Endothelin-1 Stimulates Arterial VCAM-1 Expression Via NADPH Oxidase–Derived Superoxide in Mineralocorticoid Hypertension

Lixin Li, Yi Chu, Gregory D. Fink, John F. Engelhardt, Donald D. Heistad, Alex F. Chen

Abstract—Although hypertension is a major risk factor for atherosclerosis, its underlying mechanisms remain to be delineated. We have recently reported that both endothelin-1 (ET-1) and vascular cellular adhesion molecule-1 (VCAM-1) levels, key early markers of atherosclerosis, are significantly elevated in carotid arteries of deoxycorticosterone acetate (DOCA)-salt hypertensive rats, a model known for its suppressed plasma renin levels. This study tested the hypothesis that ET-1 augments arterial VCAM-1 expression through NADPH oxidase–derived superoxide (O$_2^-$). Carotid arteries of DOCA-salt or sham-operated rats were transduced ex vivo with extracellular superoxide dismutase (EC-SOD), dominant negative HA-tagged N17Rac1 that inhibits Rac1, the small GTPase component of NADPH oxidase, or β-galactosidase (β-gal) reporter gene (5×10$^{10}$ plaque formation units [pfu]/mL), and the effect of transgene expression on O$_2^-$ and VCAM-1 levels was assayed 24 hours afterward. The arterial activity of NADPH oxidase but not xanthine oxidase was significantly higher in DOCA-salt than in sham rats, which was abolished by the selective ET$_A$ receptor antagonist ABT-627 (3×10$^{-8}$ mol/L), NADPH oxidase inhibitor apocynin (10$^{-4}$ mol/L), or dominant negative Rac1 gene transfer. The levels of O$_2^-$ and VCAM-1 were significantly increased in arteries of DOCA-salt rats, an effect that was ameliorated after EC-SOD or dominant negative Rac1 but not β-gal reporter gene transfer. ABT-627 and apocynin also significantly reduced elevated VCAM-1 levels in ET-1–treated arteries of normal rats and arteries of DOCA-salt rats. The results of this study indicate that ET-1 stimulates arterial VCAM-1 expression by producing O$_2^-$ from an ET$_A$ receptor/NADPH oxidase pathway in low-renin mineralocorticoid hypertension. (Hypertension. 2003;42:997-1003.)

Key Words: endothelin ■ atherosclerosis ■ hypertension, mineralocorticoid ■ oxidative stress

Hypertension is an established risk factor for atherosclerosis. Experimental and clinical evidence demonstrates that the renin-angiotensin system contributes to the pathogenesis of atherosclerosis. Angiotensin (Ang) II induces the expression of vascular cellular adhesion molecule-1 (VCAM-1), a key early marker in the development of atherosclerotic lesions (fatty streaks and fibrous plaques), in Ang II–induced hypertensive rats. In contrast, endothelin-1 (ET-1) expression and level are significantly higher in aortic and mesenteric arteries of deoxycorticosterone acetate (DOCA)-salt hypertension, a model known for its suppressed plasma renin levels. Recently, we have reported that both ET-1 and VCAM-1 are significantly elevated in carotid arteries of DOCA-salt hypertensive rats. However, a direct causal relationship between vascular ET-1 and VCAM-1 in mineralocorticoid hypertension has never been demonstrated to date.

Our recent studies have shown that ET-1 increases superoxide (O$_2^-$) levels by activating ET$_A$ receptor/NADPH oxidase pathway in carotid arteries of DOCA-salt rats. In addition, we have also demonstrated that enhanced arterial VCAM-1 expression is suppressed by gene transfer of manganese superoxide dismutase (Mn-SOD) in this model, suggesting that O$_2^-$ plays an important role in mediating VCAM-1 expression. Indeed, O$_2^-$ has been shown to stimulate VCAM-1 expression through activation of redox-sensitive transcription factor nuclear factor (NF)-κB. Based on the above experimental observations, we tested the hypothesis that ET-1 augments arterial VCAM-1 expression through NADPH oxidase–derived O$_2^-$ in DOCA-salt hypertensive rats in the present study.

Because NADPH oxidase is a key enzymatic source for O$_2^-$ in this model and the effective pharmacological interventions that can be applied to its inhibition is rather limited because of the complexity of the enzyme with multiple subunits, we used a replication-incompetent adenoviral vector encoding a dominant negative HA-tagged N17Rac1 gene that abrogates Rac1, the small GTPase

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component required for NADPH oxidase activation. In addition, since recent studies have shown that gene transfer of extracellular SOD (EC-SOD), but not copper/zinc SOD (Cu/Zn-SOD) is effective to reduce both vascular $O_2^\bullet\cdot$ level and mean arterial pressure in spontaneously hypertensive rats (SHR). EC-SOD gene transfer was used to suppress arterial $O_2^\bullet\cdot$ levels in this study. Our results indicate that ET-1 stimulates VCAM-1 expression through its ET$_A$ receptors in carotid arteries of DOCA-salt rats, an effect that is dependent on $O_2^\bullet\cdot$ derived from NADPH oxidase.

**Methods**

**DOCA-Salt Hypertension**

DOCA-salt hypertension was created in adult male Sprague-Dawley rats as previously described. All the arteries used were collected between weeks 4 to 6 after DOCA implantation. All animal procedures were in accordance with the institutional guidelines of Michigan State University.

**Ex Vivo Gene Transfer**

The propagation, purification, and titration of replication-defective adenoviral vectors were as previously described. The prepared $\beta$-galactosidase ($\beta$-gal), EC-SOD, and dominant negative Rac1 vectors were stored at $-80°C$ in 0.01 mol/L Tris, 0.01 mol/L MgCl$_2$, and 10% glycerol before use. Isolated arterial segments (4 mm long) were transduced ex vivo with adenoviral vectors at a titer of $5 \times 10^8$ plaque formation units (pfu)/mL in minimal essential medium (MEM, Fisher) at 37°C for 4 hours, followed by incubation in fresh MEM for 24 hours, as previously described.

**NADPH Oxidase and Xanthine Oxidase Activity**

Isolated arterial ring segments (4 mm long) from carotid arteries of sham and DOCA-salt rats with or without treatment of the selective ET$_A$ receptor antagonist ABT-627 ($3 \times 10^{-8}$ mol/L, 24 hours, Abbott Laboratories), the NADPH oxidase inhibitor apocynin (APO, $10^{-8}$ mol/L, 24 hours, Calbiochem), or gene transfer of dominant negative Rac1 were homogenized in lysis buffer (10 mol/L phenylmethylsulfonyl fluoride, and 0.2% Triton X-100). The homogenates were centrifuged at 12,000 $\times$ g for 30 minutes and then subjected to protein assay (Bio-Rad). The enzyme activities were measured by lucigenin chemiluminescence assay ($5 \times 10^{-6}$ mol/L, lucigenin, Sigma) and indicated as the amount of $O_2^\bullet\cdot$ levels in the presence of their relative substrates NADPH ($10^{-4}$ mol/L, Sigma) or xanthine ($10^{-4}$ mol/L, Sigma), as previously described. No enzymatic activity could be detected in the absence of NADPH or xanthine. Reactions were initiated by addition of 10 to 20 $\mu$L, tissue homogenates containing 25 to 50 $\mu$g extracted protein. The enzyme activity was expressed as nmol/min per milligram of protein.

**Arterial $O_2^\bullet\cdot$ Levels**

Arterial $O_2^\bullet\cdot$ was quantified by the use of lucigenin chemiluminescence, as previously described. The concentration of lucigenin was $5 \times 10^{-5}$ mol/L to minimize the formation of nonspecific $O_2^\bullet\cdot$ through redox cycling. Isolated arterial ring segments of DOCA-salt or sham rats with or without gene transfer of EC-SOD, dominant negative Rac1, or $\beta$-gal ($5 \times 10^{10}$ pfu/mL); or treatment of ABT-627 ($3 \times 10^{-4}$ mol/L, 24 hours) were assayed for $O_2^\bullet\cdot$ levels. After reading, arterial tissues were weighed, and the subtracted readings were then converted to nmol/min per milligram of tissue. In addition, in situ detection of $O_2^\bullet\cdot$ was performed by confocal microscopy with oxidative fluorescent dye dihydroethidium (DHE, Sigma), as described previously.

**Immunohistochemistry and Western Immunoblot for Arterial VCAM-1**

Arterial VCAM-1 levels were assayed by both immunohistochemistry and Western blot analysis, as previously described. Isolated arterial ring segments (4 mm long) of DOCA-salt or sham rats were transduced with EC-SOD, dominant negative Rac1, or $\beta$-gal ($5 \times 10^{10}$ pfu/mL), or treated with ABT-627 ($3 \times 10^{-8}$ mol/L, 24 hours), BQ788 ($10^{-7}$ mol/L, 24 hours, Sigma), or apocynin ($10^{-7}$ mol/L, 24 hours). Arteries of normal rats were treated without or with ET-1 ($10^{-8}$ mol/L, 24 hours); some were pretreated with ABT-627 ($3 \times 10^{-8}$ mol/L, 1 hour) or apocynin ($10^{-7}$ mol/L, 1 hour); some were incubated with adenoviral vectors encoding EC-SOD or $\beta$-gal for 4 hours, then transferred to fresh MEM containing ET-1 ($10^{-7}$ mol/L) for 24 hours. All the experiments were performed at 37°C in MEM containing 0.1% BSA. The primary antibodies for immunostaining used were goat polyclonal antibody against VCAM-1 (1:40, Santa Cruz Biotechnology), and the secondary antibodies were biotinylated anti-goat IgG (1:750, Santa Cruz Biotechnology). For immunoblotting, the secondary antibody used was bovine anti-goat antisera (1:4000, Santa Cruz Biotechnology). To verify equal amount of protein loading (20 $\mu$g), all membranes were stained with the Coomassie brilliant blue R-250 (Sigma). The actual gel bands on radiographic films were then used against corresponding Coomassie blue bands for densitometry analyses (NIH Scion image software). The molecular size of the VCAM-1 band is $\approx 100$ kDa, as confirmed with a standard molecular weight marker (Bio-Rad) in each blot.

**Statistical Analysis**

Data are 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 two independent samples of subjects. The Bonferroni procedure was used to control type I error. Significance was established at a level of $P<0.05$.

**Results**

**Arterial NADPH Oxidase, But Not Xanthine Oxidase Activity, Is Increased in DOCA-Salt Rats**

There was a significant increase in systolic arterial blood pressure in DOCA-salt rats compared with the sham control rats ($181±4.0$ versus $120±1.0$ mm Hg, $n=27$ sham and 35 DOCA-salt rats, $P<0.01$). The activity of NADPH oxidase was significantly higher in carotid arteries of DOCA-salt rats compared with the sham rats, an effect that was suppressed by the selective ET$_A$ receptor antagonist ABT-627. Apocynin or gene transfer of dominant negative Rac1 also decreased NADPH oxidase activity (Figure 1A). However, the activity of xanthine oxidase was similar in carotid arteries between sham and DOCA-salt rats (Figure 1B).

**ET$_A$/NADPH Oxidase Increases Arterial $O_2^\bullet\cdot$ Levels in DOCA-Salt Rats**

Arterial $O_2^\bullet\cdot$ levels were also significantly higher in DOCA-salt than in sham rats, an effect that was abolished by the selective ET$_A$ receptor antagonist ABT-627. Gene transfer of dominant negative Rac1 or EC-SOD also reduced $O_2^\bullet\cdot$ to its control levels, an effect that was not observed after $\beta$-gal reporter gene transfer (Figure 2A).
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Discussion

The major new findings in the present study are (1) the activity of NADPH oxidase is increased in carotid arteries of DOCA-salt rats, which is abrogated by the ET\textsubscript{A} receptor antagonist ABT-627, and by NADPH oxidase inhibition with either apocynin or gene transfer of dominant negative Rac1; (2) ET-1 directly stimulates arterial VCAM-1 expression, an effect that is abolished by ABT-627 or apocynin; and (3) gene transfer of EC-SOD or dominant negative Rac1 ameliorates increased arterial VCAM-1 expression in DOCA-salt hypertensive rats.

Experimental and clinical studies have demonstrated that ET-1 plays a role in atherogenesis. ET-1 enhances the expression of VCAM-1, a key early marker in atherosclerosis, in TNF\textalpha-stimulated endothelial cells.\textsuperscript{21} Hypertensive patients with high plasma ET-1 levels are correlated with elevated blood VCAM-1 levels and increased risks for developing hypertension-induced organ damages.\textsuperscript{22,23} Consistent with these reports, our data showed for the first time that ET-1 treatment for 24 hours augments VCAM-1 levels directly in carotid arteries of normal rats, an effect that is mediated by the ET\textsubscript{A} receptor since its selective antagonist ABT-627 abolished the response. Similarly, the elevated arterial VCAM-1 levels in DOCA-salt rats were abrogated by ABT-627 but not the selective ET\textsubscript{B} receptor antagonist BQ788, suggesting that VCAM-1 expression in this model is mediated by ET-1 through its ET\textsubscript{A} receptors. These in vitro observations are consistent with our published data that in vivo treatment with the ET\textsubscript{A} receptor antagonist ABT-627 reduced superoxide levels and blood pressure in the same model.\textsuperscript{12} The reason that carotid artery was used is that it is a common vessel type prone to the development of atherosclerosis in hypertensive patients.\textsuperscript{24} We used in vitro ET-1 stimulation of VCAM-1 expression in normal carotid arteries in the present study to mimic the effect of the high levels of arterial ET-1 observed in DOCA-salt hypertensive rats.\textsuperscript{12} The concentration of ET-1 used was \(10^{-9}\) mol/L, which was based on our published data that ET-1 at this concentration produced similar amount of O\textsubscript{2}\textsuperscript{-} in normal carotid arteries compared with that of DOCA-salt rats.\textsuperscript{12} In addition, according to our published\textsuperscript{12} and present data, there is no significant difference of superoxide levels between 24-hour incubated and freshly isolated arteries from DOCA-salt rats.

Increased oxidative stress including superoxide has been shown to upregulate adhesion molecule expression.\textsuperscript{8} In Ang II–induced hypertensive rats, Ang II stimulates O\textsubscript{2}\textsuperscript{-} and oxidative signaling pathways involving redox-sensitive transcription factor NF-\textkappaB and upregulates its downstream genes including VCAM-1.\textsuperscript{8} Ang II is known to produce O\textsubscript{2}\textsuperscript{-} through activating the membrane-bound NADPH oxidase.\textsuperscript{18} In aldosterone-induced hypertension, intercellular adhesion molecule-1 (ICAM-1) is enhanced by an oxidase-stress–dependent mechanism.\textsuperscript{25} In DOCA-salt hypertensive rats, a model with high ET-1 levels in the carotid arteries, we have shown that ET-1 is a potent stimulant for O\textsubscript{2}\textsuperscript{-} production through an ET\textsubscript{A} receptor/NADPH oxidase pathway.\textsuperscript{12} The present study demon-

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**ET\textsubscript{A} Receptor Blockade and NADPH Oxidase Inhibition Reduce Arterial VCAM-1 Levels in DOCA-Salt Rats**

Arterial VCAM-1 levels were significantly increased in DOCA-salt rats compared with the sham control rats, an effect that was ameliorated by selective ET\textsubscript{A} receptor antagonist ABT-627 (Figure 2B) but not selective ET\textsubscript{B} receptor antagonist BQ788 (data not shown). NADPH oxidase inhibition by either apocynin or gene transfer of dominant negative Rac1 suppressed VCAM-1 expression. In addition, gene transfer of EC-SOD reduced both arterial VCMA-1 and O\textsubscript{2}\textsuperscript{-} levels (Figure 2B and Figures 3A and 3B).

**ET-1 Stimulates Arterial VCAM-1 Expression Through ET\textsubscript{A} Receptor/NADPH Oxidase–Induced O\textsubscript{2}\textsuperscript{-} in Normal Rats**

In carotid arteries of normal rats, ET-1 treatment for 24 hours significantly increased VCAM-1 levels compared with the blank-incubated control rats, an effect that was prevented by the pretreatment of ABT-627 (Figures 4A and 4B). NADPH oxidase inhibition by either apocynin or gene transfer of dominant negative Rac1 suppressed ET-1–induced VCAM-1 expression. Similarly, gene transfer of EC-SOD also reduced arterial VCMA-1 levels (Figures 4A and 4B).

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**Figure 1.** NADPH oxidase (A) and xanthine oxidase (B) activity in carotid arteries of sham and DOCA-salt rats. Arterial supernatants from sham and DOCA rats, with or without treatments as indicated, were subjected to NADPH and xanthine oxidase measurements by lucigenin chemiluminescence in the presence of their relative substrates 10\textsuperscript{-4} mol/L NADPH or xanthine (see Methods). No enzymatic activity could be detected in the absence of NADPH or xanthine. n=4 to 10 rats (A) and 6 rats (B). *P<0.05 vs sham, #P<0.05 vs DOCA control.
strated that NADPH oxidase activities were significantly elevated in carotid arteries of DOCA-salt rats, which were blocked by ET_{A} receptor antagonist ABT-627 and NADPH oxidase inhibitor apocynin. In contrast, the activity of xanthine oxidase was not increased in carotid arteries of DOCA-salt rats. Furthermore, ABT-627 and apocynin also suppressed augmented VCAM-1 levels in both ET-1–treated carotid arteries of normal rats and carotid arteries of DOCA-salt rats. The selectivity of apocynin, a methoxy-substituted catechol, on NADPH oxidase has been well...
characterized, as it impedes the assembly of the p47phox and p67phox subunits within the membrane NADPH oxidase complex. Taken together, these data suggest that ET-1–induced VCAM-1 expression is mediated by O$_2^-$, which is derived from the activated NADPH oxidase.

Since we have observed that NADPH oxidase is a key source for arterial O$_2^-$ generation in DOCA-salt hypertension, a blockade of its activity may also allow effective inhibition of VCAM-1 expression. However, NADPH oxidase is a complex enzyme that includes multiple membrane and cytosolic subunits. Pharmacological interventions are rather limited and often difficult for maximal and specific inhibition of the enzyme activity. In this study, arterial gene transfer of a dominant negative HA-tagged N17Rac1 was used in an attempt to abrogate the endogenous Rac1 expression, a key GTPase component required for NADPH oxidase activation. Our results demonstrate that gene transfer of dominant negative Rac1 markedly inhibited NADPH oxidase activity (Fig 1A), with a resultant suppression of both arterial O$_2^-$ levels (Fig 2A) and VACM-1 levels (Fig 2B) in DOCA-salt rats. These experimental observations strongly suggest that dominant negative Rac1 overexpression leads to NADPH oxidase inhibition, which were also consistent with our apocynin data. Collectively, these findings suggest that NADPH oxidase inhibition results in a concomitant reduction of both O$_2^-$ and VCAM-1 levels in carotid arterial of DOCA-salt rats.

Because ET-1–induced VCAM-1 expression appears to be mediated by superoxide, we also examined the strategy that aimed at scavenging vascular O$_2^-$ levels directly by gene transfer of EC-SOD, which has been shown to reduce vascular O$_2^-$ and mean arterial pressure in SHR. Gene transfer of EC-SOD at the titer of $5 \times 10^{10}$ pfu/mL reversed VCAM-1 levels to that of the sham control rats in carotid arteries of DOCA-salt rats. Compared with gene transfer of Mn-SOD, the mitochondrial O$_2^-$ scavenging enzyme EC-SOD...
appears to be more effective, since Mn-SOD gene transfer only partially suppressed arterial VCAM-1 levels at the same titer in DOCA-salt rats, as we previously reported.13 These data suggest that in addition to mitochondria, extracellular O$_2^-$ also plays a pivotal role in stimulating VCAM-1 expression. This is consistent with the reports that EC-SOD is a principle regulator of oxidative stress and represents an important enzymatic antioxidant defense system in vascular disease including atherosclerosis.27–29 The reason for the observed discrepant effects between these two SOD isozymes is unclear; it may be that EC-SOD has a higher affinity to cellular membrane and is cell-permeable with heparin-binding domain and/or that EC-SOD has a much longer half-life (18 hours) than Mn-SOD and CuZn-SOD (several minutes).15 Further studies are needed to determine the relative endogenous activities of all three SOD isoforms and compare their gene transfer effects on VCAM-1 expression in this and other models of hypertension.

It is of interest to note that the increased O$_2^-$ appears to be scavenged by EC-SOD gene transfer throughout the vascular walls. Recombinant EC-SOD can scavenge O$_2^-$ in endothelial and adventitial layers because of known ex vivo transgene expression at both locations,16 whereas this may not be the case inside the smooth muscle cells. The exact reason that ex vivo gene transfer led to reduced O$_2^-$ signal in the media is unknown. We speculate that because O$_2^-$ has been shown to cross erythrocyte30 and endothelial cell31 membranes through anion channels (eg, chloride channels), it may diffuse outward into the lumen and perivascular site because of its high level in smooth muscle and relative low levels in the endothelium and adventitia as it is being scavenged at both sites after gene transfer. Thus, overexpression of EC-SOD in the endothelium and adventitia may produce a "diffusion-gradient" effect through which O$_2^-$ gets into the two outside layers, whereby it is scavenged. Additionally, EC-SOD is known to possess a high affinity to cellular membrane and is cell-permeable with its heparin-binding domain.15 Future studies are needed to elucidate the underlying mechanisms on our experimental observations. Finally, the increased VCAM-1 expression appears to occur throughout the vascular walls. It is difficult to identify the specific cell types under light microscopy, although the VCAM-1 immunoreactivity appears to exist in endothelial cells, smooth muscle cells, and adventitial fibroblasts.

In summary, the findings of the present study demonstrate that ET-1 directly stimulates arterial VCAM-1 expression through its ETA receptor–mediated activation of NADPH oxidase and superoxide formation in mineralocorticoid hypertensive. Inhibition of NADPH oxidase by gene transfer of dominant negative RacI is a novel strategy that may be effective against increased arterial VCAM-1 levels associated with cardiovascular disease, including hypertension.

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