ET\textsubscript{A} Receptor Blockade Decreases Vascular Superoxide Generation in DOCA-Salt Hypertension

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Abstract—Development and progression of end-organ damage in hypertension have been associated with increased oxidative stress. Superoxide anion accumulation has been reported in deoxycorticosterone acetate (DOCA)-salt hypertension, in which endothelin-1 plays an important role in cardiovascular damage. We hypothesized that blockade of ET\textsubscript{A} receptors in DOCA-salt rats would decrease oxidative stress. Both systolic blood pressure (SBP, 210±9 mm Hg; \( P<0.05 \)) and vascular superoxide generation in vivo were increased in DOCA-salt (44.9±10.3\% of ethidium bromide–positive nuclei; \( P<0.05 \)) versus control uninephrectomized (UniNx) rats (118±3 mm Hg; 18.5±3\%, respectively). In DOCA-salt rats, the ET\textsubscript{A} antagonist BMS 182874 (40 mg/kg per day PO) lowered SBP (170±4 versus UniNx, 120±3 mm Hg) and normalized superoxide production (21.7±6 versus UniNx, 11.9±7\%). Vitamin E (200 mg/kg per day PO) decreased superoxide formation in DOCA-salt rats (18.8±7\%) but did not alter SBP. Oxidative stress in nonstimulated circulating polymorphonuclear cells (PMNs) or in PMNs treated with zymosan, an inducer of superoxide release, was similar in DOCA-salt and UniNx groups. Superoxide formation by PMNs was unaffected by treatment with BMS 182874. Western blot analysis showed increased nitrotyrosine-containing proteins in mesenteric vessels from DOCA-salt compared with UniNX. Treatment with either BMS 182874 or vitamin E abolished the differences in vascular nitrotyrosine-containing proteins between DOCA-salt and UniNX. Maximal relaxation to acetylcholine was decreased in DOCA-salt aortas (75.8±4.2\% versus UniNx, 95.4±1.9\%, \( P<0.05 \)). BMS 182874 treatment increased acetylcholine-induced relaxation in DOCA-salt aortas to 93.5±4.5\%. These in vivo findings indicate that increased vascular superoxide production is associated with activation of the endothelin system through ET\textsubscript{A} receptors in DOCA-salt hypertension, in apparently blood pressure–independent fashion. (Hypertension. 2003; 42[part 2]:606–612.)

Key Words: endothelin \( \mathbf{\textit{A}} \) receptors, endothelin \( \mathbf{\textit{A}} \) deoxycorticosterone \( \mathbf{\textit{A}} \) hypertension, arterial \( \mathbf{\textit{A}} \) oxidative stress

Reactive oxygen species (ROS), such as superoxide anion (\( \text{O}_2^\cdot \)), hydrogen peroxide, and peroxynitrite (ONOO\(^{-}\)), are generated as intermediates in reduction-oxidation reactions. The major source of ROS in the vasculature is the nonmitochondrial NADPH oxidase. Under physiological conditions, ROS production is inactivated by an elaborate cellular and extracellular antioxidant defense system, of which glutathione peroxidase is a major component. In pathological conditions, increased generation of ROS and/or depletion of the antioxidant capacity results in increased bioavailability of ROS, referred to as oxidative stress.\(^{1}\)

There is increasing evidence that oxidative stress plays a pathological role in hypertension.\(^{2}\) Several recent studies have provided compelling evidence for increased ROS generation in the vascular tissues of hypertensive rats. Enhanced \( \text{O}_2^\cdot \) production has been demonstrated in mesenteric arterioles of SHR in vivo.\(^{3}\) Likewise, increased \( \text{O}_2^\cdot \) generation has been reported in cultured aortic endothelial cells from SHR compared with WKY.\(^{4}\) Oxidative stress has been implicated in a variety of other hypertensive models including Angiotensin II (Ang II)–induced hypertension,\(^{5,6}\) Dahl salt-sensitive hypertension,\(^{7}\) and in human essential hypertension.\(^{8}\) By promoting NO inactivation, lipid peroxidation, DNA damage, and protein modification, oxidative stress plays a key role in endothelial dysfunction and end-organ damage. Furthermore, ROS activate many redox-sensitive, growth-related intracellular signaling pathways in vascular smooth muscle and endothelial cells, which is particularly important in altered proliferation and hypertrophy, contributing to vascular remodeling, a characteristic feature of hypertensive disease.\(^{9,10}\)

Cytokines, growth factors, and vasoactive agents such as Ang II regulate the activity and expression of enzymes involved in ROS production.\(^{1}\) In Ang II–dependent models of hypertension, vascular production of \( \text{O}_2^\cdot \) is increased through activation of vascular NADPH oxidase.\(^{5,6}\) Indeed,
antioxidant treatment has been shown to have beneficial effects in Ang II–induced hypertension by decreasing blood pressure and reducing end-organ damage.6,11

In double transgenic rats (dTGR) harboring the human renin and angiotensinogen genes, endothelin-1 (ET-1) receptor blockade with bosentan interferes with ROS-dependent inflammatory processes and ameliorates end-organ damage in dTGR.12 These data implicate ET-1 in the production of ROS by Ang II. Increased vascular O$_2^-$ production also occurs in deoxycorticosterone acetate (DOCA)-salt hypertension,13–17 an experimental model in which ET-1 plays an important role in cardiovascular damage.18–23 Because the DOCA-salt model displays a marked decrease in plasma renin activity, it provides an opportunity to study the contribution of ET-1 to oxidative stress, without interference of the renin-angiotensin system. Accordingly, in this study, we tested the hypothesis that in addition to its vasoactive and growth-promoting actions, ET-1 plays a role in the vascular production of ROS in DOCA-salt hypertension.

**Methods**

**Animal Experiments**

Experimental protocols followed standards and policies of the University of Sao Paulo’s Animal Care and Use Committee. Male Wistar rats from the Institute of Biomedical Science’s animal facility were used in this study. All animals had ad libitum access to both standard laboratory rat chow and tap water and were housed individually in a room under constant temperature (24°C) and a 12-hour/12-hour light/dark cycle. DOCA-salt hypertension was induced as previously described,22 and rats were randomized into 3 groups: (1) DOCA-salt and uninephrectomized control (UniNX) rats; (2) DOCA-salt and UniNX rats treated with the ETA antagonist BMS 182874 (40 mg/kg per day PO per gavage); (3) DOCA-salt and UniNX rats treated with vitamin E (α-tocopherol, 200 mg/kg per day PO per gavage). Systolic blood pressure (SBP) was measured weekly in unanesthetized animals by an indirect tail-cuff method (pneumatic transducer, PowerLab 4/S, AD Instruments Pty Ltd). At the end of the 5th week of treatment, rats were submitted to the experimental procedures described below.

**Intravital Fluorescence Microscopy**

Intravital fluorescence microscopy was used to estimate the O$_2^-$ production as previously described.24 Briefly, rats were anesthetized with chloral hydrate (400 to 450 mg/kg SC), and the mesentery was arranged for microscopic observation in vivo, in situ. The preparation was kept at 37°C and was continuously superfused (1.0 mL/min) with a Krebs solution, saturated with a 95% N$_2$/5% CO$_2$ gas mixture. Single unbranched arterioles (15 to 25 μm) were selected for this study. The mesenteric microcirculation was visualized through an intravital microscope (Axioskop, Zeiss) with a ×20 water immersion objective lens by using a digital color charge-coupled device (CCD) camera (ZVS-47EC, Zeiss). Transilluminated and fluorescent images were recorded by a computer system (KS-300, Kontron) for posterior analysis. After an initial 30-minute stabilization period, when the mesenteric preparation was superfused with a standard buffer, a background autofluorescence image in the selected tissue area was recorded. The preparation was then superfused with a buffer solution containing hydroethidine (HE; 10.0 μmol/L, Polysciences) for 60 minutes. The number of nuclei labeled with ethidium bromide (EB-positive nuclei) along arterioles (NEB) was determined every 15 minutes after the onset of HE superfusion. At the end of the experiments, the tissue was superfused with absolute ethanol for 5 minutes followed by EB superfusion to establish the total number of nuclei along the vessel wall (NT). The EB-positive number was counted (double-blind) and expressed as a percentage of EB-positive nuclei=(NEB/NT)×100 (%).

**Measurement of O$_2^-$ Production by Circulating Polymorphonuclear Cells**

Polymorphonuclear cells (PMNs) were isolated by a combined sedimentation and density centrifugation procedure, according to the method previously described.25 PMNs were resuspended with Hank’s balanced salt solution supplemented with 10 mmol/L HEPES (pH 7.4), and the isolated cells were counted. Cell suspensions were diluted to a concentration of 10$^6$ cells/mL. The production of O$_2^-$ by PMN cells was measured based on the SOD-inhibitable spectrophotometric detection of reduced cytochrome C. The production of O$_2^-$ was studied in stimulated cells and in the presence of zymosan (100 particles/cell).

**Western Blot Analysis for Nitrotyrosine**

Mesenhytrom homogenate proteins (20 μg) were separated by SDS-PAGE (10% polyacrylamide) and electrophoretically transferred to a nitrocellulose membrane. After blocking non-specific sites with 0.2% casein, the membranes were incubated overnight at 4°C with primary mouse monoclonal antibodies raised against nitrotyrosine (NT)-modified KLH (Keyhole Limpet Hemocyanin; 500 ng/mL; Upstate). Membranes were washed with Tris-buffered saline containing 0.2% Tween 20 and incubated with alkaline phosphatase–conjugated rabbit anti-mouse antibody. A chemiluminescence assay kit (ImmunoStar; Bio-Rad) was used to detect immunoreactive NT-containing proteins, and the intensity of all bands was estimated by densitometric analysis with a Chemosizer 5500 system (Alpha Innotech).

**Relaxant Response of Aortic Artery Rings to Acetylcholine**

Aortic rings, 4 mm in length, were cut and mounted between two steel hooks to measure the isometric tension as earlier described.22 Vessels were submitted to a tension of 1.5 g, which was adjusted every 15 minutes during a 60-minute equilibration period before the addition of a given drug. At the beginning of the experiments, the aortas were stimulated with 0.1 μmol/L norepinephrine, and the integrity of the endothelium was assessed by the presence of relaxation in response to 1 μmol/L acetylcholine. Concentration-response curves to acetylcholine (1 nmol/L to 10 μmol/L) were performed in endothelium-intact aorta precontracted with 0.3 μmol/L phenylphrine from both DOCA-salt and UniNX rats. Endothelium-independent relaxation was evaluated with sodium nitroprusside (0.01 nmol/L to 10 μmol/L).

**Data Analysis**

Results are expressed as mean ± SEM; n indicates the number of animals. The concentration of the agonist producing a half-maximal response (EC$_{50}$) was determined after logit transformation of the normalized concentration-response curves and is reported as the negative logarithm of the mean of individual values for each tissue by the use of the Prism GraphPad 4.04 software. Statistical significance was evaluated by ANOVA or Student t test, as appropriate; a probability value <0.05 was considered significant.

**Results**

**Blood Pressure Measurement**

SBP increased progressively after DOCA treatment and salt loading (Figure 1). At 5 weeks of treatment, SBP in DOCA-salt rats was higher (P<0.05) than in UniNX. Treatment with the ETA antagonist BMS 182874 significantly reduced but did not prevent SBP elevation in DOCA-salt rats (Figure 1A). At 5 weeks of treatment with BMS 182874, SBP was lower in DOCA-salt (P<0.05) but not in UniNX rats compared with vehicle-treated groups. Vitamin E had no effect on SBP in DOCA-salt or UniNX rats (Figure 1B).
Nonstimulated PMNs generated small amounts of ·O₂⁻, and zymosan-stimulated PMNs had an increased generation of ·O₂⁻. Production of ·O₂⁻ by both nonstimulated and zymosan-stimulated PMNs was similar in DOCA-salt and UniNX. BMS 182874 treatment did not affect the generation of ·O₂⁻ in nonstimulated or zymosan-stimulated circulating cells from DOCA-salt or UniNX.

Nitrotyrosine-Containing Proteins
Western blot analysis showed increased nitrotyrosine-containing proteins in mesenteric arteries from DOCA-salt as compared with UniNX (Figure 3A, n=4). Both BMS 182874 (Figure 3B, n=4) and vitamin E (Figure 3C, n=4) treatment abolished the difference in nitrotyrosine-containing proteins between DOCA-salt and UniNX.

Aortic Relaxation by Acetylcholine
As shown in Figure 4, maximal relaxation to acetylcholine was decreased in DOCA-salt aorta (75.8±4.2%; −Log EC₅₀: 6.9±0.1; n=14) compared with UniNX (95.4±1.9%; −Log EC₅₀: 7.3±0.05; n=11). Treatment with BMS 182874 improved relaxation to acetylcholine in DOCA-salt aorta (93.5±4.5%; −Log EC₅₀: 7.4±0.1; n=8). Endothelium-independent relaxation by sodium nitroprusside was similar between DOCA-salt and UniNX (data not shown).

Discussion
Development and progression of end-organ damage in hypertension have been associated with increased vascular oxidative stress. The present in vivo study provides new evidence of the functional role of ET-1 on oxidative stress by demonstrating that increased vascular ·O₂⁻ production in DOCA-salt hypertension is mediated by ET-1 through activation of ET₄ receptors.

The significance of ET-1 in cardiovascular disease and its contribution to hypertension and vascular remodeling has been recently reviewed. However, little is known about the precise role of ET-1 on oxidative stress. Whether ET-1 plays a role in mediating oxidative stress or is affected by it is not clear. ET-1 augments ·O₂⁻ generation in endothelial cells. Moreover, suppression of ET-1 secretion under oxidative stress observed in endothelial cells is proposed to be a compensatory mechanism to inhibit vasoconstriction and proliferation during oxidative stress. Increased oxidative stress has been reported in DOCA-salt rats, suggesting an experimental model characterized by increased expression of ET-1. Our findings in this study support the role of ET-1 in ROS production through stimulation of ET₄ receptors. Hydroethidine has been used as a tool to detect spontaneous oxidative changes in the microcirculation in vivo conditions.

Although hydroethidine oxidation to EB is caused more rapidly by ·O₂⁻ than by other ROS, we cannot exclude the possibility that other ROS may also contribute to the increased fluorescence signal observed in our study. However, ·O₂⁻ is the most likely ROS involved in vascular oxidative stress as recently reported by Li et al, who demonstrated increased ET-1–mediated oxidative stress in carotid arteries from DOCA-salt rats by using dihydroethidium and lucigenin in vitro.

Besides vascular cells, other sources of ·O₂⁻ may contribute to oxidative stress in hypertension. Peripheral PMN leukocytes, which generate ·O₂⁻, may contribute to the oxidative stress in patients with essential hypertension. Interestingly, there were no differences in ·O₂⁻ formation...
either in nonstimulated or zymosan-stimulated PMNs between DOCA-salt and UniNX, showing that oxidative stress in DOCA-salt hypertension is apparently unrelated to changes in \( \text{O}_2^- \) formation by circulating PMNs.

ROS are generated as intermediates in redox processes and may interact with different groups of compounds. For instance, \( \text{O}_2^- \) reacts with NO to produce ONOO\(^-\), a highly cytotoxic compound, which can, in turn, react with DNA, lipids, and aromatic amino acids such as tryptophan and tyrosine. Tyrosine residues, either free or protein bound, can be nitrated by ONOO\(^-\), resulting in the formation of 3-nitrotyrosine. However, this reaction is not exclusive, since nitrotyrosine residues can also be formed from other nitrogen-derived species different than ONOO\(^-\).

Increased nitrotyrosine-containing proteins, a hallmark of oxidative stress, was demonstrated in mesenteric vessels from DOCA-salt rats in this study. The improvement of nitrotyrosine accumulation by BMS 182874 treatment reinforces the role of ETA-mediated ROS production, and specifically ONOO\(^-\) formation.

An important characteristic of oxidative stress is the impairment of endothelium-dependent vasodilation caused by enhanced NO inactivation by ROS. Furthermore, nitrotyrosine accumulation has been implicated in NO sequestration and inactivation. Thus, decreased NO availability may contribute to impaired endothelium-dependent relaxation in DOCA-salt hypertension. Previous findings demonstrated that the reduction of vascular \( \text{O}_2^- \) generation by SOD

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**Table 1: Effect of BMS 182874 Treatment on \( \text{O}_2^- \) Production by PMNs Obtained From UniNX and DOCA-Salt Rats**

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Nonstimulated</th>
<th>Zymosan-Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>UniNX (6)</td>
<td>0.2±0.1</td>
<td>2.2±0.4*</td>
</tr>
<tr>
<td>DOCA-salt (6)</td>
<td>0.3±0.2</td>
<td>1.7±0.2*</td>
</tr>
<tr>
<td>UniNX BMS 182874 (6)</td>
<td>0.4±0.1</td>
<td>2.0±0.6*</td>
</tr>
<tr>
<td>DOCA-salt BMS 182874 (6)</td>
<td>0.3±0.2</td>
<td>1.9±0.3*</td>
</tr>
</tbody>
</table>

Results (as nmol \( \text{O}_2^-/10^6 \) cell · min\(^{-1}\)) are expressed as mean±SEM. *\( P < 0.05 \) vs nonstimulated cells.

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**Figure 2.** Top, Representative images from intravital fluorescence microscopy show transillumination images (left) and EB fluorographs (right) of mesenteric arterioles 60 minutes after onset of hydroethidine superfusion in UniNX, DOCA-salt, BMS 182874–treated DOCA-salt, and vitamin E–treated DOCA-salt rats. Bottom, Time course of the EB-positive nuclei along mesenteric arterioles of DOCA-salt and UniNX rats treated with BMS 182874 (A) or vitamin E (B). Results are mean±SEM. *\( P < 0.05 \) vs UniNX, **\( P < 0.05 \) vs DOCA-salt rats.
mimetics ameliorates endothelium-dependent relaxation in DOCA-salt.\textsuperscript{15} In our study, besides normalizing nitrotyrosine accumulation, BMS 182874 treatment also corrected the impaired relaxation to acetylcholine in DOCA-salt rats. Taken together, these observations indicate that improvement of endothelial function by blockade of ETA receptor in DOCA-salt hypertension may be related to a decrease in ROS generation. However, since our experiments on vascular function were performed in the absence of indomethacin, it is possible that BMS 182874 corrects other altered mechanisms such as the imbalance in vasodilator and vasoconstrictor cyclooxygenase products, which contributes to endothelial dysfunction in DOCA-salt hypertension.

Significant antihypertensive effects and improvement of antioxidant status in experimental hypertension have been reported by several studies with SOD mimetics, vitamins C and E.\textsuperscript{13,37,39–45} In the present study, vitamin E treatment failed to lower blood pressure in DOCA-salt rats but prevented the overproduction of \textsuperscript{1}O\textsubscript{2}\textsuperscript{–} in vivo as well as vascular nitrotyrosine accumulation. Free radical scavenging by vitamins may be a mechanism contributing to decreased oxidative stress. Furthermore, it has been recently demonstrated that vitamins modulate NADPH oxidase and SOD activities.\textsuperscript{43} \textalpha-Tocopherol supplementation prevented development of increased blood pressure, reduced lipid peroxides in plasma and blood vessels, and enhanced total antioxidant status, including SOD activity, in hypertensive rats.\textsuperscript{40,41} Differences in the blood pressure–lowering effects of vitamins and other antioxidants suggest that mechanisms other than \textsuperscript{1}O\textsubscript{2}\textsuperscript{–} scavenging may also be involved in the actions of these

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure_3.png}
\caption{Representative Western blot and densitometric group data of nitrotyrosine-containing proteins in mesenteric vessels from vehicle-treated UniNX and DOCA-salt rats (A) and effects of BMS 182874 (B) and vitamin E (C) treatments. *P<0.05 vs UniNX.}
\end{figure}
compounds. Indeed, the decrease in blood pressure by Tem-
po is mediated largely by an NO-independent sympathoin-
hibition in DOCA-salt rats.\textsuperscript{44}

The effect of ET\textsubscript{A} blockade on blood pressure in the
DOCA-salt model has been previously demonstrated.\textsuperscript{66} Treatment
with BMS 182874 attenuates but does not prevent hypertension in DOCA-salt rats, whereas in UniNX blockade of ET\textsubscript{A} had no effects on blood pressure. One can speculate that reduction in blood pressure might be responsible for reducing oxidative stress. Since vitamin E, which did not lower blood pressure in DOCA-salt rats, produced similar effects to BMS 182874 on vascular O\textsubscript{2}\textsuperscript{-} generation and nitrotyrosine protein accumulation, we can speculate that decrease of blood pressure by ET\textsubscript{A} blockade is not directly related to improvement of oxidative stress in DOCA-salt rats and may rely on other actions of ET-1 not related to ROS generation.

**Perspectives**

The reduction of increased vascular oxidative stress in vivo by blockade of ET\textsubscript{A} receptors in DOCA-salt rats supports an important role for ET-1 in ROS generation in DOCA-salt hypertension. Since oxidative stress influences specific signal
pathways and redox-sensitive genes that coordinate several integrated responses in the cardiovascular system, including growth of vascular smooth muscle, inflammatory process, cardiac hypertrophy, and impairment of endothelium-dependent relaxation,\textsuperscript{2} and because each of these alterations represents characteristic features of ET-1 actions, oxidative stress may play an important role in cardiovascular changes in mineralocorticoid hypertension as a result of ET-1 overexpression/actions. These processes appear to be independent of blood pressure elevation. These data also provide a rationale for the use of ET\textsubscript{A} receptor blockade in some forms of human hypertension.

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