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

Lixin Li, Elahe Crockett, Donna H. Wang, James J. Galligan, Gregory D. Fink, Alex F. Chen

Abstract—Enhanced vascular cell adhesion molecule-1 (VCAM-1) expression directly contributes to vascular dysfunction in hypertension. Decreased NO and/or increased superoxide are causative factors for such an event in the vessel wall. The present study was undertaken to determine whether gene transfer of endothelial NO synthase (eNOS) or manganese superoxide dismutase (MnSOD) affects VCAM-1 levels in arteries from hypertensive rats. Isolated carotid and femoral arteries from deoxycorticosterone acetate (DOCA)-salt hypertensive rats were transduced for 4 hours with adenoviral vectors encoding eNOS, MnSOD, or β-galactosidase reporter genes. Recombinant eNOS or MnSOD expression was evident morphologically and quantitatively 24 hours after gene transfer. Immunohistochemistry, ELISA, and Western blot techniques were used to determine VCAM-1 expression and levels. In addition, endogenous eNOS and MnSOD and in situ superoxide levels were analyzed by immunoblotting and fluorescence confocal microscopy, respectively. Arterial VCAM-1 expression was significantly higher in DOCA-salt hypertensive rats than in sham-operated rats; this expression was accompanied by decreased MnSOD but unaltered endogenous eNOS levels. VCAM-1 expression was significantly lower in MnSOD- and eNOS-transduced hypertensive arteries, with a concomitant reduction of superoxide level. These results suggest that gene transfer of MnSOD or eNOS suppresses arterial VCAM-1 expression in DOCA-salt hypertension by reducing the superoxide level. (Arterioscler Thromb Vasc Biol. 2002;22:249-255.)

Key Words: endothelial NO synthase ■ gene transfer ■ hypertension ■ superoxide ■ vascular cell adhesion molecule-1

Although hypertension is one of the key risk factors for atherosclerosis, the underlying molecular and cellular mechanisms remain to be delineated. Enhanced adhesion molecule expression is known to contribute directly to vascular dysfunction in hypertension. Vascular cell adhesion molecule-1 (VCAM-1) is an early marker of endothelial activation and dysfunction, leukocyte infiltration, and vascular remodeling in the development of early atherosclerotic lesions (fatty streaks and fibrous plaques). Although VCAM-1 is structurally similar to intercellular adhesion molecule-1 (ICAM-1), plays a critical role in early atherogenesis.

Enhanced adhesion molecule expression has been ascribed to an imbalance between oxidative stress and antioxidant activity. Experimental evidence suggests that NO and superoxide are 2 key regulating factors for the expression of adhesion molecules, including VCAM-1. In angiotensin II (Ang II)-induced hypertension, Ang-II stimulates vascular NADPH oxidase to produce superoxide, which not only inactivates NO and impairs vasomotor function but also contributes to atherogenesis by the activation of VCAM-1.

The elevated superoxide is present throughout the atherosclerotic vessel wall, and a major source of superoxide is from the electron transport chain in mitochondrial respiration.

Two recent studies have indicated that there is also a substantial increase of vascular superoxide with reduced NO release in deoxycorticosterone acetate (DOCA)-salt hypertension, a model well known for its suppressed plasma renin and angiotensin levels. However, the level of vascular VCAM-1 expression has never been investigated in this model of hypertension.
Therefore, the present study was undertaken to address the following 3 unanswered questions: (1) Is there an increase of VCAM-1 in carotid and femoral arteries of DOCA-salt hypertensive rats? (2) Are there altered levels of endogenous endothelial NO synthase (eNOS) and manganese superoxide dismutase (MnSOD), the mitochondrial scavenging isozyme? (3) What are the effects of ex vivo gene transfer of eNOS and MnSOD on VCAM-1 and superoxide in DOCA-salt hypertension? Our results showed that VCAM-1 and superoxide levels were significantly increased in carotid and femoral arteries, with a concomitant decrease in the endogenous MnSOD level; these responses were ameliorated after MnSOD or eNOS gene transfer.

**Methods**

**DOCA-Salt Hypertension**

DOCA-salt hypertension was created in male Sprague-Dawley rats as previously described. Briefly, rats (250 to 275 g, Charles River Laboratories, Portage, Ind) underwent uninephrectomy (flank incision, left side), and a silicone rubber DOCA implant (200 mg/kg) was placed subcutaneously between the shoulder blades. Sham-operated rats were also uninephrectomized but received no implant. DOCA-salt rats received 1.0% NaCl and 0.2% KCl in water to drink, and sham-operated rats received tap water. All animals were fed standard rat chow and had ad libitum access to food and drinking solution. Hypertension develops gradually in this model, with arterial pressure rising gradually but steadily over a 4-week period. DOCA-salt hypertension was created in male Sprague-Dawley rats (115 ± 1 versus 170 ± 3 mm Hg for sham-operated group 1 versus 170 ± 3 mm Hg for sham-operated group).

**Preparation of Adenoviral Vectors**

The propagation, purification, and titration of replication-incompetent adenoviral vectors were routinely performed as previously described. The prepared β-galactosidase (β-Gal), eNOS, and MnSOD vectors were stored at -80°C in 0.01 mol/L Tris, 0.01 mol/L MgCl₂, and 10% glycerol before use.

**Ex Vivo Gene Transfer**

Isolated arterial segments (4 mm) were transduced with adenoviral vectors with titers between 10¹⁰ to 10¹¹ plaque-forming units (pfu)/mL as indicated in MEM at 37°C in 0.01 mol/L Tris, 0.01 mol/L MgCl₂, and 0.02% azide. Arteries transduced with the β-Gal marker gene served as controls.

**Immunohistochemistry for VCAM-1 and Recombinant Protein Expression**

Immunohistochemistry was performed as described. Briefly, cross sections of the vessel (6 μm thin) were fixed in ice-cold acetone for 10 minutes, and endogenous peroxidase was inhibited with 0.3% (vol/vol) hydrogen peroxide for 30 minutes. Sections were blocked with 5% horse serum/PBS-Tween 20 (pH 7.4) for 20 minutes and then washed out. Dilute substrate in diethanolamine buffer (pH 7.4) and incubated at 37°C for 30 minutes in a dark humidified container. Fluorescence in vessel sections was then detected by a Zeiss 210 confocal microscope with a 590-nm long-pass filter. Images of the vessels that were treated with saline were measured first. After the basal settings of the confocal microscope were adjusted, images of the treated vessels were collected digitally.

**Western Immunoblot for VCAM-1, eNOS, and MnSOD**

The detailed procedures have been described previously. Arteries were cleaned and homogenized in lysis buffer (100 mmol/L K, HPO₄, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.2% Triton X-100). Equal amounts of protein preparations (10 µg in 25 µL buffer) were run on SDS-polyacrylamide (7.5% for VCAM-1 and eNOS, 12% for MnSOD) gels, electrotransferred to polyvinylidene difluoride membranes, and blotted with a primary antibody against VCAM-1 (1:500), eNOS (1:1500), and MnSOD (1:150). The secondary antibodies used were bovine anti-goat antisera for VCAM-1 and rabbit anti-mouse for eNOS (1:4000). Blots were developed using ECL plus reagent (Amersham Pharmacia Biotech). Prestained protein marker was used for molecular mass determinations. To confirm equal protein loading of all samples, membranes were stained with Coomassie brilliant blue R-250 (Sigma). Molecular band intensity was determined by densitometry (NIH image software).

**In Situ Detection of Superoxide**

In situ detection of superoxide anion was performed by confocal microscopy with the use of the oxidative fluorescent dye dihydroethidium, as described previously. Dihydroethidium is freely permeable to cell membranes and fluoresces red when oxidized to ethidium bromide (EtBr) by superoxide. Unfixed frozen vessel sections (30 µm) with or without gene transfer were placed on glass slides and submerged in 10⁻⁶ mol/L dihydroethidium (Sigma) in PBS buffer (pH 7.4) and incubated at 37°C for 30 minutes in a dark humidified container. Fluorescence in vessel sections was then detected by a Zeiss 210 confocal microscope with a 590-nm long-pass filter. Images of the vessels that were treated with saline were measured first. After the basal settings of the confocal microscope were adjusted, images of the treated vessels were collected digitally.

**Statistical 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. The Bonferroni procedure was used to control type I error when needed. Significance was established at P < 0.05.

**Results**

**Arterial VCAM-1 Expression in DOCA-Salt Hypertension**

There was a significant elevation of average systolic blood pressure in DOCA-salt rats compared with the sham-operated rats (115 ± 1 versus 170 ± 3 mm Hg for sham-operated group.17 The prepared β-galactosidase (β-Gal), eNOS, and MnSOD vectors were stored at -80°C in 0.01 mol/L Tris, 0.01 mol/L MgCl₂, and 10% glycerol before use.
versus DOCA-salt group, respectively \( P < 0.001 \); \( n = 42 \) each). VCAM-1 immunoreactivity was mainly localized in the intima and the intima-media border of the vessels in DOCA-salt rats (Figure 1; sections are typical of 5 separate experiments). The difference in VCAM-1 expression of fresh isolated vessels between the DOCA-salt and sham-operated groups was determined quantitatively by ELISA and Western blotting, showing that the VCAM-1 level was significantly increased in carotid and femoral arteries from the DOCA-salt group (Figure 2; \( P < 0.05 \) and \( P < 0.01 \) versus sham-operated group, \( n = 5 \) to 9 rats).

**Endogenous eNOS and MnSOD Levels in Arteries of DOCA-Salt Hypertension**

As shown in Figure 3, there was no significant difference in endogenous eNOS levels in carotid and femoral arteries between DOCA-salt and sham-operated rats. However, endogenous MnSOD levels were reduced by \( \approx 60\% \) and \( 40\% \) in hypertensive carotid and femoral arteries, respectively \( (P<0.05 \) versus sham-operated group, \( n=4 \)).

**Arterial eNOS and MnSOD Levels After Gene Transfer**

Twenty-four hours after gene transfer, total eNOS and MnSOD (endogenous plus recombinant proteins) were significantly increased in transduced carotid and femoral arteries compared with nontransduced control arteries; both were taken from DOCA-salt hypertensive rats (Figure 4; \( P<0.05 \) and \( P<0.01 \) versus control group, \( n=3 \) to 5). In MnSOD-transduced vessels, an increase in viral vector titer (ie, \( 5 \times 10^{10} \) pfu/mL) resulted in a further elevation of total MnSOD, suggesting a “gene-dose” effect. Immunohistochemical staining showed that positive eNOS and MnSOD immunoreactivities were localized in adventitial and intimal layers of the transduced arteries in DOCA-salt rats compared with the intimal staining in nontransduced arteries (data not shown).

**Gene Transfer on Superoxide Level in DOCA-Salt Hypertension**

After incubation with the superoxide-sensitive dye dihydroethidium, there was a marked increase in EtBr fluorescence (ie, red color), reflecting an increase in superoxide, throughout the vessel wall of a DOCA-salt carotid artery (Figure 5A) compared with the vessel wall of a sham-operated artery (Figure 5B). Twenty-four hours after the gene transfer of eNOS (Figure 5C) or MnSOD (Figure 5D) at \( 5 \times 10^{10} \) pfu/mL, EtBr fluorescence was attenuated (sections were representative of 4 separate experiments). Compared with no incubation, incubation of nontransduced vessels for 24 hours did not increase the superoxide level (data not shown). Similar results were observed in femoral arteries (data not shown).

**Gene Transfer on VCAM-1 Expression in DOCA-Salt Hypertension**

At a titer of \( 5 \times 10^{10} \) pfu/mL (high titer), gene transfer of either MnSOD or eNOS significantly reduced VCAM-1 levels in the carotid and femoral arteries of DOCA-salt rats (Figure 6; \( P<0.05 \) versus nontransduced control arteries, \( n=3 \) to 5). However, at a titer of \( 10^{10} \) pfu/mL (low titer), only MnSOD gene transfer was able to reduce VCAM-1 expression significantly. In contrast, \( \beta \)-Gal reporter gene transfer had no effect on VCAM-1 expression \( (P>0.05 \) versus nontransduced control arteries, \( n=3 \)). In the arteries of sham-operated rats,
VCAM-1 levels remained constant before and after the vascular gene transfer of \( /H9252\)-Gal, eNOS, or MnSOD (please see online data supplement at http://atvb.ahajournals.org).

**Discussion**

The present study investigated the effect of ex vivo gene transfer of eNOS and MnSOD on the regulation of arterial VCAM-1 expression and superoxide production in a rat model of DOCA-salt hypertension. The major new findings of the present study are as follows: (1) VCAM-1 expression was significantly increased in carotid and femoral arteries of DOCA-salt hypertensive rats, (2) endogenous MnSOD, but not eNOS, was reduced with increased superoxide level in these vessels, and (3) gene transfer of MnSOD and eNOS suppressed arterial VCAM-1 expression with a concomitant reduction of superoxide in DOCA-salt hypertensive rats. These results suggest that superoxide plays an important role in stimulating vascular VCAM-1 expression in DOCA-salt hypertensive rats. Our results further showed that the increase in superoxide was accompanied by a reduced endogenous MnSOD level in carotid and femoral arteries, 2 different vessel types that are prone to the development of atherosclerosis in hypertensive patients. These observations suggest that in addition to Ang II–induced hypertension, superoxide may also contribute to the enhanced arterial VCAM-1 expression in salt-sensitive hypertension. This assumption is further supported by our findings that MnSOD gene transfer to these vessels attenuated VCAM-1 expression and superoxide level. Therefore, superoxide-induced oxidative stress may be a general stimulus for VCAM-1 expression in hypertension associated with either a high or low renin-angiotensin state. In addition, the

**Figure 3.** Western blot analysis of endogenous eNOS and MnSOD in rat carotid and femoral arteries. Equal amounts of protein (10 \( \mu \)g) were used for each group. The molecular band sizes are \(~135\) kDa for eNOS and \(~50\) kDa for MnSOD. Cumulative densitometry was used for protein quantification with NIH image software (Scion Image). The protein level in the sham-operated group was expressed as 100%. *\( P<0.05\) for DOCA-salt vs sham-operated groups (n=4 rats in each group).

**Figure 4.** Western blot analysis of total eNOS and MnSOD in carotid and femoral arteries of DOCA-salt rats with or without gene transfer. The molecular band sizes are \(~135\) kDa for eNOS and \(~50\) kDa for MnSOD. Cumulative densitometry was used for protein quantification with NIH image software (Scion Image). The protein level in nontransduced arteries of DOCA-salt rats was expressed as 100%. AdeNOS and AdMnSOD represent adenoviral vectors encoding eNOS and MnSOD genes, respectively. *\( P<0.05\) and **\( P<0.01\) for transduced vs nontransduced controls (n=3 to 5 rats in each group).
observed increase in superoxide levels may not be related to the stimulation of NADPH oxidase by Ang II, inasmuch as this model of hypertension is well known for its suppressed plasma renin and Ang II levels.16

NO possesses important antihypertensive and antiatherogenic effects, including vasodilatation,22 inhibition of VCAM-1 expression,23 NF-κB activation,24 platelet adhesion, and smooth muscle proliferation.22 NO-dependent vasodilatation is markedly impaired in atherosclerosis and hypertension.22 In patients with essential hypertension or salt-sensitive hypertension, agonist-induced NO release in the forearm is attenuated.24

Figure 5. Fluorescent confocal micrographs showing in situ detection of superoxide in rat carotid arteries. Arterial sections were labeled with the oxidative dye dihydroethidium, which fluoresces red when oxidized to EtBr by superoxide (see Methods). Fluorescent intensity was markedly elevated across the vessel wall in a carotid artery from a DOCA-salt rat (A), as indicated by red color, compared with a vessel from a sham-operated rat (B). Gene transfer of eNOS (C) or MnSOD (D) at 5 × 10^10 pfu/mL markedly attenuated the fluorescence intensity in vessel sections from the DOCA-salt rat. The sections shown are typical of 4 separate experiments. Bar = 0.05 mm.

Because of the pivotal role of NO in vascular protection, we hypothesized that gene transfer of eNOS would be able to inhibit VCAM-1 expression, either directly or by neutralizing the effect of superoxide. We found that eNOS gene transfer was able to reduce the arterial VCAM-1 level by 50% and, at the same time, to attenuate the superoxide level in DOCA-salt hypertensive rats. These findings were consistent with an earlier study28 in atherosclerotic carotid arteries of the cholesterol-fed rabbits in which VCAM-1 expression and inflammatory cell infiltration were effectively ameliorated by neuronal NO synthase gene transfer.

The results of our eNOS gene transfer experiments seemed to be contrary to the conventional assumption that the generation of more NO would be inactivated by superoxide. However, these findings were in agreement with recent studies that have convincingly demonstrated that ex vivo and in vivo gene transfer of eNOS or neuronal NO synthase restores NO-mediated arterial relaxations that were impaired by increased superoxide production in hypertensive,29,30 atherosclerotic,31–34 or diabetic35–38 animals. These experimental observations support the novel concept that NO generated by recombinant NO synthase, as a result of gene transfer, provides an effective means of inactivating superoxide and, thereby, improving vascular function, including endothelium-dependent relaxation, in vessels with elevated superoxide.39

In the present study, we found that compared with eNOS gene transfer, MnSOD gene transfer is more effective in suppressing arterial VCAM-1 expression. Our data showed that at a vector titer of 5 × 10^10 pfu/mL, eNOS gene transfer decreased VCAM-1 levels by 50%, an effect that was achieved with MnSOD gene transfer at a titer 5-fold lower. When the titer was increased to the same as used for eNOS, gene transfer of MnSOD reduced the VCAM-1 level further (up to 70%). The difference in the efficacy of MnSOD and eNOS gene transfer may be explained in part by our findings that endogenous MnSOD, but not eNOS, was significantly reduced in the hypertensive vessels. Accordingly, MnSOD gene transfer may restore the functional capacity of the antioxidant enzyme in scavenging elevated superoxide. In contrast, a larger amount of NO may be required from eNOS...
gene transfer to neutralize the stimulatory effect of superoxide on VCAM-1 and directly inhibit its expression. It is interesting to notice that although recombinant eNOS and MnSOD were expressed only in the endothelium and adventitia after gene transfer, they were effective in reducing the increased superoxide observed throughout the vessel wall in DOCA-salt hypertensive rats. Although the exact mechanisms for these experimental observations remain to be determined, we speculate that the NO generated by eNOS gene transfer, in either the endothelium or adventitia, may neutralize superoxide in the adjacent smooth muscle layer because NO is highly diffusible. This speculation is consistent with a previously published study\(^29\) in which gene transfer of eNOS resulted in a significant improvement of Ang II–induced impairment of endothelium-dependent relaxations to acetylcholine, despite increased superoxide levels across the vessel wall. Although recombinant superoxide dismutase (SOD) can scavenge superoxide in endothelial and adventitial layers because of its large molecular weight, this may not be the case inside the smooth muscle cells. However, because superoxide has been shown to cross erythrocyte\(^40\) and endothelial cell\(^41\) membranes via anion channels (e.g., chloride channels), we speculate that 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 MnSOD in the endothelium and adventitia may produce a “diffusion-gradient” effect through which superoxide gets into the 2 outside layers, whereby it is scavenged.

Previous studies have reported that vascular gene transfer of CuZnSOD or extracellular SOD did not improve superoxide-impaired endothelium-dependent relaxation in animal models of Ang II–induced hypertension,\(^29\) atherosclerosis,\(^12\) or diabetes.\(^33\) On the other hand, studies from other groups have shown that gene transfer of MnSOD and CuZnSOD normalized such impaired vasomotor function in diabetic vessels\(^37\) and atherosclerotic vessels without plaque formation.\(^38\) On the basis of the different results of these studies, we decided to examine the effect of MnSOD gene transfer, because mitochondria may also be a key source of superoxide production in addition to the cytosol and extracellular space.\(^13\) Our results of MnSOD gene transfer on vascular VCAM-1 and superoxide levels are in agreement with the latter reports. Although the exact reasons are unclear for these different experimental observations, the source of superoxide production (e.g., cytosolic versus mitochondrial), the disease model studied (e.g., Ang II versus DOCA-salt hypertension), the stages of disease examined (e.g., vessels before and after atherosclerotic plaque formation), and the animal species used (e.g., rabbit versus rat) are factors to be considered to explain such discrepancies. In addition, the effect of MnSOD gene transfer on NO-mediated endothelium-dependent relaxation in DOCA-salt hypertension remains to be determined.

In summary, the results of the present study demonstrate that in the carotid and femoral arteries of DOCA-salt hypertensive rats, there was a significant increase in VCAM-1 expression and superoxide level. Our data have also shown, for the first time, that the endogenous MnSOD level was decreased in DOCA-salt hypertension. Finally, gene transfer of MnSOD and eNOS was effective in reducing VCAM-1 and superoxide levels in a model of hypertension associated with a low renin state. Gene therapy strategies aimed at reducing superoxide-induced oxidative stress may be useful in ameliorating vascular dysfunction and atherogenesis in hypertension.\(^42\)

Acknowledgments

This work was supported by American Heart Association/Midwest Affiliate Scientist Development Grant 0130537Z, American Diabetes Association Regular Research Award 7-01-RA-10, Juvenile Diabetes Research Foundation Innovative Grant 5-2001-311, a Michigan State University Foundation Capacity Building Grant (all to Dr Chen), and a Program Project Grant (No. 1592) of the Michigan Life Sciences Corridor Fund (to Drs Wang and Chen).

References

18. Chen AF, O’Brien T, Tsutsui M, Kinoshita H, Pompilii VJ, Crotty TB, Spector DJ, Katusic ZS. Expression and function of recombinant endo-
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

Lixin Li, Elahe Crockett, Donna H. Wang, James J. Galligan, Gregory D. Fink and Alex F. Chen


Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2002 American Heart Association, Inc. All rights reserved.

Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://atvb.ahajournals.org/content/22/2/249

Data Supplement (unedited) at:

http://atvb.ahajournals.org/content/suppl/2002/01/31/22.2.249.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:

http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:

http://atvb.ahajournals.org//subscriptions/
Fig. I. Western blot analysis of VCAM-1 in carotid and femoral arteries of sham-operated rats with or without gene transfer. Equal amount of protein (10 µg) was used for each group. The molecular band size for VCAM-1 is ~100 kD. Cumulative densitometry was used for protein quantifications with a NIH image software (Scion Image). The protein level in non-transduced control arteries was expressed as 100%. *P > 0.05 vs. non-transduced controls, n = 3 rats in each group. The titer of virus vector used was 10^{10} pfu/ml.