Xanthine oxidase and mitochondria contribute to vascular superoxide anion generation in DOCA-salt hypertensive rats

Emilie C. Viel, Karim Benkirane, Danesh Javeshghani, Rhian M. Touyz, and Ernesto L. Schiffrin

1Hypertension and Vascular Research Unit, Lady Davis Institute for Medical Research, Sir Mortimer B. Davis-Jewish General Hospital, McGill University, Montreal, Quebec; and 2Kidney Research Centre, Ottawa Health Research Institute, University of Ottawa, Ottawa, Ontario, Canada

Submitted 20 March 2008; accepted in final form 12 May 2008

VASCULAR DISEASE is characterized by the increased generation of ROS, specifically superoxide anion (O$_2^-$), and is observed in hypertension, atherosclerosis, and the response to injury (44). Vascular O$_2^-$ production in angiotensin II-induced hypertension is generated by NAD(P)H oxidase (28, 44, 45). There is evidence of a contribution of NAD(P)H oxidase to O$_2^-$ production in a low-renin endothelin (ET)-I-dependent hypertensive model, the DOCA-salt hypertensive rat (41, 44). This model of experimental hypertension is characterized by the overexpression of ET-1 in resistance arteries (21, 22) and an elevation of O$_2^-$ levels in the vascular wall (43). Although the relationship between ET$_A$ and ET$_B$ receptors and NAD(P)H oxidase has been highlighted by several studies (2, 19, 23, 24), inhibition of NAD(P)H oxidase activity only partially affected O$_2^-$ generation in DOCA-salt rats (4), which may reflect controversies in apocynin specificity for NAD(P)H oxidase. Treatment with an ET$_A$ antagonist abolished O$_2^-$ generation (23, 24), suggesting the possible contribution of additional sources of O$_2^-$ other than NAD(P)H oxidase in this model. Xanthine oxidase (XO) and mitochondria produce O$_2^-$ and may contribute to vascular dysfunction in hypertension (10) and are thus candidates for an additional source of generation of O$_2^-$ in DOCA-salt rats.

The inhibition of nitric oxide (NO)-dependent function in endothelial cells that contain bound XO indicates that cell-bound XO can impair vascular cell function and produce O$_2^-$ in a sequestered microenvironment (11). XO is responsible for increased ROS production in aortic endothelial cells in response to oscillatory shear stress (26), and treatment with the XO inhibitor allopurinol inhibited neointimal hyperplasia and improved vascular function in diabetic rats (15). XO induces hypertrophic responses in human vascular smooth muscle cells (VSMCs) (27), and its expression is upregulated in atherosclerotic plaques (32). Under normal conditions, the mitochondrial electron transport chain (METC) is a major source of superoxide, converting up to 5% of molecular O$_2$ to O$_2^-$ (46). Because of its subcellular localization, the mitochondrial enzyme Mn-SOD is considered the first line of defense against oxidative stress (11). The importance of O$_2^-$ generation in the effects of ET-1 has been shown by gene transfer of Mn-SOD to carotid arteries in DOCA-salt rats, which results in reduced O$_2^-$ production in response to ET-1 (24). Similarly, ET-1 stimulated p38 MAPK, JNK, and ERK5 through mitochondria-dependent ROS generation in human VSMCs (45). Flow-induced dilation and H$_2$O$_2$ formation in coronary resistance arteries resulted from O$_2^-$ generated from mitochondrial respiration (25). Finally, damage to mitochondrial DNA leading to mitochondrial dysfunction has been reported in atherosclerosis and hypertension (36).

We hypothesized that ET-1-induced XO activity and mitochondrial oxidative phosphorylation contribute to vascular ROS generation in low-renin ET-1-dependent hypertension. To test this hypothesis, we blocked, in vivo, the activity of XO, the oxidative phosphorylation chain, and ET receptors to investigate their relative contribution to O$_2^-$ production in the aorta and mesenteric resistance arteries from DOCA-salt rats.
MATERIALS AND METHODS

Animals. Experiments were conducted in unilaterally nephrectomized (UniNx) male Sprague-Dawley rats (Charles River Laboratories, St. Constant, QC, Canada), weighing 180–200 g, treated or not with DOCA, following the recommendations of the Canadian Council on Animal Care and with the approval of the Animal Care Committee of the Clinical Research Institute of Montreal and the Lady Davis Institute. DOCA implants (800 mg/kg) were prepared by mixing DOCA in silicone rubber, resulting in a given dose of 250 mg/kg, as previously described (22). DOCA rats (n = 8 rats/treatment) received 1% saline as drinking water, whereas UniNx rats (n = 11) were given tap water. DOCA-salt-treated rats were fed a powder diet (Agribrands) containing apocynin (1.5 mmol/l, Sigma, St. Louis, MO), allopurinol (100 mg·kg⁻¹·day⁻¹, Sigma), boseantan (100 mg·kg⁻¹·day⁻¹, ET₄₅₅ antagonist, Actelion, Basel, Switzerland), or BMS-182874 (BMS; 40 mg·kg⁻¹·day⁻¹) or vehicle for 30 min. For the study of the METC, tissue sections from UniNx rats within 30 min of staining to avoid signal was measured as arbitrary units and compared with that found in vessels from UniNx rats. Dual blockade of ETA and ETB receptors with bosentan and hydralazine completely prevented their increase.

Liver XO activity. Liver XO activity was determined based on hepatic uric acid synthesis in response to xanthine. The rat liver was first extracted and concentrated as previously described (16). Then, 50 μl of liver extract were incubated in 50 mM potassium phosphate buffer (pH 7.5) containing 0.3 mM EDTA and 300 μM xanthine for 15 min at room temperature in a spectrophotometer (Smartspec Plus, Bio-Rad). XO activity was calculated according to the following formula: Unit activity/ml = [(ΔA₃₉₀ nm/min test − ΔA₃₉₀ nm/min blank) (3) (df)/((12.2) (0.1), where ΔA₃₉₀ nm is the absorbance at 290 nm; 3 is the total volume of the assay (in ml); df is the dilution factor; 12.2 is the extinction coefficient of uric acid at 290 nm (in m⁻¹·cm⁻¹); and 0.1 is the volume of tissue extract (in ml).

Western blot analysis. The whole aorta and mesenteric artery were cleaned of fat, frozen in liquid nitrogen, and stored at −80°C until use. The vessels were homogenized in lysis buffer (20 mM MOPS, 1% Triton, 4% SDS, 10% glycerol, 5.5 mM leupeptin, 5.5 mM pepstatin, 200 KIU aprotinin, 1 mM Na₃VO₄, 10 mM NaF, 100 mM ZnCl₂, 20 mM b-glycerophosphate, and 20 mM PMSF), kept on ice for 15 min, and centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was collected and the protein concentration was determined using the Bio-Rad protein assay. Western blot analysis was performed on polyvinylidene difluoride membranes as previously described (1). Rabbit anti-ETA antibody or rabbit anti-ETB antibody (1:500) (Alomone Labs, Jerusalem, Israel) and rabbit anti-XO antibody (1:500) (Labsvision, Fremont, CA) were used to detect protein expression. The signal was scanned by Chemicon (Bio-Rad) and quantified by the QuantityOne program. One UniNx rat sample from the aorta was set at 100%, and further UniNx and DOCA-salt samples were compared with this 100% sample. Results were expressed as percentages of UniNx values in the aorta.

Statistical analysis. Results are expressed as means ± SE. Differences among groups were tested by one-way ANOVA followed by the Newman-Keuls post hoc test. P < 0.05 was considered statistically significant.

RESULTS

Body weight, SBP, and heart and aorta weight. Body weight, SBP, and heart and aorta weight were measured after 3 wk of treatment. In DOCA-salt rats, SBP was significantly increased and body weight was significantly reduced (P < 0.001) compared with UniNx rats (Table 1). All treatments significantly reduced but did not normalize SBP. The weight of the heart and aorta increased with DOCA-salt treatment. Heart weight did not change, whereas aorta weight was reduced by BMS but not by the other treatments.

Lipid peroxidation. Lipid peroxidation, a marker of systemic oxidative stress measured by plasma TBARS as MDA, was significantly higher in DOCA-salt rats (P < 0.001) compared with UniNx rats (Fig. 1). Aprocylin and allopurinol did not reduce TBARS in DOCA-salt rats, whereas boseantan, BMS, and hydralazine completely prevented their increase.

ET receptor-induced O₂⁻• production. Aorta and mesenteric resistance artery O₂⁻• generation were investigated by oxidative fluorescent microtopography. O₂⁻• generation was increased to a greater degree in media of mesenteric arteries than in the aorta (Fig. 2, A and B) from DOCA-salt rats compared with UniNx rats. Dual blockade of ET₄ and ET₆ receptors with boseantan or selective ET₄ receptor blockade with BMS decreased the production of O₂⁻• compared with untreated DOCA-salt rats in both vascular tissues. However, BMS had greater effects on mesenteric arteries than boseantan. To correlate differences in O₂⁻• production observed in both vascular tissues with the abundance of ET receptors, ET receptor expres-
sion was measured by Western blot analysis. The ETB protein level was higher in DOCA-salt rats in the aorta compared with mesenteric arteries (Fig. 3B). ETA expression was decreased in mesenteric arteries from DOCA-salt rats (P \leq 0.05) and was found to be less expressed in the aorta from UniNx rats than in DOCA-salt rats (P \leq 0.01).

Vascular XO-derived O$_2$•$^-$ generation. Aortic XO-derived O$_2$•$^-$ was significantly increased in DOCA-salt rats compared with UniNx rats (P < 0.001; Fig. 4A). Apocynin, allopurinol, and hydralazine prevented the activation of aortic XO, whereas bosentan and BMS had no effect. Mesenteric artery production of O$_2$•$^-$ by XO, which was higher than that of the aorta, was significantly higher in DOCA-salt rats compared with UniNx rats (P < 0.001). This increase was prevented by all the treatments used (Fig. 4B). Western blot analysis of XO showed greater protein expression in mesenteric arteries from DOCA-salt rats compared with UniNx rats from both vasculatures (P \leq 0.01), but no significant differences were observed between UniNx and DOCA-salt rats within the aorta (Fig. 4C).

Plasma uric acid concentration, creatinine concentration, and XO activity. To further evaluate the involvement of XO in the DOCA-salt model, plasma uric acid was measured (Table 1). There were no significant differences of plasma uric acid levels between UniNx and DOCA-salt rats. Uric acid levels were significantly reduced (P < 0.05) after treatment of DOCA-salt rats with allopurinol and bosentan, whereas uric acid increased with BMS treatment. Plasma creatinine in DOCA-salt rats was reduced by apocynin, allopurinol, and hydralazine. XO activity was reduced in the liver of DOCA-salt rats compared with UniNx rats (Fig. 4D).

Role of mitochondrial oxidative phosphorylation in vascular O$_2$•$^-$ generation. Rotenone treatment did not affect aortic O$_2$•$^-$ production (Fig. 5A). TTFA-treated aortic rings exhibited a 50% reduction in fluorescence, whereas CCCP treatment decreased the signal by 25%. Mesenteric artery rings from DOCA-salt rats treated with rotenone showed significant differences of fluorescence compared with untreated rings from DOCA-salt rats (Fig. 5B). TTFA reduced the signal by 70%, whereas CCCP-treated rings showed a 30% reduction in O$_2$•$^-$ production.

**DISCUSSION**

The present study provides the first evidence that XO and mitochondrial sources are involved in vascular O$_2$•$^-$ production in a low-renin ET-1-dependent hypertension model, the DOCA-salt hypertensive rat. We showed that 1) the production of O$_2$•$^-$ by XO is greater in mesenteric resistance arteries than in the aorta in DOCA-salt rats, 2) ETA receptors stimulate vascular NO production to induce vascular oxidative stress, and 3) mitochondrial oxidative phosphorylation complexes can contribute to ET-1-induced vascular O$_2$•$^-$ production. Dual blockade of ETA receptors demonstrated a possible antagonism of ETB on ETA receptor function, since bosentan treatment resulted in the partial inhibition of O$_2$•$^-$ generation derived from XO, in contrast to ETA receptor-selective blockade.

NAD(P)H oxidase has been suggested to be the most important ROS source in the vascular wall of DOCA-salt rats (23, 26, 36, 42). Low-grade inflammation occurs in the vascular wall of DOCA-salt rats (14) and may exert a key regulatory role on XO gene expression (4). XO contributes to vascular endothelial dysfunction by inhibiting NO-dependent cGMP production in VSMCs (14), which underlies in part the impairment in endothelium-dependent vasorelaxation found in DOCA-salt rats (33). Thus, XO is a potential candidate as an important source of vascular O$_2$•$^-$ in DOCA-salt rats. Xanthine oxidoreductase reduces O$_2$ to produce O$_2$•$^-$, whereas xanthine dehydrogenase reduces NAD$^+$ to produce NADH. The conversion of xanthine oxidoreductase into XO, which transforms hypoxanthine to xanthine and xanthine to uric acid, is controlled by growth factors, cellular pH, or O$_2$ availability (9). Levels of plasma uric acid were affected by blocking XO or

---

**Table 1. Systolic blood pressure, heart and aorta weight, and biological parameters**

<table>
<thead>
<tr>
<th></th>
<th>UniNx</th>
<th>DOCA-Salt</th>
<th>Apocynin</th>
<th>Allopurinol</th>
<th>Bosentan</th>
<th>BMS-182874</th>
<th>Hydralazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>330±12</td>
<td>225±16*</td>
<td>243±6‡</td>
<td>264±30‡†</td>
<td>265±7‡</td>
<td>239±29</td>
<td>245±43</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>117±5</td>
<td>207±17*</td>
<td>146±7§</td>
<td>161±6‡†</td>
<td>166±23§</td>
<td>172±10§</td>
<td>129±4‡†</td>
</tr>
<tr>
<td>Heart weight/tibia length, mg/mm</td>
<td>35.8±2.1</td>
<td>45.4±2.0*</td>
<td>46.8±1.6</td>
<td>42.4±1.9</td>
<td>47.9±1.5</td>
<td>50.1±0.6</td>
<td>42.3±2.5</td>
</tr>
<tr>
<td>Aorta weight/tibia length, mg/mm</td>
<td>0.95±0.02</td>
<td>1.29±0.06*</td>
<td>1.23±0.05</td>
<td>1.38±0.03</td>
<td>1.31±0.07</td>
<td>1.16±0.02‡</td>
<td>1.22±0.06</td>
</tr>
<tr>
<td>Plasma uric acid, mg/dl</td>
<td>3.7±0.1</td>
<td>4.1±0.3</td>
<td>4.6±0.2</td>
<td>3.3±0.2†</td>
<td>3.2±0.2†</td>
<td>8.3±0.5‡</td>
<td>3.5±0.1</td>
</tr>
<tr>
<td>Plasma creatinine, µmol/l</td>
<td>25.5±2.8</td>
<td>35.2±3.1*</td>
<td>23.0±1.6†</td>
<td>23.2±3.4†</td>
<td>35.3±4.3</td>
<td>41.7±5.3</td>
<td>20.0±2.6‡</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. UniNx, unilaterally nephrectomized rats; DOCA-salt, DOCA-salt hypertensive rats. *P < 0.001, DOCA-salt vs. UniNx rats; †P < 0.05, treated vs. DOCA-salt rats; ‡P < 0.001, treated vs. DOCA-salt rats (all by one-way ANOVA followed by the Newman-Keuls post hoc test).
ET<sub>A</sub> and ET<sub>B</sub> receptors, independently of BP reduction and in the absence of significant differences of plasma uric acid levels between control and DOCA-salt rats. Allopurinol is capable of reducing tissue injury without measurably affecting XO activity (12), which may suggest that allopurinol could exert protective effects through other mechanisms such as free radical scavenging actions. The ET<sub>A</sub>-selective antagonist BMS increased uric acid by 180% compared with DOCA-salt rats.

Fig. 2. Representative fluorescent confocal micrographs showing in situ O<sub>2</sub><sup>−</sup> detection in the aorta (A) and mesenteric arteries (B) from UniNx and DOCA-salt rats treated with or without Bos and BMS. Red fluorescence represents O<sub>2</sub><sup>−</sup> production, and green fluorescence represents the autofluorescence of elastin fibers. Scale bar = 50 μm. Data are representative of 4 separate experiments. Results are means ± SE. *P < 0.001 vs. UniNx rats; †P < 0.01 vs. DOCA-salt rats.

Fig. 3. Graphs showing ET<sub>A</sub> receptor (A) and ET<sub>B</sub> receptor (B) expression in the aorta and mesenteric arteries from UniNx and DOCA-salt rats. Results are means ± SE. n = 5 animals/group. *P < 0.05 vs. UniNx counterparts; †P < 0.01 vs. aorta counterparts.
This probably results from increased ET-1 binding to renal tubular ETB receptors with the associated stimulation of natriuresis and diuresis that is accompanied by the decreased secretion of uric acid and consequently increased uric acid concentration in plasma (39). Increased ET-1-mediated oxidative stress through ETA receptors has been reported in mesenteric resistance (34) and carotid arteries (24), aortas (5), and veins (23) of DOCA-salt rats. The role of ETB receptors in ROS generation (15) is more controversial. ETA/B blockade reduced O$_2^•$/H$_2$O$_2$ production in mesenteric arteries to a lesser extent than of ETA-selective blockade, which could suggest that ETB receptors may antagonize the function of ETA receptors.

Our results suggest, for the first time, that although XO activity is lower in DOCA-salt hypertensive rats in the liver, where it can be measured, it is involved in vascular ROS production in this experimental hypertensive rodent model. Discrepancies with other studies (5, 23, 24) may be related to the tissue-specific O$_2^•$/H$_2$O$_2$ generation produced through XO that is much higher in small resistance arteries than in conduit vessels, which can be attributed to the increased XO expression in mesenteric arteries shown in the present study. Our results extend a previous proposal of the existence of a loop regulation of redox enzymes (43). By not only inhibiting NAD(P)H oxidase but also by scavenging radicals such as hydroxyl radicals, peroxy nitrite, and O$_2^•$/H$_2$O$_2$ with apocynin, XO is affected in both the aorta and mesenteric arteries, suggesting that O$_2^•$/H$_2$O$_2$ produced by XO may be dependent on radicals generated by other sources of oxidative stress. Furthermore, we showed that ET receptors, particularly ET$_A$ receptors, are involved in the stimulation of XO-derived O$_2^•$/H$_2$O$_2$ from resistance arteries. Although the protein levels of ET receptors do not explain the difference of XO-derived O$_2^•$/H$_2$O$_2$ between UniNx and DOCA-salt rats in mesenteric arteries, these differences might reflect the affinity of the receptors for the peptide. In addition, we showed that ET$_A$ receptors are more highly expressed in mesenteric arteries than in the aorta, which could explain the differences observed in both tissues of UniNx rats. ET$_A$ receptors may have a direct effect on XO or an indirect effect via other redox-sensitive system (34). ET-1 content is higher in mesenteric arteries than in the aorta in DOCA-salt rats (47), which could explain the fact that XO-derived O$_2^•$/H$_2$O$_2$ in the aorta was not affected by ET receptor blockade. Interestingly, XO has been shown to regulate the ET-1 promoter (16). Hypothetically, this transcriptional regulation could suggest a feedback loop and support our observation that O$_2^•$/H$_2$O$_2$ originating from XO is regulated by the ET system.

The involvement of XO in DOCA-salt rats was also supported by the BP results in this study. The contribution of vascular O$_2^•$/H$_2$O$_2$ to elevate BP depends in part on its ability to quench endogenous NO and to activate vasoconstrictor signaling mechanisms in VSMCs (42, 43). Several studies (5, 42) have shown that antioxidants reduce BP in experimental hypertensive models. Our study is the first in which DOCA-salt rats received a XO inhibitor. Data have been previously reported on the effect of XO activity on BP (20, 29). We were unable to find evidence of enhanced XO activity by measuring uric acid plasma or enzymatic activity in the liver from DOCA-salt rats. However, BP and vascular O$_2^•$/H$_2$O$_2$ were reduced after...
treatment with allopurinol, demonstrating that XO is an important player in DOCA-salt hypertension. The decreased SBP did not affect lipid peroxidation in apocynin- or allopurinol-treated rats, whereas the reduction of lipid peroxidation (oxidative stress) in bosentan- and BMS-treated rats may contribute to SBP lowering (30).

In addition to redox enzyme sources, nonenzymatic \( \text{O}_2^{=} \)/H\(_2\text{O}_2 \) production occurs in tissues. The four different complexes of the METC participate in the production of mitochondria-derived \( \text{O}_2^{=} \) (45) that can be released into the cytoplasm by voltage-dependent anion channels (13). Complexes I and III have been found to be responsible for most of the \( \text{O}_2^{=} \) produced in several conditions including heart, lung, and nervous system diseases (45). By blocking complex I (NADH dehydrogenase) with rotenone and complex II (succinate dehydrogenase) with TTFA, two complexes independent from each other, we identified the limiting step of the METC induced by ET-1. Our results showed that complex II appears to be critical for ROS formation in DOCA-salt rats, which is supported by previous work (31). Rotenone inhibits complex I in the proximity of the ubiquinone binding site, one of several sites available (3). The lack of effect of rotenone does not rule out the possibility that complex I can be involved in vascular ROS production. Complex IV (cytochrome c oxidase) may be important for vascular \( \text{O}_2^{=} \) production stimulated by ET-1 in DOCA-salt rats. The present data are supported by reports in which a reduction of ET-1-induced p38 MAPK phosphorylation was found when complex II and IV were inhibited (44), indicating a role of METC in ROS-dependent signaling pathways. The central role played by mitochondria in ROS production in hypertension has been reviewed recently (6).

In summary, our data suggest, for the first time, that the ET-1/ET\(_A\) receptor pathway is involved in the XO-derived \( \text{O}_2^{=} \) detected in DOCA-salt hypertension and that ET-1 may stimulate METC to further contribute to vascular ROS formation in this model of hypertension. In addition, we have shown that XO is dependent on other redox sources, supporting the concept of ROS-triggering ROS formation (6). ET-1 plays a critical role in the pathogenesis of salt-sensitive hypertension, in part through ROS production in vascular tissues. Vascular bed localization seems to be an important factor in the sources involved in ROS production in each tissue. Because \( \text{O}_2^{=} \) production, which contributes to vascular disease, is abrogated by inhibitors of XO and mitochondrial oxidative phosphorylation, targeting the production of \( \text{O}_2^{=} \) dependent on XO activity and mitochondrial oxidation may represent new strategies to prevent vascular disease in hypertension.
ACKNOWLEDGMENTS

We are grateful to André Turgeon, Suzanne Diebold, and Annie Vallée for excellent technical support.

GRANTS

This work was supported by Canadian Institutes of Health Research (CIHR) Grant 37917 (to E. L. Schiffrin), the Canada Research Chair Program of the Government of Canada, and the Canada Fund for Innovation (to E. L. Schiffrin and R. M. Touyz). E. C. Viel and K. Benkiran were supported by studentships from CIHR.

REFERENCES


