Gene transfer of extracellular superoxide dismutase protects against vascular dysfunction with aging

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Brown, Kathryn A., Yi Chu, Donald D. Lund, Donald D. Heistad, and Frank M. Faraci. Gene transfer of extracellular superoxide dismutase protects against vascular dysfunction with aging. Am J Physiol Heart Circ Physiol 290: H2600–H2605, 2006.—Aging is an independent risk factor for cardiovascular disease, but mechanisms leading to vascular dysfunction have not been fully elucidated. Recent studies suggest that oxidative stress may increase in blood vessels during aging. Levels of superoxide are influenced by the activity of SODs. The goal of this study was to examine the effect of extracellular superoxide dismutase (ECSOD) on superoxide levels and vascular function in an animal model of aging. Aortas from young (4–8 mo old) and old (29–31 mo old) Fischer 344 rats were examined in vitro. Relaxation of aorta to ACh was impaired in old rats compared with young rats; e.g., 3 μM ACh produced 57 ± 4% (mean ± SE) and 84 ± 2% relaxation in old and young rats, respectively (P < 0.0001). Three days after gene transfer of adenosine expressing human ECSOD (AdECSOD), the response to ACh was not affected in young rats but was improved in old rats. There was no difference in relaxation to the endothelium-independent dilator sodium nitroprusside between young, aged, and AdECSOD-treated old rats. Superoxide levels (lucigenin-enhanced chemiluminescence) were significantly increased in aged rats compared with young rats. After gene transfer of ECSOD to aged rats, superoxide levels in aorta were similar in old and young rats. Gene transfer of an ECSOD with the heparin-binding domain deleted had no effect on vascular function or superoxide levels in old rats. These results suggest that 1) vascular dysfunction associated with aging is mediated in part by increased levels of superoxide, 2) gene transfer of ECSOD reduces vascular superoxide and dysfunction in old rats, and 3) beneficial effects of ECSOD in old rats require the heparin-binding domain of ECSOD.

oxidative stress; endothelium; acetylcholine; rat

AGING IS AN INDEPENDENT risk factor for cardiovascular disease, but mechanisms leading to vascular dysfunction have not been fully elucidated (25). Decreased bioavailability of nitric oxide (NO) may be a major cause of endothelial dysfunction in aging (32). A decrease in endothelium-derived NO predisposes blood vessels to vasoconstriction, oxidative stress, and inflammation (41). Endothelial dysfunction has emerged as an independent risk factor for cardiovascular events (20, 34).

Bioactivity of NO depends, in part, on its interaction with reactive oxygen species (ROS), particularly superoxide anion (33). Recent studies (21, 39) suggest that oxidative stress increases in blood vessels during aging. NO reacts with superoxide to form peroxynitrite (ONOO–), and ONOO– can induce protein modifications and DNA damage (5, 7, 19). Levels of superoxide are dependent, in part, on the activity of three isoforms of endogenous SOD: cytosolic CuZnSOD (SOD-1), mitochondrial MnSOD (SOD-2), and extracellular CuZnSOD (EC-SOD; SOD-3) (15). ECSOD is found throughout the vessel wall in many species and is the only isoform of SOD that is located outside of the cell, binding to cell surfaces and the extracellular matrix via its heparin-binding domain (HBD) (16, 28, 29). The functional importance of ECSOD in vascular function during aging is not known.

The goal of this study was to examine the hypothesis that gene transfer of ECSOD, but not ECSOD lacking the HBD, reduces superoxide levels and improves vascular function during aging in rats. Rats were chosen because blood vessels of rats have low levels of endogenous ECSOD within the vessel wall, compared with other species (10). Thus effects of gene transfer of ECSOD could be examined in the presence of low levels of endogenous tissue ECSOD and after substantial increases in ECSOD with gene transfer. Overexpression of ECSOD was achieved by using adenoviral-mediated gene transfer of ECSOD.

METHODS

Animals. Male Fischer 344 rats ages 4–6 (n = 23) and 29–31 (n = 37) mo were obtained from the National Institute on Aging. The animals were housed under a 12-h:12-h light-dark cycle with free access to water and food. All experimental protocols were in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH) and approved by the Animal Care and Use Committee of the University of Iowa.

Adenoviral vectors and in vivo gene transfer. Replication-deficient adenovirus expressing either human ECSOD (AdECSOD) or ECSOD with the HBD deleted (AdECSODΔHBD), both driven by the human cytomegalovirus (CMV) promoter/enhancer, was constructed by using standard procedures in our laboratory (13). Briefly, human ECSOD cDNA contained within a plasmid (kindly provided by Dr. James Crapo of the National Jewish Medical and Research Center at Denver, CO) was cloned into the plasmid, purified using low melting-point agarose gel electrophoresis, and ligated directionally into a shuttle vector. Complementary DNA for human ECSOD with deletion of the HBD (ECSODΔHBD) was amplified using the ECSOD plasmid as a template, and the ECSODΔHBD segment was then cleaved and ligated directionally into the adenovirus containing CMV (Ad5CMV) shuttle vector.

Rats were anesthetized with halothane (2–5%), and adenovirus (0.25 ml of 1 × 1012 particles/ml in 3% sucrose in PBS) was injected into a penile vein. Three days after viral injection, rats were euthanized by injection of pentobarbital sodium (150 mg/kg ip). The aorta was quickly removed and placed in cold (4°C) oxygenated Krebs...
solution. Sections from a single aorta were used for vascular function studies and superoxide measurements.

**Plasma analysis.** Whole arterial blood (~1 ml) was obtained while vessels were being harvested. The blood was centrifuged for 15 min at 15,000 g. Plasma was isolated and stored at −80°C until analysis, at which time cholesterol and glucose were measured. Total cholesterol was measured enzymatically (Infinity cholesterol reagent, Sigma). Plasma glucose (nonfasting) was measured by an Accu-Chek Advantage glucometer and Comfort Curve test strips.

**Vasomotor function.** Thoracic aortas were cut into rings (3–4 mm in length) and mounted on stainless steel hooks at optimal resting tension (2 g). Vascular rings were suspended in an organ bath containing oxygenated (95% O2-5% CO2) Krebs solution maintained at 37°C. Tension was increased gradually to reach the optimal level (determined by unpublished observations). Data obtained from each ring were averaged so that each animal represents an n of 1 for statistical comparison. Vessels were initially contracted submaximally (50–70% of maximum) with phenylephrine. After a contractile plateau was reached, relaxation curves were generated by cumulative addition of ACh (10⁻⁶ to 10⁻⁴ M) or sodium nitroprusside (SNP; 10⁻⁹ to 10⁻⁴ M). ACh was used to assess NO-mediated endothelial function, whereas SNP was used to examine endothelium-independent relaxation. To determine whether aging affects other vascular responses, reactivity to several vasoconstrictors (serotonin, KCl, and phenylephrine) was examined.

**Measurement of superoxide.** Measurements of superoxide were made by using lucigenin-enhanced chemiluminescence, as described previously (3). Briefly, aortic rings were placed in a polypropylene tube containing PBS (0.5 ml) and lucigenin (5 μM). The tube was placed in a luminometer that reported relative light units emitted. Background counts were determined from vessel-free preparations and were subtracted from the readings obtained with vessels. Surface area of the vessel was imaged with a video camera and calculated with the use of NIH Image software to normalize superoxide levels.

**Western blot analysis.** Vessels were stored at −80°C or in RNA-later at −20°C. On the day of protein extraction, vessels were thawed and homogenized in extraction buffer and boiled. The protein extract was collected as supernatant after centrifugation (15,000 g for 15 min). Protein concentrations were determined by using a Lowry assay and then adjusted to 2.5 mg/ml with ×2 Laemmli buffer. Protein samples were boiled again, and 20 μg of protein were loaded onto 4–20% gradient SDS-PAGE gels and electrophoresed. Proteins were then transferred from the gel to a nitrocellulose membrane using a semi-dry cell. The nitrocellulose blot was blocked with PBS containing 5% nonfat milk and 1% BSA, incubated with rabbit anti-MnSOD antibody (1:1,000) or anti-CuZnSOD antibody (1:1,000) at 4°C overnight. An horseradish peroxidase-conjugated secondary antibody was added (1:10,000) and incubated at room temperature for 1 h, followed by rinses with PBS. Blots were incubated with chemiluminescent substrate for 1 min, exposed to X-ray film, and developed. Densitometry was performed on exposed film using the Image J program (NIH).

**Statistical analysis.** Data are expressed as means ± SE. Statistical analysis was performed by using repeated-measures ANOVA followed by the Tukey-Kramer post hoc test and Student’s t-test. A P value of ≤0.05 was considered significant.

**RESULTS**

**Animal characteristics.** There was no significant difference in body weight or plasma glucose (nonfasting) between young and old Fischer 344 rats (Fig. 1). Old rats had significantly higher plasma levels of cholesterol than young rats, although the levels of cholesterol were still relatively low (Fig. 1). The finding that plasma cholesterol is increased in aged Fischer 344 rats is consistent with previous studies using this model (9, 40).

**Vasomotor responses.** Relaxation to ACh was impaired in aorta from old Fischer 344 rats (29–31 mo), as reported previously (38, 39) (Fig. 2). Responses to ACh were significantly improved after adenoviral-mediated gene transfer of human ECSOD (Fig. 2, top) In contrast, transfection with ECSOD lacking the HBD had no significant effect on responses to ACh in vessels from old rats (Fig. 2, bottom). Young rats exhibited no significant vascular effects when treated with ECSOD (Fig. 2, top). Responses to SNP were similar in aortas from young and old rats and were not altered by gene transfer of either form of ECSOD (Fig. 3).

There were no significant differences in response to serotonin in aortas from young and old rats (Fig. 4). Constriction of the aorta to phenylephrine was augmented in old rats. Gene transfer of either form of ECSOD had no significant effect on the response to phenylephrine (Fig. 5).

**SOD protein expression.** Total protein was extracted from aortas of young and old rats (n = 4 for both groups). There were no significant differences in the levels of MnSOD and CuZnSOD between groups, but there was a tendency for MnSOD to be increased in the old rats (Fig. 6). These findings are consistent with previous studies (14, 39).

**Superoxide levels.** Superoxide levels, as measured using lucigenin-enhanced chemiluminescence, were significantly greater in aorta from old rats compared with young. After gene transfer of ECSOD, superoxide levels from old rats were similar to those in aorta from young rats, whereas there was no effect in aorta from young rats. Gene transfer of ECSOD lacking the heparin-binding domain had no significant effect on superoxide levels in aorta from old rats (Fig. 7).

**DISCUSSION**

There are three major findings in the present study. First, vascular dysfunction associated with aging is mediated in part...
by increased levels of superoxide. Second, gene transfer of extracellular superoxide dismutase reduces vascular superoxide and improves endothelial function in old rats. Third, beneficial effects of ECSOD in old rats require the HBD of ECSOD.

Vascular dysfunction with aging: role of superoxide. Endothelial dysfunction has been implicated in the pathogenesis and clinical course of many cardiovascular diseases and is associated with increased risk of adverse cardiovascular events (41). Endothelial dysfunction is also a predominant feature of aging (31).

The observation that endothelial vasomotor function is impaired in old rats is consistent with previous studies (11, 23, 30, 35, 38, 39) in animals and humans. We also observed heterogeneity in vascular aging, because the aorta showed evidence of dysfunction at an earlier age than was shown in the carotid artery (data not shown). Other studies have also observed a differential effect of aging on blood vessels. For example, Barton et al. (6) found that vascular function was impaired in the aorta but not the femoral artery of aged (32–33 mo old) Ro-Ro Wistar rats. Several mechanisms may contribute to impairment of vasomotor function with aging. Endothelial dysfunction with aging appears to be related to an increase in ROS, especially superoxide (24, 31, 39). Steady-state levels of superoxide increase in vessels by an increase in production and/or a decrease in degradation of the free radical. Anti-oxidant enzymes include catalase, glutathione peroxidases, and SOD. The three isoforms of SOD maintain super-

Fig. 2. Responses to ACh in aorta from Fischer 344 rats. Top: responses in old (untreated; n = 15) rats after treatment with adeno virus expressing human extracellular SOD (AdECSOD) (n = 14) and young (untreated; n = 17) rats after treatment with AdECSOD (n = 6) to ACh. *P < 0.05 vs. old + ECSOD, young, and young + ECSOD (high doses of ACh). Bottom: responses of old rats after treatment with ECSOD with the heparin-binding domain (HBD) deleted (AdECSODΔHBD) (n = 8). *P < 0.05 vs. young. Data are expressed as means ± SE.

Fig. 3. Responses to sodium nitroprusside (SNP) in aorta from Fischer 344 rats. Responses of old (n = 15) rats after treatment with AdECSOD (n = 14) or AdECSODΔHBD (n = 8) and young rats (n = 17) to the endothelium-independent dilator SNP. Data are expressed as means ± SE.

Fig. 4. Responses to serotonin (5-HT, 5-hydroxytryptamine) in aorta from Fischer 344 rats. Contractile responses of old (n = 6) rats after treatment with AdECSOD (n = 6) or AdECSODΔHBD (n = 3) and young rats (n = 10) to serotonin. Data are expressed as means ± SE.

Fig. 5. Responses to phenylephrine (PE) in aorta from Fischer 344 rats. Responses of old (n = 8) rats after treatment with AdECSOD (n = 9) or AdECSODΔHBD (n = 6) and young rats (n = 8) to PE. *P < 0.01 vs. old, old + ECSOD, old + ECSODΔHBD, and old + ECSOD. Data are expressed as means ± SE.
oxide at relatively low levels under normal conditions, but antioxidant mechanisms may eventually become inadequate during prolonged exposure to increased levels of ROS, resulting in cellular damage (8). In this study, we found a significant increase in superoxide levels in the aorta from aged rats. Because there is much variability between species and tissues, it is not clear whether the increase in oxidative damage observed with age is due to increased production of free radicals and/or a decline in activity of antioxidant systems. Consistent with our findings, several studies (17, 36) suggest that aorta from old rats and mice have protein levels of CuZnSOD and MnSOD comparable to or slightly higher than young animals. Effects of age on vascular expression of ECSOD are not clear (1, 22, 36).

Relaxation to SNP was not affected by age in these experiments. Whereas ACh stimulates endothelial NO synthase (eNOS) to produce NO that must diffuse to underlying vascular muscle to elicit relaxation, SNP acts as a direct NO donor. Our results with SNP suggest that the vascular dysfunction observed with aging is primarily due to a defect in the endothelium rather than smooth muscle.

A decrease in expression or activity of eNOS during aging could potentially contribute to impaired vasorelaxation. Several investigators (1, 12, 17), however, report a paradoxical increase in expression and/or activity of eNOS with increasing age, whereas others (4, 14, 37) have observed a decrease. If, indeed, eNOS expression declined with age, it seems likely that gene transfer of ECSOD would not restore vascular function. Our observation that ECSOD improved vasomotor responses in old rats suggests that production of NO may be relatively normal, and bioavailability of NO is decreased due to inactivation by superoxide.

Reaction of NO with superoxide may increase levels of ONOO\(^-\). ONOO\(^-\) is also responsible for the nitration of tyrosine residues in certain enzymes, specifically superoxide-reducing enzymes, thereby reducing enzyme activity and potentially further increasing superoxide levels (39). ONOO\(^-\) is known to inactivate MnSOD and CuZnSOD (2, 27). Because of the structural homology of CuZnSOD and ECSOD, we speculate that ECSOD activity may also be decreased via inactivation by ONOO\(^-\). We also suggest that inactivation of SOD by ONOO\(^-\) may contribute to increases in intracellular levels of superoxide, with a concomitant increase in SOD expression with aging (17, 36). The relative contribution of decreased SOD activity versus increased production of superoxide from sources such as vascular NAD(P)H oxidase is still not clear.

Protective effect of ECSOD in aged vessels. Within the vessel wall, ECSOD is localized primarily between the endothelium and smooth muscle and constitutes a large portion of total SOD activity in the vessel wall (15). Our laboratory (13, 26) has recently demonstrated that vasomotor function can be improved by gene transfer of ECSOD to blood vessels in rats with hypertension and inflammation.

The present data suggest that vascular dysfunction with aging in rat aorta is mediated, in part, by increased levels of superoxide. Gene transfer of ECSOD restored superoxide levels to normal and improved vascular function in the aorta of old rats. Conversely, gene transfer of ECSOD without its HBD produced no improvement of vascular function and no decrease in superoxide levels. Current data and previous results from the laboratory suggest that the HBD is necessary for normal function of ECSOD (13). Because of its location, it has been suggested that ECSOD is an important determinant of NO
levels by preventing destruction of NO released from the endothelium (18).

In conclusion, we have confirmed that aging is characterized by a decrease in endothelium-dependent relaxation. We present the first evidence that vascular function in aged rats is significantly improved after gene transfer of ECSOD; in contrast, gene transfer of ECSOD lacking the HBD has no effect on vascular function or increased levels of superoxide in this model of aging. These data demonstrate the importance of the HBD of ECSOD during aging.

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GRANTS

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