

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.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),^{4–7} in Ang II–induced hypertensive rats.⁸ In contrast, endothelin-1 (ET-1) expression and level are significantly higher in aortic and mesenteric arteries of deoxycorticosterone acetate (DOCA)-salt hypertension,^{9,10} 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 (O_2^-) levels by activating ET_A 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 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)- κ B.⁸ 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.¹⁴ 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^- level and mean arterial pressure in spontaneously hypertensive rats (SHR),¹⁵ EC-SOD gene transfer was used to suppress arterial O_2^- 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^- derived from NADPH oxidase.

Methods

DOCA-Salt Hypertension

DOCA-salt hypertension was created in adult male Sprague-Dawley rats as previously described.^{12,13} 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.^{16,17} The prepared β -galactosidase (β -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 5×10^{10} 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.^{12,13}

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 (3×10^{-8} mol/L, 24 hours, Abbott Laboratories),¹² the NADPH oxidase inhibitor apocynin (APO, 10^{-4} mol/L, 24 hours, Calbiochem), or gene transfer of dominant negative Rac1 were homogenized in lysis buffer (10^{-1} mol/L K_2HPO_4 , 10^{-3} mol/L phenylmethylsulfonyl fluoride, and 0.2% Triton X-100). The homogenates were centrifuged at 12 000g, 4°C , for 30 minutes and then subjected to protein assay (Bio-Rad). The enzyme activities were measured by lucigenin chemiluminescence assay (5×10^{-6} mol/L, lucigenin, Sigma)^{12,13} and indicated as the amount of O_2^- levels in the presence of their relative substrates NADPH (10^{-4} mol/L, Sigma) or xanthine (10^{-4} mol/L, Sigma), as previously described.¹⁸ No enzymatic activity could be detected in the absence of NADPH or xanthine. Reactions were initiated by addition of 10 to 20 μL tissue homogenates containing 25 to 50 μg extracted protein. The enzyme activity was expressed as nmol/min per milligram of protein.

Arterial O_2^- Levels

Arterial O_2^- was quantified with the use of lucigenin chemiluminescence, as previously described.^{12,13} The concentration of lucigenin was 5×10^{-6} mol/L to minimize the formation of nonspecific O_2^- through redox cycling.^{19,20} Isolated arterial ring segments of DOCA-salt or sham rats with or without gene transfer of EC-SOD, dominant negative Rac1, or β -gal (5×10^{10} pfu/mL); or treatment of ABT-627 (3×10^{-8} mol/L, 24 hours) were assayed for O_2^- levels. After reading, arterial tissues were weighed, and the subtracted readings were then converted to nmol/min per milligram of tissue through a standard curve made by cytochrome C (Sigma) reduction from O_2^- , produced from hypoxanthine (Sigma) and xanthine oxidase (Sigma).

In addition, in situ detection of O_2^- was performed by confocal microscopy with oxidative fluorescent dye dihydroethidium (DHE, Sigma), as described previously.^{12,13}

Immunohistochemistry and Western Immunoblot for Arterial VCAM-1

Arterial VCAM-1 levels were assayed by both immunohistochemistry and Western blot analysis, as previously described.¹³ Isolated arterial ring segments (4 mm long) of DOCA-salt or sham rats were transduced with EC-SOD, dominant negative Rac1, or β -gal (5×10^{10} pfu/mL), or treated with ABT-627 (3×10^{-8} mol/L, 24 hours), BQ788 (10^{-7} mol/L, 24 hours, Sigma), or apocynin (10^{-4} mol/L, 24 hours). Arteries of normal rats were treated without or with ET-1 (10^{-9} mol/L, 24 hours); some were pretreated with ABT-627 (3×10^{-8} mol/L, 1 hour) or apocynin (10^{-4} mol/L, 1 hour); some were incubated with adenoviral vectors encoding EC-SOD or β -gal for 4 hours, then transferred to fresh MEM containing ET-1 (10^{-9} 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 μg), 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 ≈ 100 kDa, as confirmed with a standard molecular weight marker (Bio-Rad) in each blot.

Statistical Analysis

Data are expressed as mean \pm 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^- Levels in DOCA-Salt Rats

Arterial O_2^- 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^- to its control levels, an effect that was not observed after β -gal reporter gene transfer (Figure 2A).

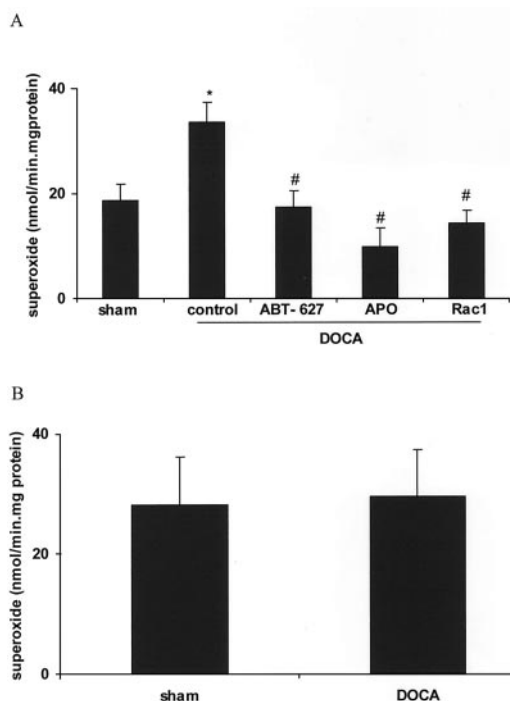


Figure 1. NADPH oxidase (A) and xanthine oxidase (B) activity in carotid arteries of sham and DOCA-salt rats. Arterial supernatants from sham and DOCA rats, with or without treatments as indicated, were subjected to NADPH and xanthine oxidase measurements by lucigenin chemiluminescence in the presence of their relative substrates 10^{-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.

ET_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_A receptor antagonist ABT-627 (Figure 2B) but not selective ET_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 VCAM-1 and O₂⁻ levels (Figure 2B and Figures 3A and 3B).

ET-1 Stimulates Arterial VCAM-1 Expression Through ET_A 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 VCAM-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 ET_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 atherosclerosis. ET-1 enhances the expression of VCAM-1, a key early marker in atherosclerosis, in TNF α -stimulated endothelial cells.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.^{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_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_B receptor antagonist BQ788, suggesting that VCAM-1 expression in this model is mediated by ET-1 through its ET_A receptors. These in vitro observations are consistent with our published data that in vivo treatment with the ET_A receptor antagonist ABT-627 reduced superoxide levels and blood pressure in the same model.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.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.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 O₂⁻ in normal carotid arteries compared with that of DOCA-salt rats.¹² In addition, according to our published¹² 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.⁸ In Ang II-induced hypertensive rats, Ang II stimulates O₂⁻ and oxidative signaling pathways involving redox-sensitive transcription factor NF- κ B and upregulates its downstream genes including VCAM-1.⁸ Ang II is known to produce O₂⁻ through activating the membrane-bound NADPH oxidase.¹⁸ In aldosterone-induced hypertension, intercellular adhesion molecule-1 (ICAM-1) is enhanced by an oxidase-stress-dependent mechanism.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 O₂⁻ production through an ET_A receptor/NADPH oxidase pathway.¹² The present study demon-

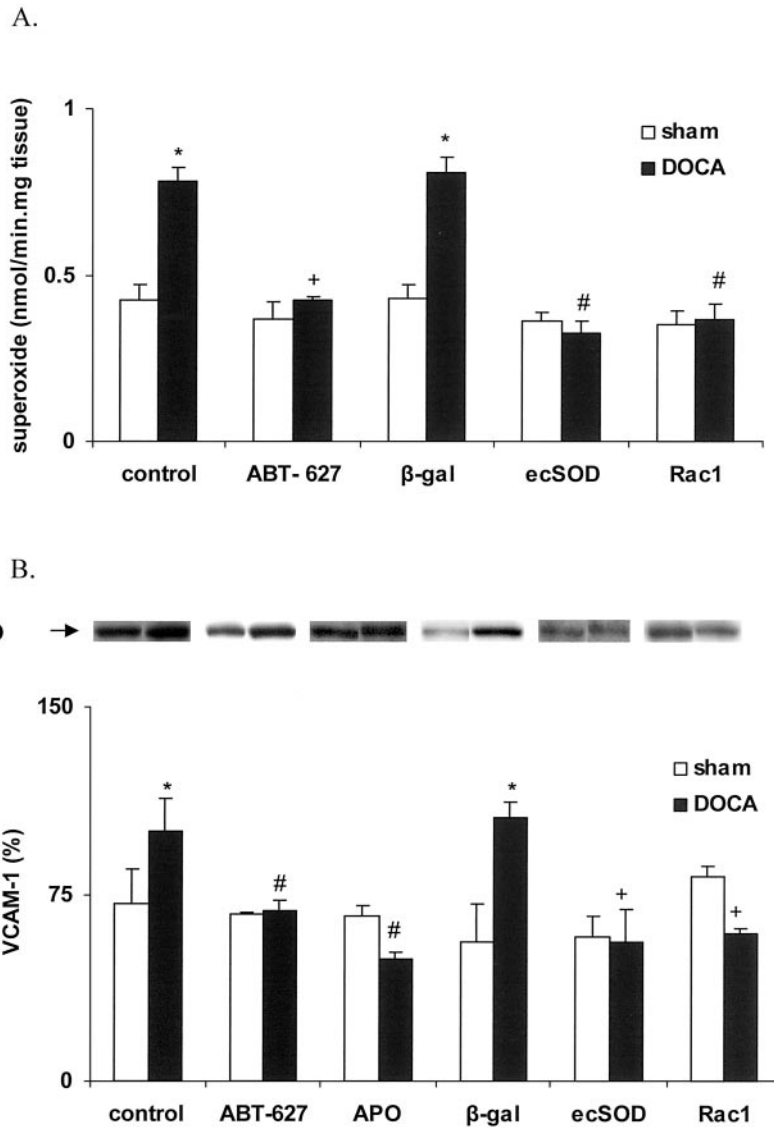


Figure 2. Superoxide (A) and VCAM-1 (B) levels in carotid arteries of sham and DOCA-salt rats. Arterial segments and homogenized supernatants, with or without treatments as indicated, were subjected to lucigenin-enhanced O₂⁻ assay and VCAM-1 immunoblotting analysis, respectively. For Western blot, equal amounts of protein (20 μg) were used for each group as confirmed by membrane staining with the Coomassie brilliant blue R-250. Molecular size for VCAM-1 is ≈100 kDa. VCAM-1 level in DOCA control group is expressed as 100%. A, n=4 to 8 rats. *P<0.05 vs sham group, #P<0.05 vs β-gal-transduced DOCA group, +P<0.05 vs DOCA control. B, n=3 to 4 rats. *P<0.05 vs sham group, #P<0.05 vs DOCA control, +P<0.05 vs β-gal-transduced DOCA group.

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

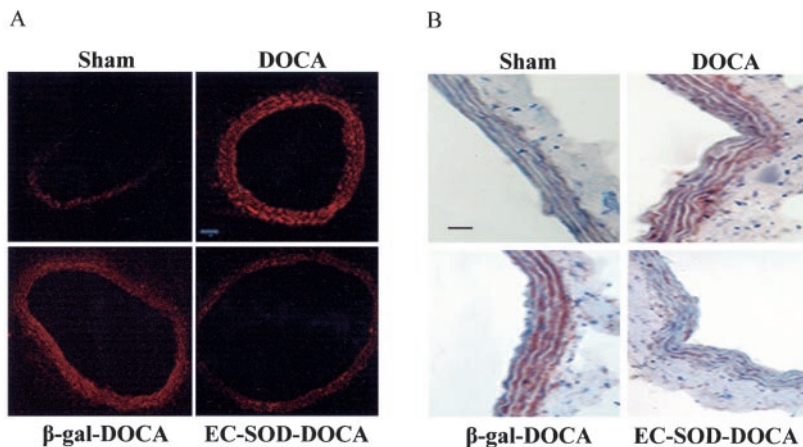


Figure 3. A, Fluorescent confocal micrographs showing in situ detection of superoxide in rat carotid arteries. Arterial cross sections were labeled with the superoxide-sensitive dye dihydroethidium (DHE), which fluoresces red when oxidized to ethidium bromide by superoxide (see Methods). Sections shown are typical of 3 separate experiments. Bar=0.05 mm. B, Immunohistochemical detection of VCAM-1 expression in cross sections of carotid arteries of DOCA-salt and sham rats. Positive VCAM-1 immunoreactivity is indicated by dark brown staining. Sections shown are typical of 4 separate experiments. Bar=0.1 mm.

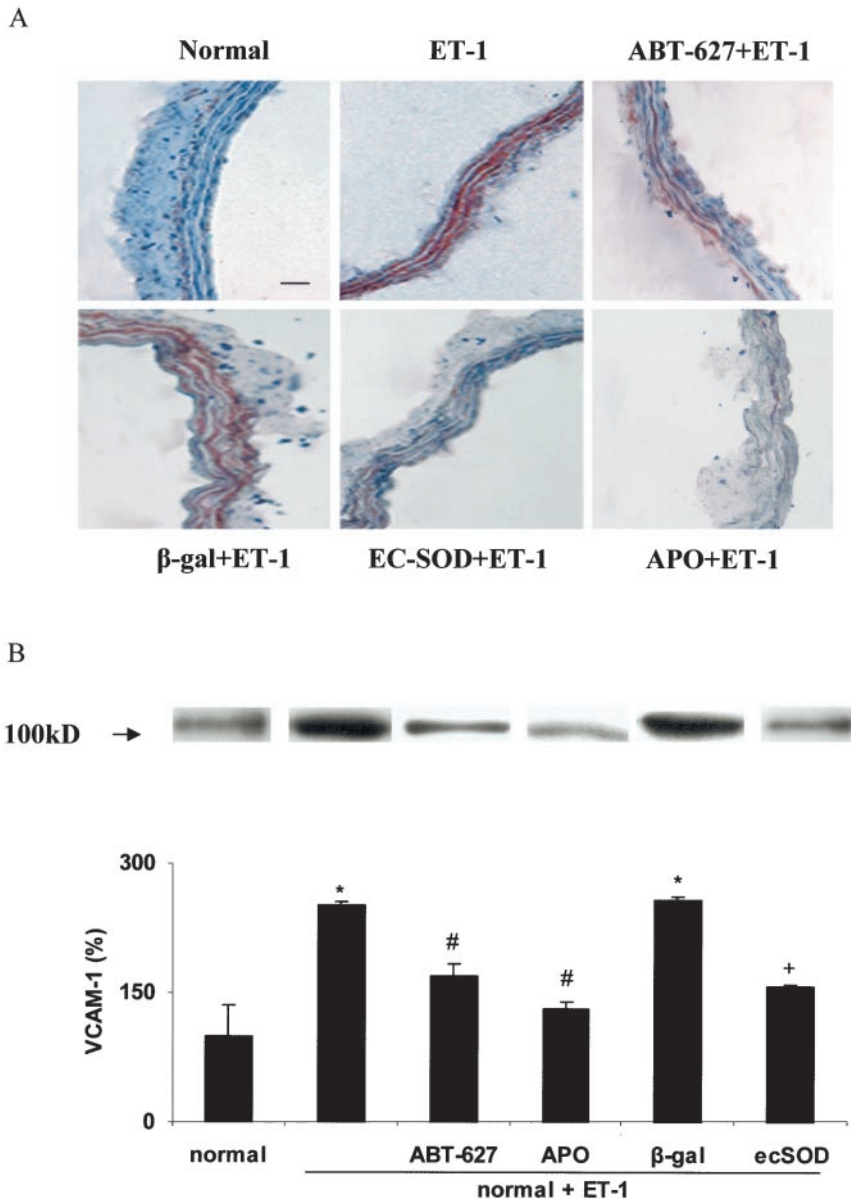


Figure 4. A, Immunohistochemical detection of VCAM-1 expression in cross sections of carotid arteries of normal rats. Positive VCAM-1 immunoreactivity is indicated by dark brown staining. Sections shown are typical of 4 separate experiments. Bar=0.1 mm. B, Western blot analysis of VCAM-1 protein levels in carotid arteries of normal rats. Equal amounts of protein (20 μg) were used for each group as confirmed by membrane staining with the Coomassie brilliant blue R-250. Molecular size for VCAM-1 is ≈100 kDa. Protein level in control group is expressed as 100%. A, n=3 to 4 rats. *P<0.05 vs normal, #P<0.05 vs normal+ET-1, +P<0.05 vs β-gal+ET-1.

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 O₂⁻, which is derived from the activated NADPH oxidase.

Since we have observed that NADPH oxidase is a key source for arterial O₂⁻ 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 O₂⁻ levels (Fig 2A) and VCAM-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 O₂⁻ 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 O₂⁻ levels directly by gene transfer of EC-SOD, which has been shown to reduce vascular O₂⁻ and mean arterial pressure in SHR.¹⁵ Gene transfer of EC-SOD at the titer of 5×10¹⁰ 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 O₂⁻ 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 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.^{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 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,¹⁶ 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 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 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.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 ET_A receptor-mediated activation of NADPH oxidase and superoxide formation in mineralocorticoid hypertension. 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

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References

- Lusis AJ. Atherosclerosis. *Nature*. 2000;407:233–241.
- Alderman MH, Madhavan S, Ooi WL, Cohen H, Sealey JE, Laragh JH. Association of the renin-sodium profile with the risk of myocardial infarction in patients with hypertension. *N Engl J Med*. 1991;324:1098–1104.
- Cambien F, Poirer O, Lecerf L, Evens A, Cambou JP, Arveiler D, Luc G, Bard JM, Bara L, Richard S. Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. *Nature*. 1992;359:641–644.
- Kriegelstein CF, Granger DN. Adhesion molecules and their role in vascular disease. *Am J Hypertens*. 2001;14:44S–54S.
- Price DT, Loscalzo J. Cellular adhesion molecules and atherogenesis. *Am J Med*. 1999;107:85–97.
- Ley K, Huo Y. VCAM-1 is critical in atherosclerosis. *J Clin Invest*. 2001;107:1209–1210.
- Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, Davis V, Gutierrez-Ramos J-C, Connelly PW, Milstone DS. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *J Clin Invest*. 2001;107:1255–1262.
- Tummala PE, Chen XL, Sundell CL, Laursen JB, Hammes CP, Alexander RW, Harrison DG, Medford RM. Angiotensin II induces vascular cell adhesion molecule-1 expression in rat vasculature: a potential link between the renin-angiotensin system and atherosclerosis. *Circulation*. 1999;100:1223–1229.
- Lariviere R, Thibault G, Schiffrin EL. Increased endothelin-1 content in blood vessels of deoxycorticosterone acetate-salt hypertensive but not in spontaneously hypertensive rats. *Hypertension*. 1993;21:294–300.
- Schiffrin EL, Lariviere R, Li JS, Sventek P. Enhanced expression of the endothelin-1 gene in blood vessels of DOCA-salt hypertensive rats: correlation with vascular structure. *J Vasc Res*. 1996;33:235–248.
- Gavras H, Brunner HR, Laragh JH, Vaughan ED Jr., Koss M, Cote LJ, Gavras I. Malignant hypertension resulting from deoxycorticosterone acetate and salt excess: role of renin and sodium in vascular changes. *Circ Res*. 1975;36:300–309.
- Li L, Fink GD, Watts SW, Northcott C, Galligan JJ, Pagano PJ, Chen AF. ET-1 increases vascular superoxide via ET_A-NADPH oxidase pathway in low renin hypertension. *Circulation*. 2003;107:1053–1058.
- Li L, Crockett E, Wang D, Galligan JJ, Fink GD, Chen AF. Gene transfer of endothelial NO synthase and manganese superoxide dismutase on arterial vascular cell adhesion molecule-1 expression and superoxide production in deoxycorticosterone acetate-salt hypertension. *Arterioscler Thromb Vasc Biol*. 2002;22:249–255.
- Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res*. 2000;86:494–501.
- Chu Y, Iida S, Lund DD, Weiss RM, DiBona GF, Watanabe Y, Faraci FM, Heistad DD. Gene transfer of extracellular superoxide dismutase reduces arterial pressure in spontaneously hypertensive rats: role of heparin-binding domain. *Circ Res*. 2003;92:461–468.
- Chen AF, O'Brien T, Tsutsui M, Kinoshita H, Pompili VJ, Crotty TB, Spector DJ, Katusic ZS. Expression and function of recombinant endothelial nitric oxide synthase gene in canine basilar artery. *Circ Res*. 1997;80:327–335.
- Chen AF, Jiang S, Crotty TB, Tsutsui M, Smith LA, O'Brien T, Katusic ZS. Effects of in vivo adventitial expression of recombinant endothelial nitric oxide synthase gene in cerebral arteries. *Proc Natl Acad Sci U S A*. 1997;94:12568–12573.
- Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADP and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res*. 1994;74:1141–1148.
- Munzel T, Afanas'ev IB, Kleschyov AL, Harrison DG. Detection of superoxide in vascular tissue. *Arterioscler Thromb Vasc Biol*. 2002;22:1761–1768.
- Li Y, Zhu H, Kuppusamy P, Roubaud V, Zweier JL, Trush MA. Validation of lucigenin (bis-N-methylacridinium) as a chemiluminescent probe for detecting superoxide anion radical production by enzymatic and cellular systems. *J Biol Chem*. 1998;273:2015–2023.
- Ishizuka T, Takamizawa-Matsumoto M, Suzuki K, Kurita A. Endothelin-1 enhances vascular cell adhesion molecule-1 expression in

- tumor necrosis factor alpha-stimulated vascular endothelial cells. *Eur J Pharmacol.* 1999;369:237–245.
22. Ferri C, Bellini C, Desideri G, Giuliani E, De Siati L, Cicogna S, Santucci A. Clustering of endothelial markers of vascular damage in human salt-sensitive hypertension: influence of dietary sodium load and depletion. *Hypertension.* 1998;32:862–868.
 23. Cottone S, Mule G, Amato F, Riccobene R, Vadala A, Lorito MC, Raspanti F, Cerasola G. Amplified biochemical activation of endothelial function in hypertension associated with moderate to severe renal failure. *J Nephrol.* 2002;15:643–648.
 24. Ravera M, Ratto E, Vettoretti S, Viazzi F, Leoncini G, Parodi D, Tomolillo C, Del Sette M, Maviglio N, Deferrari G, Pontremoli R. Microalbuminuria and subclinical cerebrovascular damage in essential hypertension. *J Nephrol.* 2002;15:519–524.
 25. Pu Q, Fritsch Neves MF, Virdis A, Touyz RM, and Schiffrin EL. Endothelin antagonism on aldosterone-induced oxidative stress and vascular remodeling. *Hypertension.* 2003;42:49–55.
 26. Meyer JW, Schmitt ME. A central role for the endothelial NADPH oxidase in atherosclerosis. *FEBS Lett.* 2000;472:1–4.
 27. Oury TD, Day BJ, Crapo JD. Extracellular superoxide dismutase: a regulator of nitric oxide bioavailability. *Lab Invest.* 1996;75:617–636.
 28. Fukai T, Galis ZS, Meng XP, Parthasarathy S, Harrison DG. Vascular expression of extracellular superoxide dismutase in atherosclerosis. *J Clin Invest.* 1998;101:2101–2111.
 29. Landmesser U, Spiekermann S, Dikalov S, Tatge H, Wilke R, Kohler C, Harrison DG, Hornig B, Drexler H. Vascular oxidative stress and endothelial dysfunction in patients with chronic heart failure: role of xanthine oxidase and extracellular superoxide dismutase. *Circulation.* 2002;106:3073–3078.
 30. Lynch RE, Fridovich I. Permeation of the erythrocyte stroma by superoxide radical. *J Biol Chem.* 1978;253:4697–4699.
 31. Brzezinska AK, McLeod J, Chilian WM. Chloride channels conduct superoxide (O_2^-) radical across the plasma membrane of vascular endothelial cells. *Circulation.* 2001;104(suppl II):II-99. Abstract.