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 cell 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 (O2·-). 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 O2·- 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 ETA 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 O2·- 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 O2·- from an ETA receptor/NADPH oxidase pathway in low-renin mineralocorticoid hypertension. (Hypertension. 2003;42:900-906.)

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

Hypertension is an established risk factor for atherosclerosis.1 Experimental and clinical evidence demonstrates that the renin-angiotensin system contributes to the pathogenesis of atherosclerosis.2-3 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),4-5 in Ang II–induced hypertensive rats.6 In contrast, endothelin-1 (ET-1) expression and level are significantly higher in aortic and mesenteric arteries of deoxycorticosterone acetate (DOCA)-salt hypertension,8,9 a model known for its suppressed plasma renin levels.11 Recently, we have reported that both ET-1 and VCAM-1 levels are significantly elevated in carotid arteries of DOCA-salt hypertensive rats.12,13 However, a direct causative relation 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 (O2·-) levels by activating ETA receptor/NADPH oxidase pathway in carotid arteries of DOCA-salt rats.12 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,13 suggesting that O2·- plays an important role in mediating VCAM-1 expression. Indeed, O2·- has been shown to stimulate VCAM-1 expression through activation of redox-sensitive transcription factor nuclear factor (NF)-κB.8 Based on the above experimental observations, we tested the hypothesis that ET-1 augments arterial VCAM-1 expression through NADPH oxidase–derived O2·- in DOCA-salt hypertensive rats in the present study.

Because NADPH oxidase is a key enzymatic source for O2·- 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...
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 (CuZn-SOD) is effective to reduce both vascular \(O_2^\cdot\) level and mean arterial pressure in spontaneously hypertensive rats (SHR). EC-SOD gene transfer was used to suppress arterial \(O_2^\cdot\) levels in this study. Our results indicate that ET-1 stimulates VCAM-1 expression through its ETA receptors in carotid arteries of DOCA-salt rats, an effect that is dependent on \(O_2^\cdot\) 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-incompetent adenoviral vectors were as previously described. The prepared \(\beta-gal\)-galactosidase (\(\beta\)-gal), EC-SOD, and dominant negative Rac1 vectors were stored at \(-80^\circ C\) 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 \(5 \times 10^{10}\) plaque formation units (pfu)/mL in minimal essential medium (MEM, Fisher) at \(37^\circ C\) 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 ETA receptor antagonist ABT-627 (3 \(\times 10^{-6}\) mol/L, 24 hours, Abbott Laboratories), the NADPH oxidase inhibitor apocynin (AP0, 10 \(\mu\)mol/L, 24 hours, Calbiochem), or gene transfer of dominant negative Rac1 were homogenized in lysis buffer (10 mmol/L, 24 hours, Calbiochem), or gene transfer of dominant negative Rac1 vectors were stored at \(-80^\circ \) for 24 hours. All the arteries used were collected 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 \(\pm\)4.0 versus 120 \(\pm\)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 ETA 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 \(O_2^\cdot\) Levels in DOCA-Salt Rats**

Arterial \(O_2^\cdot\) levels were also significantly higher in DOCA-salt than in sham rats, an effect that was abolished by the selective ETA receptor antagonist ABT-627. Gene transfer of dominant negative Rac1 or EC-SOD also reduced \(O_2^\cdot\) to its control levels, an effect that was not observed after \(\beta\)-gal reporter gene transfer (Figure 2A).
ETα 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 ETα receptor antagonist ABT-627 (Figure 2B) but not selective ETβ receptor antagonist BQ788 (data not shown). NADPH oxidase inhibition by 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 O2 levels (Figure 2B and Figures 3A and 3B). 

ET-1 Stimulates Arterial VCAM-1 Expression Through ETα Receptor/NADPH Oxidase–Induced O2 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).

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 ETα 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 TNFα-stimulated endothelial cells. 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.

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 ETα 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 ETβ receptor antagonist BQ788, suggesting that VCAM-1 expression in this model is mediated by ET-1 through its ETα receptors. These in vitro observations are consistent with our published data that in vivo treatment with the ETα receptor antagonist ABT-627 reduced superoxide levels and blood pressure in the same model. The reason that carotid artery was used is that it is a common vessel type prone to the development of atherosclerosis in hypertensive patients. 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. The concentration of ET-1 used was 10−8 mol/L, which was based on our published data that ET-1 at this concentration produced similar amount of O2 in normal carotid arteries compared with that of DOCA-salt rats. In addition, according to our published 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. In Ang II–induced hypertensive rats, Ang II stimulates O2 and oxidative signaling pathways involving redox-sensitive transcription factor NF-κB and upregulates its downstream genes including VCAM-1. Ang II is known to produce O2 through activating the membrane-bound NADPH oxidase. In aldosterone-induced hypertension, intercellular adhesion molecule-1 (ICAM-1) is enhanced by an oxidase-stress–dependent mechanism. 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 O2 production through an ETα receptor/NADPH oxidase pathway. The present study demon-
strated that NADPH oxidase activities were significantly elevated in carotid arteries of DOCA-salt rats, which were blocked by ETα 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.26 Taken together, these data suggest that ET-1–induced VCAM-1 expression is mediated by \( \text{O}_2^– \), which is derived from the activated NADPH oxidase.

Since we have observed that NADPH oxidase is a key source for arterial \( \text{O}_2^– \) generation in DOCA-salt hypertension,12 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.14 Our results demonstrate that gene transfer of dominant negative Rac1 markedly inhibited NADPH oxidase activity (Fig 1A), with a resultant suppression of both arterial \( \text{O}_2^– \) 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 \( \text{O}_2^– \) 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 \( \text{O}_2^– \) levels directly by gene transfer of EC-SOD, which has been shown to reduce vascular \( \text{O}_2^– \) and mean arterial pressure in SHR.15 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 \( \text{O}_2^– \) 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. These data suggest that in addition to mitochondria, extracellular O₂⁻ 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. 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). 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 O₂⁻ appears to be scavenged by EC-SOD gene transfer throughout the vascular walls. Recombinant EC-SOD can scavenge O₂⁻ in endothelial and adventitial layers because of known ex vivo transgene expression at both locations, whereas this may not be the case inside the smooth muscle cells. The exact reason that ex vivo gene transfer led to reduced O₂⁻ signal in the media is unknown. We speculate that because O₂⁻ has been shown to cross erythrocyte and endothelial cell 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 O₂⁻ 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. 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 ET₁ receptor–mediated activation of NADPH oxidase and superoxide formation in mineralocorticoid hypertensive. Inhibition of NADPH oxidase by gene transfer of dominant negative Rac1 is a novel strategy that may be effective against increased arterial VCAM-1 levels associated with cardiovascular disease, including hypertension.

Acknowledgments
This work was supported in part by the National Institutes of Health grants 1 P01, HL70687-01 to Drs G.D. Fink and A.F. Chen; American Diabetes Association grants 9806347X and 0130537Z; American Diabetes Association Research Award 7-01-RA-10; Juvenile Diabetes Research Foundation Innovative grant 5-2001-311; and Michigan State University Intramural Research Grants Program grant 41140 (all to Dr A.F. Chen). Dr Lixin Li is an awardee of the American Heart Association/Midwest Affiliate Physician-Scientist Postdoctoral Fellowship (0225408Z).

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Hypertension. published online September 29, 2003;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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