Vascular Effects of the Human Extracellular Superoxide Dismutase R213G Variant

Yi Chu, PhD; Abdullah Alwahdani, MD; Shinichiro Iida, MD, PhD; Donald D. Lund, PhD; Frank M. Faraci, PhD; Donald D. Heistad, MD

Background—Extracellular superoxide dismutase (ECSOD) is a major extracellular antioxidant enzyme. We have demonstrated that vascular effects of ECSOD require an intact heparin-binding domain. A common genetic variant with a substitution in the heparin-binding domain (ECSOD R213G) was reported recently to be associated with ischemic heart disease. The goal of this study was to examine vascular effects of ECSOD R213G.

Methods and Results—A recombinant adenovirus (Ad) that expresses ECSOD R213G was constructed. ECSOD R213G and ECSOD proteins bound to collagen type I in vitro, but binding to aorta ex vivo was 10-fold greater with ECSOD than ECSOD R213G. Three days after intravenous injection of AdECSOD R213G or AdECSOD in spontaneously hypertensive rats (SHR), immunostaining demonstrated binding of ECSOD to carotid arteries and kidneys but minimal binding of ECSOD R213G. Binding to aorta and carotid artery was 2.5- to 3-fold greater with ECSOD than ECSOD R213G by immunoblotting. Arterial pressure was significantly reduced by AdECSOD but not by AdECSOD R213G. Responses to acetylcholine and basal levels of nitric oxide in carotid arteries were impaired in SHR compared with normotensive Wistar-Kyoto rats and were improved after AdECSOD but not AdECSOD R213G. Levels of superoxide and nitrotyrosine in aorta were higher in SHR than Wistar-Kyoto rats and were greatly reduced after AdECSOD but not AdECSOD R213G.

Conclusions—In contrast to ECSOD, ECSOD R213G has no significant protective effect on arterial pressure, vascular function, or vascular levels of oxidative stress in SHR. These findings may provide a mechanistic basis for association studies that suggest that human beings carrying ECSOD R213G are predisposed to vascular diseases. (Circulation. 2005;112:1047-1053.)

Key Words: free radicals ■ hypertension ■ nitric oxide ■ risk factors ■ gene therapy

Levels of superoxide in blood vessels are elevated in several vascular diseases. Superoxide inactivates nitric oxide (NO), resulting in a reduction in NO bioavailability and production of peroxynitrite. Extracellular SOD (ECSOD or SOD 3) is a major extracellular antioxidant enzyme distributed in the extracellular matrix of many tissues and especially blood vessels. A fundamental property of ECSOD is its affinity, through its heparin-binding domain (HBD), for heparan sulfate proteoglycans located on cell surfaces and in extracellular matrix.

Substitution of arginine-213 by glycine (R213G), which results from a C-to-G transversion at the first base of codon 213, is a common human gene variant in the HBD of ECSOD. Plasma concentrations of ECSOD are increased greatly in the 2% to 5% of the population that carries ECSOD R213G. This alteration in the HBD reduces affinity for heparin but does not affect the enzymatic activity of ECSOD. Binding to bovine aortic endothelial cells in culture is 50-fold less by ECSOD R213G than by normal ECSOD.

Three association studies have been reported for ECSOD R213G. Affected individuals in Sweden did not have major phenotypic abnormalities, but there was a trend for increased triglycerides and body weight. In Japan, patients with diabetes on hemodialysis carrying ECSOD R213G had an increase in 5-year mortality rate, with significantly higher death rates from ischemic heart disease and cerebrovascular disease than those of noncarriers. A recent large study in Denmark suggested a 2.3-fold increase in risk of ischemic heart disease in heterozygotes carrying ECSOD R213G, with a 9-fold increase after adjustment for plasma levels of ECSOD. In contrast to these association studies, no studies have been reported to directly demonstrate vascular effects of ECSOD R213G.

We reported recently that gene transfer of ECSOD reduces arterial pressure and improves vascular and renal function in...
spontaneously hypertensive rats (SHR) and that the HBD is necessary for the effects. In the present study, we constructed a replication-deficient adenoviral vector that expresses human ECSOD R213G. Effects of gene transfer of ECSOD R213G were compared with normal ECSOD to test the hypothesis that ECSOD R213G may have less effect than ECSOD on arterial pressure and vascular function in SHR. Rats were chosen as the experimental model because blood vessels in rats contain little endogenous ECSOD, so that effects of ECSOD and ECSOD R213G can be observed with low background levels of endogenous ECSOD. Recombinant viruses were injected intravenously, with the expectation that ECSOD and ECSOD R213G protein would be produced in the liver and released into the circulation.

**Methods**

**Animals**
Male SHR and Wistar-Kyoto (WKY) rats (Harlan, Indianapolis, Ind), weighing 320 to 360 g, 20 weeks old, were used in the study. Procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH) and were approved by the Animal Care and Use Committee of the University of Iowa. Rats were anesthetized with sodium pentobarbital (50 mg/kg IP). Adenovirus (5x10^11 particles in phosphate-buffered saline [PBS] that contained 3% sucrose) or vehicle was injected into the penile vein. Rats began to awaken within half an hour after injection. Arterial pressure was measured by using indwelling catheters before and 3 days after injection of viruses in anesthetized or conscious rats, as has been described previously in detail.

**Recombinant Adenoviral Vectors**
A replication-deficient adenovirus that expresses normal human ECSOD (AdECSOD) was described previously. The shuttle plasmid for construction of AdECSOD was used as template for site-directed mutagenesis to produce AdECSOD R213G. Primer pairs underlined: sense, 5'-GACCTTGACGCAAGAGAAAGGGGGGAGCGGACAGGCAGTAAGCCCTTCATGTTCCTGCTATGAGTCGCG-3' antisense, 5'-CTCTGGCTCTCCGCGGCGCCTTCTCTGCTATGAGTCGCG-3'. The site-directed mutagenesis reaction was performed with the use of the QuickChange II XL kit (Strategene). After transformation of the mutant sequence by DNA sequencing, production and amplification of AdECSOD R213G were performed according to standard procedures. The ratio of viral particles to plaque-forming units was 50.1 for all viruses used in the study.

**Binding of ECSOD Proteins In Vitro**
To obtain recombinant ECSOD proteins, HeLa cells (70% confluent) were incubated with an adenovirus expressing ECSOD, AdECSOD R213G, or ECSODΔHBD at 20 PFU/cell for 3 hours. After removal of unbound viruses by extensive washing, cells were incubated in Dulbecco-modified Eagle’s medium with 10% fetal bovine serum for 3 days. Cell-free culture medium was then harvested by filtration through a 0.22-μm, low-protein binding filter. The ECSOD activity of the media was determined, as described below, to be 70 U/mL. We examined binding to collagen type I protein (Sigma), which is a major type of collagen in blood vessels. Collagen type I, dissolved in 0.1 mol/L acetic acid, was blotted on a nitrocellulose membrane. After washing, blots were blocked in 5% nonfat milk plus 2% bovine serum albumin in PBS and incubated for 2 hours with cell- and virus-free culture medium that contained equal concentrations of ECSOD, AdECSOD R213G, or ECSODΔHBD (70 and 7 U/mL). Blots were washed with PBS and then incubated with anti-human ECSOD rabbit antisemur (obtained from Dr James Crapeo of National Jewish Medical Research Center, Denver, Colo). Blots were washed with PBS and incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:20 000 in the blocking solution). Blots were washed, incubated with chemiluminescent substrate (Femto, Pierce), and exposed to x-ray film. Density of bands on x-ray film was measured by using Quantity One (BioRad) and quantified against ECSOD standards (in serial 5-fold dilutions).

Using confocal microscopy, we also observed binding of ECSOD to MS-1 cells (a mouse endothelial cell line obtained from American Type Culture Collection) in vitro. There was little or no detectable binding of ECSOD R213G (data not shown).

**Binding of ECSOD Proteins to Aorta Ex Vivo**
Thoracic aortae were excised from SHR rats and cut into 3- or 7-mm-long segments. After blood and fat were removed from lumen and adventitia, respectively, pairs of segments were incubated with culture medium, which contained equal concentrations of ECSOD, AdECSOD R213G, or ECSODΔHBD proteins, as well as control medium, at 37°C for 60 minutes. Segments were then washed with PBS to remove unbound protein. The 3-mm segment was embedded in OCT compound for immunostaining, and the 7-mm segment was submersed in phosphate buffer (0.5 mol/L, pH 7.4) containing a cocktail of protease inhibitors and 0.3 mol/L KBr for quantification of binding of ECSODs, using immunoblotting. Quantification of binding by immunoblotting was performed as described above.

Segments of aorta in OCT were cut into 7-μm sections. Sections on slides were fixed in 2% paraformaldehyde for 15 minutes, blocked in 10% bovine serum albumin, and incubated with anti-human ECSOD antiserum described above at 4°C overnight. After washing, sections were incubated with Alexa 488-conjugated goat anti-rabbit IgG antibody (Molecular Probes). After washing, slides were mounted with Vectashield (Vector Laboratories) and examined using confocal microscopy by a blinded observer. At least 5 random images were taken for each sample.

**Binding of ECSOD Versus ECSOD R213G to Tissues After In Vivo Gene Transfer**
Three days after in vivo gene transfer, carotid arteries, kidneys, and livers from SHR rats were isolated and processed for immunostaining for ECSOD R213G or ECSOD as described previously. Briefly, segments of the organs were embedded in OCT, sectioned, and processed as described above except that the secondary antibody was horseradish peroxidase-conjugated and sections were stained with 3,3′-diaminobenzidine as substrate. Carotid arteries and aortas were cleaned of outer fat tissue, and rings were rinsed thoroughly in PBS to remove blood. Immunoblotting to quantify ECSOD was performed as described above.

**Vasomotor Function and Basal Levels of NO in Carotid Arteries**
Vasomotor function of carotid arteries was examined as described previously. Briefly, rings of carotid artery, after equilibration to resting tension of 1.0 g, were contracted with KCl (70 mmol/L). After rinse, responses to phenylephrine were measured, and cumulative concentration response curves for acetylcholine or sodium nitroprusside were generated after preconstriction of rings with the EC50 dose of phenylephrine. Basal activity of NO was estimated by examining contraction to the NO synthase inhibitor Nω-nitro-l-arginine (L-NNA) (10^-6 to 10^-4 mol/L), in the presence of a threshold concentration of phenylephrine (3x10^-8 mol/L).

**Measurements of ECSOD Enzymatic Activity, Superoxide, and Nitrotyrosine**
Enzymatic activity of ECSOD was measured as described previously. Briefly, plasma ECSOD or ECSOD R213G protein was enriched by extraction with 37.5% chloroform/62.5% ethanol (vol/vol), and the aqueous phase of extracted plasma was then assayed for SOD activity with nitroblue tetrazolium reduction. Levels of superoxide and nitrotyrosine in aorta were measured as described previously.
binding of ECSOD than ECSOD<sub>R213G</sub> to the aorta ex vivo, with no detectable binding of ECSODΔHBD (Figure 1C).

Thus, these findings suggest that the HBD is essential for binding of ECSOD in blood vessels, and the single–amino acid substitution results in impairment of binding of ECSOD<sub>R213G</sub> to vessels ex vivo.

It has been reported recently that binding of ECSOD to collagen type I requires the HBD. Collagen type I, blotted on nitrocellulose membrane, was incubated with ECSOD, ECSOD<sub>R213G</sub>, or ECSODΔHBD protein (70 U/mL; similar results were obtained with 7 U/mL). Binding to collagen type I was 30% greater for ECSOD than ECSOD<sub>R213G</sub>, whereas ECSODΔHBD did not bind to collagen (Figure 1D).

### Binding of ECSOD<sub>R213G</sub> Versus ECSOD to Tissues After In Vivo Gene Transfer

Plasma levels of SOD activity were increased significantly 3 days after injection of an adenovirus expressing either ECSOD or ECSOD<sub>R213G</sub> (Figure 2A). Normal ECSOD was bound to the endothelium and some subendothelial smooth muscle of the carotid artery and glomeruli and surfaces of tubules in the kidney (Figure 2B). There was much less binding of ECSOD<sub>R213G</sub> to the carotid artery and no detectable binding to the kidney (Figure 2B). Staining of liver was similar after gene transfer of ECSOD<sub>R213G</sub> and ECSOD, as expected. Quantification of binding by immunoblotting indicated 3-fold more binding to the aorta and 2.5-fold more binding to the carotid artery with ECSOD than with ECSOD<sub>R213G</sub> (Figure 2C). Thus, findings both in vivo and ex vivo suggest that binding of ECSOD<sub>R213G</sub> to cells or tissues is greatly impaired, even though binding to collagen type I in vitro is only 30% less for ECSOD<sub>R213G</sub> than ECSOD.

### Effects of ECSOD<sub>R213G</sub> Versus ECSOD on Arterial Pressure of SHR

In conscious SHR, AdECSOD reduced mean arterial pressure (MAP), whereas AdECSOD<sub>R213G</sub> or AdΔgαl had no significant effect on MAP (Figure 3). There was no significant change in heart rate before and after injection of viruses in any group (data not shown). Similar findings, with reduction of MAP by ECSOD and no reduction by ECSOD<sub>R213G</sub>, were observed in anesthetized SHR (Data Supplement Figure I).

### Effects of ECSOD<sub>R213G</sub> Versus ECSOD on Vasomotor Function

Vasomotor responses were compared in SHR after in vivo gene transfer of ECSOD, ECSOD<sub>R213G</sub>, or AdΔgαl. Contraction of the carotid artery to phenylephrine and relaxation to sodium nitroprusside were not different in WKY and in SHR, before or after injection of the viruses (Data Supplement Figure II).

Relaxation of the carotid artery to acetylcholine was impaired in SHR after control virus injection (AdΔgαl), as compared with WKY (Figure 4A). The important finding was that relaxation to acetylcholine, which is endothelium dependent, was significantly improved after AdECSOD but not AdECSOD<sub>R213G</sub> injection (Figure 4A).

We also determined whether ECSOD increased basal NO by examining constriction to an NO synthase (NOS) inhibi-
Constriction to L-NNA was augmented after AdECSOD but not AdECSODR213G injection, to the level of constriction produced in WKY (Figure 4B). The findings suggest that the basal level of NO in SHR was increased by ECSOD but not ECSODR213G. These findings suggest that gene transfer of ECSODR213G, in contrast to ECSOD, does not improve endothelial function or increase basal levels of NO in SHR.

Effects of ECSODR213G Versus ECSOD on Levels of Superoxide and Nitrotyrosine in Aorta

The level of superoxide in aortae of SHR rats (not shown) or SHR rats injected with control virus (Figure 5A) was 2-fold higher than that in WKY rats. AdECSOD greatly reduced the level of superoxide in SHR (P<0.001). AdECSODR213G also reduced superoxide (P<0.01 versus SHR with control virus), but the effect was much smaller as compared with AdECSOD (P<0.001) (Figure 5A). These findings suggest that gene transfer of ECSOD reduces vascular levels of superoxide, but ECSODR213G has only modest effects.

We quantified levels of nitrotyrosine, a marker for peroxynitrite (which is the product of NO and superoxide), in aorta by using immunoblotting of protein extracts. The level of nitrotyrosine was higher in control SHR than in WKY rats and was reduced by AdECSOD but not AdECSODR213G (Figure 5B).

Discussion

In the present study, we examined vascular effects of the R213G variant of human ECSOD by using adenovirus-mediated gene transfer. The major findings of this study are as follows: (1) Binding of ECSODR213G, as compared with normal ECSOD, is impaired in blood vessels ex vivo and in vivo, although there is substantial binding of ECSODR213G to collagen type I in vitro. (2) ECSODR213G, in contrast to ECSOD, has minimal effects on vascular oxidative stress in SHR, measured as levels of superoxide, and fails to reduce vascular nitrotyrosine. (3) ECSODR213G, in contrast to ECSOD, does not reduce arterial pressure or improve vasomotor function or basal levels of NO in SHR.

Binding of the ECSODR213G Variant: Role of HBD

Our finding that binding to blood vessels after in vivo gene transfer is less for the ECSODR213G variant than for normal ECSOD is consistent with previous studies and other findings in the present study. First, ECSODR213G binds strongly to heparin, although with a lower affinity (eluted at 0.35 mol/L of NaCl) than the normal ECSOD (eluted at 0.55 mol/L of NaCl). Second, intravenous heparin releases less ECSODR213G than ECSOD, which suggests that ECSODR213G binds, although less than normal ECSOD, to the endothelium of vessels. Third, ECSODR213G binds to bovine aortic endothelial cells 50-fold less than ECSOD in vitro, as quantified.
by ELISA. Fourth, as suggested in the present study, binding to collagen type I in vitro is only modestly less for ECSODR213G than for normal ECSOD. Finally, very low levels of binding were detected after incubation of ECSODR213G with aorta ex vivo, both by immunoblotting and immunofluorescence with confocal microscopy. Thus, our findings and those of others suggest profound impairment of binding of ECSODR213G to blood vessels.

With regard to binding of ECSODR213G versus ECSOD, the difference is 10-fold in the aorta after incubation with proteins ex vivo but only 3-fold in the aorta and 2.5-fold in the carotid artery after gene transfer in vivo. At least 2 factors may contribute to a different magnitude of effects ex vivo and in vivo. First, in relation to binding to aorta ex vivo, ECSOD binds to both adventitia and endothelium, whereas ECSODR213G has slight binding to endothelium and no detectable binding to adventitia (Figure 1B). In contrast, ECSOD or ECSODR213G in blood has access to endothelium after gene transfer in vivo, with probably less access to adventitia than ex vivo (Figure 2B). We speculate that the difference in binding between ECSOD and ECSODR213G would be larger when both endothelium and adventitia are accessible to the protein than when only endothelium is accessible for binding of the protein. Second, after prolonged exposure of vessels to blood, ECSODR213G may be more likely to bind to the endothelium than after 1 hour of incubation ex vivo. Thus, the difference between ECSOD and ECSODR213G in binding to vessels may be smaller in vivo than ex vivo.

Whereas ECSODR213G binds to collagen type I in vitro, there was no detectable binding of ECSODR213G in the medial layer (where collagen type I is present) after in vivo gene transfer. The explanation for this variance may be that ECSODR213G and ECSOD are large molecules (homotetramers with ≈135 kDa) and thus may not readily penetrate through endothelium and internal elastic lamina to reach the media. Thus, ECSOD may have minimal access to collagen type I for binding after gene transfer in vivo.

Binding mediated by the HBD is characteristic of ECSOD, and its physiological importance has been demonstrated in this study and our previous studies in several disease models. Similar effects also have been demonstrated by using chimeric recombinant SODs, HB-SOD and SOD2/3, in which the HBD of ECSOD was added to CuZnSOD and MnSOD, respectively, to increase cellular binding to achieve antihypertensive and higher antiinflammatory activities. Thus, impairment in cellular binding in this and other studies suggests that ECSODR213G carriers may have increased risk for vascular dysfunction caused by oxidative stress, although evidence for this abnormality has not yet been reported.

Despite these profound changes in cellular binding of the R213G variant, ECSODR213G nevertheless binds to collagen type I. Because collagen type I is a major type of collagen released by vascular muscle and is highly regulated and implicated in vascular diseases, this finding suggests some degree of preservation of function by ECSODR213G in relation to activities and metabolism of collagen type I. A recent study reports that ECSODR213G binds to collagen type I with an affinity 12-fold lower than that of normal ECSOD. Despite the difference in the degree of binding observed previously and in the present study, which may have resulted from different methods of detection, both studies indicate that ECSODR213G indeed binds to collagen type I.

Mechanisms for Vascular Effects of Normal ECSOD But Not the R213G Variant

On the basis of several approaches, as described above, we found attenuated binding of ECSODR213G as compared with ECSOD, in vessels after in vivo gene transfer. ECSODR213G did not affect vascular function, although it reduced vascular superoxide levels modestly, which suggests the presence of ECSODR213G in vessels. Thus, it is likely that failure of ECSODR213G to produce a detectable effect on vascular function results from its subthreshold binding to blood vessels.
These experiments and previous studies indicate that there are several mechanisms by which gene transfer of normal ECSOD affects blood vessels in SHR. First, ECSOD reduces levels of superoxide, which are increased in SHR. Second, ECSOD reduces formation of peroxynitrite (as assayed by levels of nitrotyrosine) by reaction of superoxide and nitric oxide, which may damage vessels because of its high oxidation potential. Third, ECSOD increases basal levels of nitric oxide, as a consequence of reduction of levels of superoxide, but probably not by unmasking differences in eNOS. Vascular expression of eNOS, as quantified by real-time reverse transcriptase–polymerase chain reaction, is not different between SHR and W KY. Although the relative contributions of these mechanisms toward improved endothelial function in SHR are not delineated in the present study, it is likely that increased NO plays a major role. A recent unpublished study indicates that antihypertensive effects of ECSOD are absent in eNOS−/− mice and therefore it appears that NO is the main effector of ECSOD (R. Brandes, MD, unpublished data, 2004, personal communication).

In this study, we have injected recombinant adenoviruses intravenously. ECSOD and ECSODR213G were produced by liver, secreted into circulating blood, and bound to endothelium. There is an important difference in localization of ECSOD and ECSODR213G in our study and in human beings. Endogenous ECSOD in blood vessels in human beings is expressed mainly by vascular smooth cells, whereas after in vivo gene transfer, ECSOD or ECSODR213G are bound almost exclusively to endothelium. Thus, gene transfer in vivo does not duplicate the physiology of ECSOD or ECSODR213G in human beings. Nevertheless, this does not detract from our goal of comparing vascular effects of ECSODR213G versus ECSOD.

Implications of the Study
In the present study, we have characterized binding of ECSODR213G to blood vessels ex vivo and in vivo. We obtained direct evidence that even though ECSODR213G binds in vitro to collagen type I, only 30% less than ECSOD, it binds poorly to endothelium and does not affect vascular function or arterial pressure in SHR. Our findings are consistent with an emerging concept that only tissue-bound ECSOD is vasoprotective, and the status of bound ECSOD versus free ECSOD in plasma modulates risk for some vascular diseases in human beings. For example, patients with heart failure have low levels of heparin-releasable (thus, endothelium-bound) ECSOD. ECSOD may become more important as an antioxidant when high levels of oxidative stress are present, such as in diabetes and renal failure with hemodialysis or in chronic hypertension (as in this study). Our findings may provide a mechanistic basis for findings of previous association studies and suggest that the R213G variant of ECSOD may be an independent risk factor for vascular diseases that are associated with increased superoxide in blood vessels.

In conclusion, the major implication of the findings in this study is that in human beings carrying the R213G variant of ECSOD, blood vessels may be deficient in ECSOD activity (due to attenuated binding of ECSODR213G) and thus may be predisposed to dysfunction from oxidative stress.

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References


**CLINICAL PERSPECTIVE**

Extracellular superoxide dismutase (ECOD) is a major antioxidant enzyme, and the HBD is required for vascular effects of ECOD. There is a common genetic variant within the HBD (ECODR213G) in ≈5% of population. Recently, ECODR213G was reported to be associated with increased risk of ischemic heart disease. In this study, the investigators examined vascular effects of ECODR213G using a novel approach of adenovirus-mediated gene transfer. In contrast to ECOD, ECODR213G binds poorly to blood vessels and has no significant protective effect against hypertension, endothelial dysfunction, or vascular oxidative stress in rats with genetic hypertension. The findings may provide a mechanistic basis for the finding that human beings carrying ECODR213G are predisposed to vascular disease.
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