Endothelin-1 Increases Vascular Superoxide via EndothelinA–NADPH Oxidase Pathway in Low-Renin Hypertension

Lixin Li, MD; Gregory D. Fink, PhD; Stephanie W. Watts, PhD; Carrie A. Northcott, MS; James J. Galligan, PhD; Patrick J. Pagano, PhD; Alex F. Chen, MD, PhD

Background—Angiotensin II–induced hypertension is associated with NAD(P)H oxidase–dependent superoxide production in the vessel wall. Vascular superoxide level is also increased in deoxycorticosterone acetate (DOCA)–salt hypertension, which is associated with a markedly depressed plasma renin activity because of sodium retention. However, the mechanisms underlying superoxide production in low-renin hypertension are undefined.

Methods and Results—This study investigated (1) whether and how endothelin-1 (ET-1), which is increased in DOCA-salt hypertensive rats, contributes to arterial superoxide generation and (2) the effect of gene transfer of manganese superoxide dismutase and endothelial nitric oxide synthase. Both superoxide and ET-1 levels were significantly elevated in carotid arteries of DOCA-salt rats compared with that of the sham-operated controls. ET-1 concentration-dependently stimulated superoxide production in vitro in carotid arteries of normotensive rats. The increase in arterial superoxide in both ET-1–treated normotensive and DOCA-salt rats was reversed by a selective ETA receptor antagonist, ABT-627, the flavoprotein inhibitor diphenyleneiodonium, and the NADPH oxidase inhibitor apocynin but not by the nitric oxide synthase inhibitor L-arginine methyl ester or the xanthine oxidase inhibitor allopurinol. Furthermore, in vivo blockade of ETA receptors significantly reduced arterial superoxide levels, with a concomitant decrease of systolic blood pressure in DOCA-salt rats. Ex vivo gene transfer of manganese superoxide dismutase or endothelial nitric oxide synthase also suppressed superoxide levels in carotid arteries of DOCA-salt rats.

Conclusions—These findings suggest that ET-1 augments vascular superoxide production at least in part via an ET A /NADPH oxidase pathway in low-renin mineralocorticoid hypertension. (Circulation. 2003;107:1053-1058.)

Key Words: endothelin • NADPH oxidase • superoxide • hypertension

Oxidative stress and the inactivation of nitric oxide (NO) by vascular superoxide anion (O2−) play a critical role in the pathogenesis of vascular disease, including hypertension.1,2 Arterial O2− is elevated in angiotensin II (Ang II)–induced hypertension,3 attributable to a large extent to NADPH oxidase activation by Ang II.4-5 However, an excess of vascular O2− production has also been found in deoxycorticosterone acetate (DOCA)–salt hypertension,6-9 a model with a markedly depressed plasma renin activity because of sodium retention.10 Humoral mechanisms responsible for O2− production in mineralocorticoid hypertension remain to be delineated.

In contrast to Ang II–induced hypertension, endothelin-1 (ET-1) has been shown to contribute to the pathogenesis of salt-sensitive hypertension in animals and humans,11 secondary to a low-renin state.12,13 ET-1 may be one of the most potent vasoconstrictors produced in the blood vessel wall to date.14 We have now found that the level of ET-1 is increased in the arteries of DOCA-salt hypertensive rats. The vasoactive effects of ET-1 are mediated through 2 receptor types, ET A and ET B.15 ET A receptors play an important role in the development of DOCA-salt–induced hypertension, whereas ET B receptors may protect against vascular and renal injuries in this model.16 ET-1 is able to activate NADPH oxidase in endothelial cells17 and stimulates O2− production in pulmonary smooth muscle cells.18 Therefore, we hypothesized that ET-1 activates NADPH oxidase to produce vascular O2− in DOCA-salt hypertensive rats. Our results suggest that ET-1 produces O2− via an ET A/NADPH oxidase pathway in carotid arteries of normotensive and DOCA-salt hypertensive rats. Because recent studies have suggested that endothelial NO synthase (eNOS) may also contribute to O2− production when its essential cofactor BH4 is below the optimal level (ie, “uncoupled” eNOS),2 we used eNOS gene transfer in the present study in addition to NOS inhibition to distinguish the sources of O2− generation. Gene transfer of manganese...
superoxide dismutase (MnSOD) was also used to test the hypothesis that mitochondria may be a key source for $O_2^-$ formation. Our data indicate that local expression of these recombinant proteins significantly reduced vascular $O_2^-$ levels.

Methods

DOCA-Salt Hypertensive Rats and In Vivo Pharmacological Intervention

DOCA-salt hypertension was created in adult male Sprague-Dawley rats as previously described.21,22 Starting at week 3, some of the DOCA-salt rats received ABT-627 (Abbott Laboratories), a selective ET<sub>A</sub> receptor antagonist, 2 mg · kg body wt<sup>-1</sup> · d<sup>-1</sup> in drinking water for 2 weeks.20 Blood pressure was measured in conscious rats by the noninvasive tail-cuff method. The vessels were collected between weeks 4 and 5 after DOCA implantation. Animal procedures were in accordance with the institutional guidelines of the Michigan University.

Ex Vivo Gene Transfer

The preparation of adenoviral vectors was as described.9,21,22 Related arterial segments (4 mm) were transduced without (negative control) or with adenoviral vectors encoding eNOS, MnSOD, or β-galactosidase (β-gal) gene (positive control) at 5 × 10<sup>10</sup> plaque formation units (pfu)/mL in minimal essential medium at 37°C for 4 hours, followed by incubation in fresh medium for 24 hours.9

Vascular $O_2^-$ Levels

Vascular $O_2^-$ was assayed with oxidative dihydroethidium fluorescence and lucigenin (5 μmol/L) chemiluminescence.9,23 To determine the effects of ET-1, ET<sub>A</sub> receptor antagonist, completely reversed the effect of ET-1 on $O_2^-$ production (Figure 2, n=5 versus 6). There was a significant increase in average systolic blood pressure (Figure 3A, 176±4 versus 117±2 mm Hg, n=5, **P<0.01 versus control) and arterial $O_2^-$ levels (Figure 3B, *P<0.05). The preparation of adenoviral vectors was as described.9,21,22 Isoelectric focusing, xanthine oxidase, and NOS-mediated $O_2^-/H_2O_2$ formation. Our data indicate that local expression of these recombinant proteins significantly reduced vascular $O_2^-$ levels.

ET-1 Immunoassay

Vascular ET-1 levels were determined by a chemiluminescence-based immunoassay with a commercial kit (R&D Systems). Briefly, arteries from sham, DOCA, or normal rats treated with ET-1 were frozen in liquid nitrogen, homogenized in 1 mol/L acetic acid (1 mL/50 mg tissue) containing 1.5×10<sup>-3</sup> mol/L pepstatin, and immediately boiled for 10 minutes. After being chilled, the homogenate was centrifuged at 20,000 g for 30 minutes at 4°C, and the supernatant was assayed for ET-1 content.

Data Analysis

Data were expressed as mean±SEM. Repeated-measures ANOVA was used for comparison of multiple values obtained from the same subject, whereas factorial ANOVA was used for comparing data obtained from 2 independent samples of subjects. Bonferroni’s procedure was used to control type I error. A value of P<0.05 was considered significant.

Results

ET-1 Levels in Carotid Arteries of DOCA-Salt Rats and Normal Rats After ET-1 Treatment

ET-1 levels in carotid arteries were significantly higher in DOCA-salt rats than in sham controls. Similarly, ET-1 levels in arteries of normal rats treated with ET-1 for 4 hours were also significantly increased compared with the vessels with-
with a concomitant decrease in arterial $\text{O}_2^-$ levels in the same group of DOCA-salt rats (Figure 3B, $n=5$, $P<0.05$ versus DOCA).

**Role of NADPH Oxidase, NOS, and Xanthine Oxidase on Arterial $\text{O}_2^-$ Levels**

DPI (10^{-4} mol/L), a flavoprotein inhibitor, and apocynin (10^{-3} mol/L), a selective NADPH oxidase inhibitor, significantly reduced arterial $\text{O}_2^-$ levels in DOCA-salt rats (Figure 4; see also Figure 6G). In contrast, allopurinol (10^{-4} mol/L) had no effect, and L-NAME (10^{-4} mol/L) increased arterial $\text{O}_2^-$ levels. Furthermore, both DPI and apocynin but not allopurinol significantly attenuated $\text{O}_2^-$ levels in arteries of normal rats treated with ET-1 (10^{-9} mol/L) (Figure 4, $n=5$ to 8, $*P<0.05$ and $**P<0.01$ versus control).

**Gene Transfer of eNOS and MnSOD on $\text{O}_2^-$ Levels in Carotid Arteries of DOCA-Salt Rats**

Arterial segments from sham or DOCA-salt rats were transduced with adenoviral vectors encoding eNOS, MnSOD, or β-gal for 4 hours at titers of 0 (control) and $5 \times 10^{10}$ pfu/mL and then transferred to fresh medium overnight before $\text{O}_2^-$ assay. Ex vivo gene transfer of either MnSOD or eNOS significantly decreased the arterial $\text{O}_2^-$ levels in DOCA-salt rats compared with the nontransduced controls of DOCA-salt rats that underwent the same medium incubation for 24 hours. In contrast, gene transfer of β-gal had no effect on $\text{O}_2^-$ levels (Figure 5, $n=4$ to 8, $*P<0.05$ versus DOCA, $**P<0.01$ versus sham).

**In Situ Detection of Vascular Superoxide**

In the presence of the superoxide-sensitive dye dihydroethidium, the ethidium bromide (EtBr) fluorescence (ie, red color) was markedly higher throughout the vessel wall of the ET-1–treated arteries of normal rat (Figure 6B) and arteries of DOCA-salt rats (Figure 6F) compared with the vessels from normal rats (Figure 6A) and sham rats (Figure 6E). The superoxide fluorescent intensity was dramatically suppressed in the arteries of DOCA-salt rats treated with DPI in vitro (Figure 6G) and arteries of DOCA-salt rats treated with ABT-627 for 2 weeks in vivo (Figure 6H) compared with the vessels from the control DOCA-salt rats (Figure 6F). Gene transfer of MnSOD (Figure 6I) and eNOS (Figure 6J) attenuated EtBr fluorescence in arteries of DOCA-salt rats. Both ABT-627 (Figure 6C) and DPI (Figure 6D) also suppressed the EtBr fluorescence in ET-1–treated arteries of normal rats.

**Discussion**

The major new findings of this study are that ET-1 stimulates $\text{O}_2^-$ production, via an ET$_A$/NADPH oxidase pathway, in carotid arteries of DOCA-salt hypertensive rats and that in vivo ET$_A$ receptor blockade attenuates systolic blood pressure and arterial $\text{O}_2^-$ levels. In addition, gene transfer of MnSOD or eNOS significantly reduces the increased arterial $\text{O}_2^-$ levels in this low-renin hypertension model.
It was recently reported that ET-1 activates NADPH oxidase and induces superoxide production in cultured endothelial and smooth muscle cells. Convincing evidence indicates that the major enzymatic sources for vascular superoxide formation are NADPH oxidase, xanthine oxidase, and uncoupled NOS. In DOCA-salt hypertensive rats, aortic NADPH oxidase activity was significantly increased compared with their normotensive controls. In the present study, we examined (1) the effect of ET-1 on superoxide production both in vitro in normal rats and in vivo in DOCA-salt hypertensive rats and (2) whether this effect is mediated by NADPH oxidase, xanthine oxidase, or uncoupled NOS. Our results indicate that (1) ET-1 stimulates arterial $O_2^-$ production in a concentration-dependent manner in normal rats; (2) apocynin but not L-NAME or allopurinol inhibits the $O_2^-$ production in both ET-1–stimulated arteries of normal rats and arteries of DOCA-salt rats; and (3) the selective ET$_A$ receptor antagonist ABT-627 suppresses superoxide production in vitro in ET-1–treated arteries of normal rats and in vivo in arteries of DOCA-salt hypertensive rats. Collectively, these data suggest that ET-1 stimulates arterial $O_2^-$ production in DOCA-salt hypertension, and NADPH oxidase but not xanthine oxidase or uncoupled NOS may play a major role in $O_2^-$ production in this model. The selectivity of apocynin, a methoxy-substituted catechol, on NADPH oxidase has been well characterized, because it impedes the assembly of the p47phox and p67phox subunits within the membrane NADPH oxidase complex.

There are at least 2 vascular ET-1 receptors, ET$_A$ and ET$_B$. ET-1 exerts its vasoactive effects mainly through the activation of the G protein–coupled ET$_A$ receptors on vascular smooth muscle cells, whereas ET$_B$ may exert protective effects in DOCA-salt hypertension. There is an exaggerated vascular and renal injury in ET$_B$ receptor–deficient rats of DOCA-salt hypertension, and such injuries were significantly improved after the treatment with ABT-627, a selective ET$_A$ receptor antagonist. In the present study, we demonstrated that arterial $O_2^-$ levels were increased significantly in DOCA-salt rats compared with the sham controls, an effect that was reversed after in vivo ABT-627 treatment in DOCA-salt rats, with a concomitant reduction of blood pressure. These findings are consistent with recent studies showing that $O_2^-$ production in rebound pulmonary hypertension was mediated by ET$_A$ receptors and that tempol, a superoxide scavenger, normalized blood pressure in spontaneously hypertensive rats. However, it is important to note that although in vivo blockade of ET$_A$ receptors by ABT-627 for 2 weeks suppressed the arterial $O_2^-$ to control levels, the blood pressure was only partially reduced. These results suggest that ET$_A$ receptor–mediated activation of NADPH oxidase by ET-1 is only one of the contributing factors for the $O_2^-$–induced blood pressure increase in this model of hypertension. In addition, they also suggest that the reduced vascular $O_2^-$ levels were only partially responsible for decreasing blood pressure and that in vivo ET$_A$ receptor blockade may also result in reduced smooth muscle tension. Furthermore, the possible influence of ET$_B$ receptors on ET-1–induced $O_2^-$ production in arteries and veins is not clear and is a subject currently being investigated. Finally, it
is also of interest to note that hypertension per se may not be a major stimulus for augmented vascular superoxide because norepinephrine-induced hypertension is not associated with an increase in vascular superoxide levels. Thus, $O_2^-$ production may be a result of the effects of different vasoactive agents in different types of hypertension. Although Ang II is a key stimulus of vascular $O_2^-$ production in high-angiotensin hypertension, ET-1 may play a major role for increasing vascular $O_2^-$ in low-renin hypertension, such as the DOCA-salt model.

Several pathophysiological conditions in addition to hypertension have been associated with increased superoxide production. These include atherosclerosis, hypercholesterolemia, diabetes, and heart failure; cigarette smoking has also been implicated. In most of these cases, the increase in vascular $O_2^-$ has been shown to impair endothelium-dependent NO-mediated vascular relaxation by inactivating endogenous NO. In addition, superoxide has also been shown to affect the sensitivity of blood vessels to vasoconstrictors, and it triggers expression of vascular adhesion molecules and vascular remodeling. The reaction rate between $O_2^-$ and NO is linear and extremely rapid. For this reason, the local balance between $O_2^-$, NO, and SOD in the vascular wall is dynamic, and relatively minor changes in the levels of any of these factors may substantially alter vascular tone. Localized gene transfer to the vessel wall may be an effective means of increasing NO and/or reducing $O_2^-$ levels. Indeed, our results demonstrate that gene transfer of MnSOD or eNOS significantly reduced arterial $O_2^-$ levels in DOCA-salt hypertensive rats. We chose MnSOD for vascular gene transfer because (1) our recent study indicates that endogenous MnSOD is significantly reduced in the carotid arteries of DOCA-salt hypertensive rats and that gene transfer of MnSOD restored the functional capacity of the antioxidant enzyme in scavenging elevated $O_2^-$ and (2) mitochondria may be a major location in which vascular $O_2^-$ is produced. In agreement with our findings, gene transfer of MnSOD has been shown to normalize superoxide-induced impairment of endothelium-dependent relaxation, whereas gene transfer of cytosolic Cu/Zn-SOD or extracellular SOD did not.

Conversely, recent studies have demonstrated that both ex vivo and in vivo gene transfer of eNOS or nNOS restored NO-mediated arterial relaxation, which was impaired by increased $O_2^-$ in hypertensive, atherosclerotic, or diabetic animals. Furthermore, in vivo gene transfer of eNOS to spontaneously hypertensive rats has resulted in direct blood pressure reduction. Consistent with these studies, our data showed that gene transfer of eNOS significantly decreased $O_2^-$ levels in carotid arteries in DOCA-salt rats. Taken together, these experimental observations support the novel concept that NO generated by recombinant NOS, as a result of vascular gene transfer, provides an effective means of inactivating $O_2^-$ and thereby improving vasomotor function.

In conclusion, the present study demonstrates that ET-1 is a potent stimulus for arterial $O_2^-$ produced in low-renin DOCA-salt hypertension, an effect that is at least partially mediated by the ET$_A$ receptor/NADPH oxidase pathway. Vascular gene transfer of MnSOD and eNOS is an effective strategy in reducing $O_2^-$ levels in this model. These findings may provide a mechanistic basis for therapeutic interventions aimed at reducing superoxide-induced vascular dysfunctions associated with increased ET-1 levels in low-renin hypertension.

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References


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