important role in cardiovascular damage associated with DOCA-salt hypertension [6-8]. We [17] and others [19] have recently demonstrated that ET-1 induces vascular $O_2^{\bullet-}$ overproduction via ET_A receptors in DOCA-salt-treated rats. In the present study, we have observed that ET-1-induced oxidative stress appears to be a systemic process, since plasma levels of TBARS were greater in DOCA-salt rats and were normalized by the ET_A receptor antagonist BMS182874.

Some studies have reported that NADPH oxidase, a multi-subunit enzyme comprising gp91^{phox}, p22^{phox}, p47^{phox} and p67^{phox}, is the major source of ET-1-stimulated ROS formation [9,11,18-20], whereas others have suggested NADPH-oxidase-independent enzymatic sources [24]. In the present *in vivo* study, we sought to determine whether NADPH oxidase is in fact a target for ET-1/ET_A receptor in DOCA-salt hypertension. We found that NADPH oxidase contributes to oxidative stress in DOCA-salt hypertension through a pathway independent of ET_A receptor activation, since ET_A receptor antagonism did not influence NADPH-dependent generation of $O_2^{\bullet-}$, measured by lucigenin-enhanced chemiluminescence, in tissues from DOCA-salt rats. p22^{phox} and p47^{phox} subunits are critically involved in fully functional NADPH oxidase. Increased vascular production of $O_2^{\bullet-}$, associated with up-regulation of p22^{phox} expression, has been shown in DOCA-salt hypertension [9]. Supporting our functional data, we also identified increased p22^{phox} gene and protein expression in vessels from DOCA-salt rats which was unaltered by ET_A antagonism. Gene as well as total protein expression of vascular p47^{phox} were

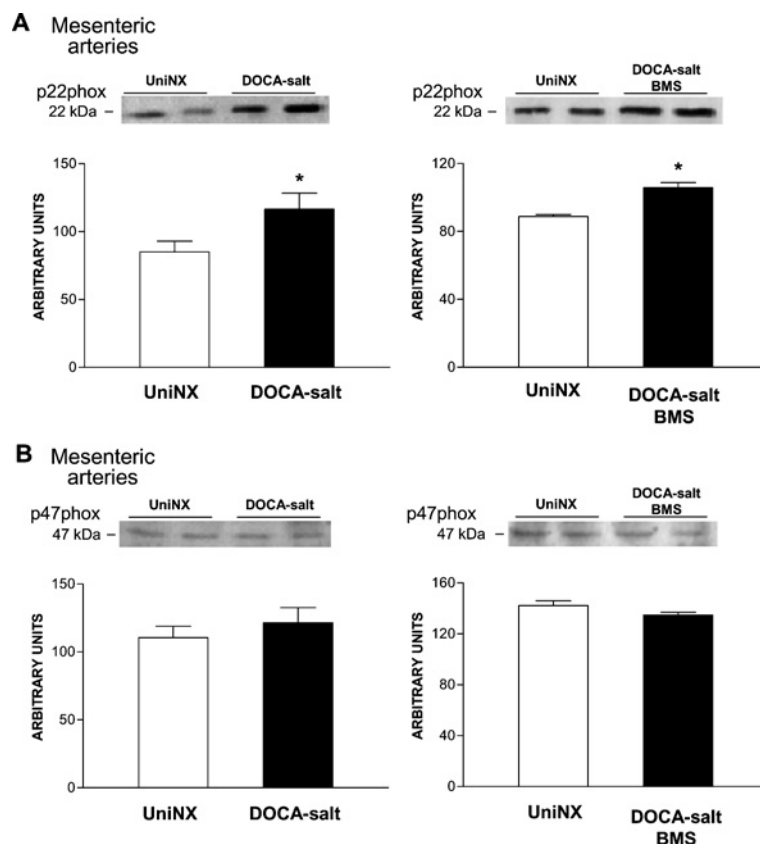


Figure 5 Protein expression of p22^{phox} (A) and p47^{phox} (B) in mesenteric arteries from UniNX rats, DOCA-salt rats and DOCA-salt rats treated with BMS182874

Upper panels, representative immunoblots of p22^{phox} and p47^{phox}. Lower panels, densitometric data for protein expression of p22^{phox} and p47^{phox}. Values are means \pm S.E.M. ($n = 4$ in each group). * $P < 0.05$ compared with UniNX rats.

not altered in DOCA-salt rats. Interestingly, translocation of the p47^{phox} cytosolic subunit to the membrane, which plays a pivotal role in NADPH oxidase activation, was increased in vessels from DOCA-salt rats, further confirming up-regulation of NADPH oxidase in DOCA-salt rats. It should be noted that ET_A blockade did not influence the increased vascular p47^{phox} translocation in DOCA-salt rats. Similar results were observed in hearts from DOCA-salt rats (results not shown). Taken together, these results strongly suggest that increased NADPH oxidase activity and p22^{phox} expression are independent of the ET-1 system in DOCA-salt hypertension. These results are consistent with previous reports in human VSMCs showing that sources other than NADPH oxidase contribute to ET-1-induced ROS production [29]. On the other hand, studies performed in veins and coronary arteries from DOCA-salt rats has demonstrated that blockade of ET_A receptors decreases the lucigenin luminence signal [18–20]. Overall these observations suggest regional heterogeneity of NADPH oxidase contribution to ET-1-induced oxidative stress.

In addition to NADPH oxidase, several enzymatic cell systems produce O₂^{•-} and its derivatives. Xanthine oxidase metabolizes hypoxanthine, xanthine and NADH to form O₂^{•-} and H₂O₂ [25]. Xanthine-generated ROS was also evaluated in the present study and our results clearly show that this pro-oxidant enzyme was not involved in ET-1-induced oxidative stress in DOCA-salt rats.

Under certain circumstances, eNOS can generate O₂^{•-}. eNOS utilizes L-arginine as a substrate to synthesize NO in a BH₄ (tetrahydrobiopterin)-dependent manner. If the concentration of L-arginine or BH₄ is low, or if BH₄ is oxidized, eNOS becomes uncoupled and generates significant amounts of O₂^{•-} [26]. This occurs in hypertension, where activation of NADPH oxidase leads to oxidation of BH₄ and production of large amounts of O₂^{•-} from eNOS [27]. The overexpression of eNOS demonstrated in aortas from DOCA-salt rats could contribute to O₂^{•-} production in this model of hypertension. However, the fact that BMS182874 does not change the increased eNOS expression provides evidence for the dissociation between

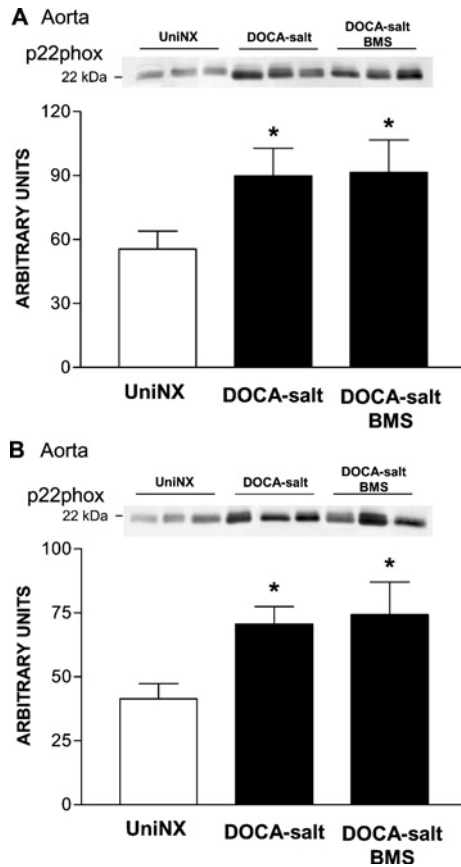


Figure 6 Expression of p22^{phox} in total protein (A) and membrane fraction (B) of aortic arteries from UniNX, DOCA-salt rats and DOCA-salt rats treated with BMS182874. Upper panels, representative immunoblots are shown. Lower panels, densitometric data for protein expression of p22^{phox}. Values are means ± S.E.M. ($n = 6-8$). * $P < 0.05$ compared with UniNX rats.

ET-1-induced oxidative stress and eNOS in DOCA-salt rats.

It has been demonstrated that human ET-1 gene causes chronic inflammation with an induction of vascular iNOS expression [28]. The induction of iNOS expression might cause a local imbalance between vascular NO and ROS, resulting in oxidative stress. In spite of the fact that DOCA-salt hypertension is characterized by an overactivation of the ET-1 system, aortic iNOS expression was not increased; however, BMS182874 decreased basal iNOS expression. This effect of ET_A antagonism may counteract the increased ROS activity under the condition of a primary activated ET system.

COX exists in two isoforms, COX-1 and COX-2. There is evidence that COX-2 may act as a source of arachidonic-acid-derived free radicals during DOCA-salt hypertension [29]. In agreement with others [30], in the present study we found an increased expression of COX-2 in aortas from DOCA-salt rats that may, in part, mediate oxidative stress in this model of hypertension.

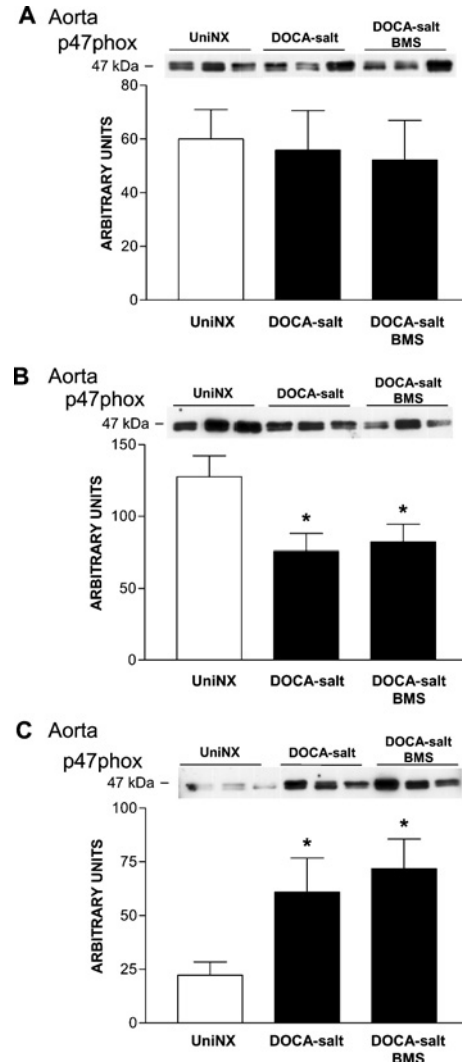


Figure 7 Expression of p47^{phox} in total protein (A) and cytosolic (B) and membrane (C) fractions of aortic arteries from UniNX, DOCA-salt rats and DOCA-salt rats treated with BMS182874.

Upper panels, representative immunoblots are shown. Lower panels, densitometric data for protein expression of p47^{phox}. Values are means ± S.E.M. ($n = 6-8$). * $P < 0.05$ compared with UniNX rats.

However, enhanced COX-2 expression was not affected by ET_A receptor antagonism. COX-2 may represent a potential mechanism of ROS production in DOCA-salt hypertension independent of the ET-1 system/ET_A receptors.

The degree of oxidative stress and severity of cardiovascular damage may depend on the imbalance between excessive ROS production and decreased antioxidant defence. Consistent with this notion, oxidative stress induced by glutathione depletion causes severe hypertension in rats [4]. Of the many antioxidant defences, the glutathione redox cycle appears to be very important

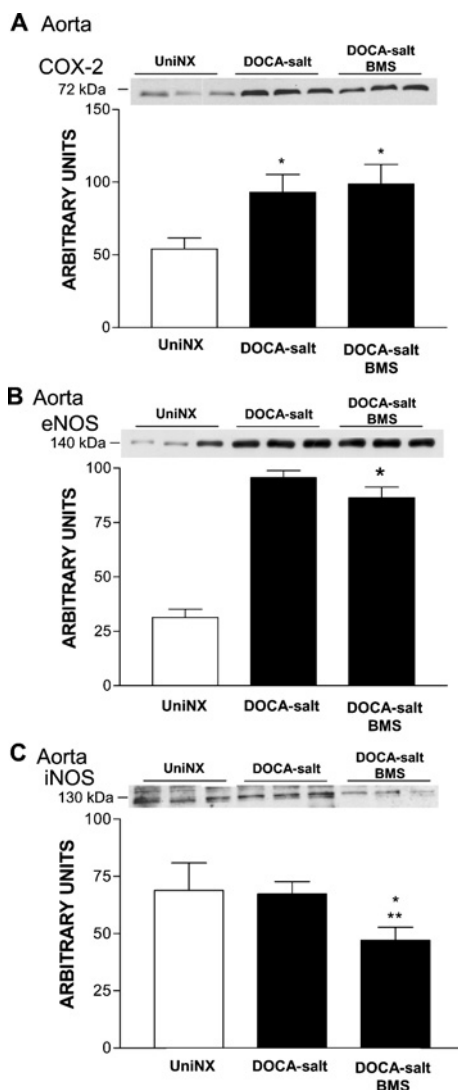


Figure 8 Expression of aortic COX-2 (A), eNOS (B) and iNOS (C) in UniNX, DOCA-salt rats and DOCA-salt rats treated with BMS182874

Upper panels, representative immunoblots are shown. Lower panels, densitometric data for protein expression. Values are means \pm S.E.M. ($n = 6-8$). * $P < 0.05$ compared with UniNX.

[31]. Glutathione is the most abundant non-protein intracellular thiol, with multiple roles as an antioxidant agent [3]. Glutathione peroxidase, an important enzyme that uses the reduced form of glutathione, not only removes H_2O_2 formed after the SOD-catalysed dismutation reaction, but also detoxifies lipid hydroperoxides. In several *in vitro* studies, glutathione peroxidase alone was demonstrated to confer an important protection against oxidative damage, even greater than SOD, catalase or the combination of SOD and catalase [32]. In this process, glutathione is converted into its oxidized form, which must be reduced by the combination of glutathione reductase and NADPH. In our present study, we also

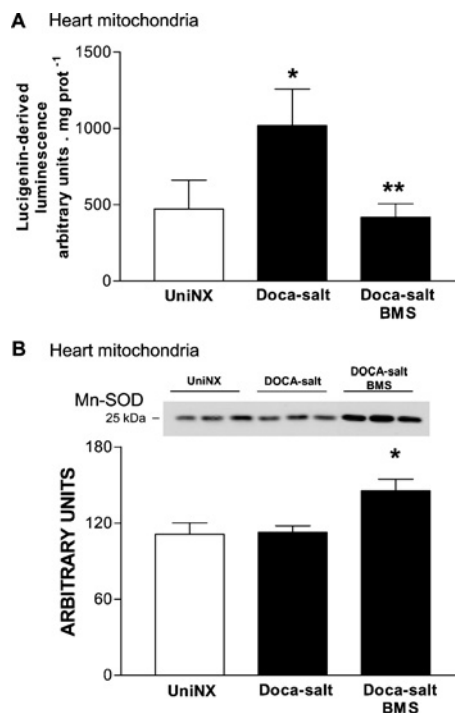


Figure 9 Succinate-dependent $O_2^{\cdot-}$ production (A) and expression of MnSOD (B) in heart intact mitochondria from UniNX rats, DOCA-salt rats and DOCA-salt rats treated with BMS182874

In (B), a representative immunoblot is shown in the upper panel, and densitometric data for MnSOD expression are presented graphically. Values are means \pm S.E.M. ($n = 4-7$). * $P < 0.05$ compared with UniNX rats; ** $P < 0.05$ compared with DOCA-salt rats.

analysed the total glutathione content and glutathione peroxidase and reductase activities as an index of cellular antioxidant defence. As we found no changes in this antioxidant defence system neither in vascular nor in non-vascular tissues from DOCA-salt hypertensive rats, it seems that oxidative stress in DOCA-salt hypertension is probably not related to changes in glutathione homeostasis.

Mitochondrial function has been implicated in oxidative stress [2]. We demonstrated recently [33] that inhibition of mitochondrial complex II and IV blunted ET-1-induced ROS generation in human VSMCs. In accordance with these observations, in the present study we found that ET_A antagonism reduced mitochondrial-dependent generation of ROS in hearts from DOCA-salt rats. Although we did not assess mitochondrial function in vessels, we propose that similar phenomena may occur in vascular tissue, since oxidative patterns are comparable in the heart, small arteries and aorta in DOCA-salt hypertensive rats, as we have reported previously [34]. Nevertheless, a detailed study investigating the role of mitochondrial-derived ROS in the development of hypertension is warranted. Taken together, our findings

suggest that ROS production by ET-1 in DOCA-salt hypertension is mediated, at least in part, by mitochondria-dependent mechanisms. The deleterious effects resulting from the ROS generation in the mitochondria are, to a large extent, prevented by antioxidant systems. The mitochondrial matrix contains a specific form of SOD, with manganese in the active site, which eliminates the $O_2^{\bullet-}$ formed in the matrix or on the inner side of the inner membrane [35]. Although the expression of MnSOD was not altered in DOCA-salt hypertension, BMS182874 improved mitochondrial antioxidant defence. These novel findings suggest that BMS182874 may have beneficial antioxidant effects by up-regulating MnSOD.

Major findings from the present study demonstrate that DOCA-salt hypertension is associated with increased generation of ROS and that the ET-1-dependent component of oxidative stress in this model may not involve NADPH oxidase. This is supported by our results showing that the translocation of p47^{phox} from the cytosol to the membrane, a critical step in NADPH oxidase activation, is increased in DOCA-salt hypertension, but that ET_A antagonism does not alter this process. Also, increased NADPH-driven $O_2^{\bullet-}$ generation associated with increased p22^{phox} expression and enhanced oxidase activity in DOCA-salt rats were unaffected by BMS182874. In DOCA-salt hypertension, xanthine oxidase as well as eNOS and COX-2 contribute to oxidative stress, but seem to be unrelated to activation of the ET system. Our present study also showed that the glutathione system is preserved in this ET-1-dependent model of hypertension. Major novel findings of our present study support the contribution of mitochondria in ET-1-dependent oxidative stress in DOCA-salt rats. The modulation of mitochondrial function by ET-1 may be an innovative mechanism mediating the beneficial effects of ET_A receptor blockade in hypertension. Moreover, up-regulation of MnSOD expression by BMS182874 reveals a potentially new role for ET_A antagonism.

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