Gene transfer of extracellular SOD to the penis reduces $O_2^-$ and improves erectile function in aged rats

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Departments of 1Urology and 2Pharmacology, Tulane University School of Medicine, New Orleans, Louisiana 70112; 3Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322; 4Biomolecular Research Annex, University of Central Florida, Orlando, Florida 32816; and 5Division of Cardiology, Department of Medicine, The Johns Hopkins Hospital, Baltimore, Maryland 21287

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Bivalacqua, Trinity J., Jeffrey S. Armstrong, John Biggerstaff, Asim B. Abdel-Mageed, Philip J. Kadowitz, Wayne J. G. Hellstrom, and Hunter C. Champion. Gene transfer of extracellular SOD to the penis reduces $O_2^-$ and improves erectile function in aged rats. Am J Physiol Heart Circ Physiol 284: H1408–H1421, 2003. First published December 27, 2002; 10.1152/ajpheart.00770.2002.—Increased superoxide anion ($O_2^-$) may contribute to vascular dysfunction in aging. In aged cavernosal tissue, lucigenin-enhanced chemiluminescence demonstrated a threefold increase in superoxide formation, and the oxidative fluorescent probe hydroethidine indicated higher superoxide levels throughout the aged penis. This increase in superoxide was associated with impaired cavernosal nerve-mediated and agonist-induced erectile responses, increased nitrotyrosine staining, and lower cGMP levels, but no compensatory change in cavernosal extracellular (EC)-superoxide dismutase (EC-SOD) mRNA or protein. In vivo adenosine (Ad) gene transfer of EC-SOD to the penis resulted in higher expression of EC-SOD mRNA, protein, SOD activity, cGMP levels, and lower nitrotyrosine staining. Transfection with AdCMVEC-SOD resulted in a significant increase in erectile response to cavernosal nerve stimulation, ACh, and zaprinast to a magnitude similar to young rats. These data provide evidence in support of the hypothesis that erectile dysfunction associated with aging is related in part to an increase in cavernosal $O_2^-$ formation. Gene-transfer of EC-SOD reduces superoxide formation and restores age-associated erectile function and may represent a novel therapeutic target for the treatment of erectile dysfunction.

Gene therapy; aging; nitric oxide; erectile dysfunction

MALE ERECTILE DYSFUNCTION (ED) has been defined as a persistent inability to maintain penile erection sufficient for normal sexual activity. Data from the Massachusetts Male Aging Study indicate that the prevalence of ED is 39% in 40-yr-old men and 67% in 70-yr-old men (18). Although the development of ED is multifactorial in nature, it is typically associated with vascular diseases and risk factors such as atherosclerosis, hypertension, diabetes mellitus, and cigarette smoking (4, 18, 35). Normal erectile function is defined by a delicate balance between vasoconstrictor and vasodilatory systems that determine the tone of corpora cavernosal smooth muscle of the penis. The nitric oxide (NO)/cGMP system is the principal mediator of penile erection and NO is synthesized by neuronal NO synthase (nNOS) and the endothelial isoform of NOS (eNOS) (4, 11, 40). Aging is recognized to alter endothelial cell function, and the decrease in age-related erectile function has been attributed to reductions in NOS activity, impaired endothelial-dependent smooth muscle relaxation, and diminished NO bioavailability (13, 26, 29).

Oxidative stress impairs vascular function. Superoxide anion ($O_2^-$) is involved in oxidative stress and likely contributes to vascular dysfunction observed in hypertension, atherosclerosis, diabetes mellitus, as well as in the normal aging process (12, 24, 42). The antioxidant superoxide dismutase (SOD) plays an important role in protection against $O_2^-$ radicals. SOD represents a major cellular defense against superoxide and peroxynitrite formation, which has direct toxic effects, thus contributing to tissue injury, alterations in vascular tone, and organ dysfunction (43). Three SOD isozymes have been identified: cytosolic CuZn-SOD, mitochondrial Mn-SOD, and extracellular (EC)-SOD (EC-SOD), which is also a CuZn-containing enzyme (22, 23). Given its location, EC-SOD is hypothesized to play a critical role in modulating the redox state of the vascular interstitial and thereby prevents the pathophysiological effects of $O_2^- \cdot$ in the vasculature. Conceptually, the increased levels of $O_2^- \cdot$ in the endothelium and cavernosal smooth muscle may cause the decrease in NO synthesis observed in the aging penis. Thus reducing superoxide levels may be an important method to preserve NO bioactivity in the penile vasculature. More-
over, aging is associated with decreased NO bioavailability and responsiveness to endothelium-dependent vasodilator stimuli in the corpus cavernosum, and the role $O_2^-$ plays in mediating this decreased responsiveness has not been established. Therefore, the present study was undertaken 1) to investigate the levels of expression of $O_2^-$ in the penises of young and aged rats, and 2) to examine the effects of adenoviral gene transfer of EC-SOD to the penis to determine the consequence of overexpression of EC-SOD on $O_2^-$ levels, erectile function, and endothelium-dependent relaxation in the penile vascular bed of the aged rat.

**METHODS**

**Adenovirus vectors.** Two replication-deficient recombinant adenoviruses (Ad) were used for the gene transfer, both driven by a cytomegalovirus (CMV) promoter: 1) nuclear-targeted β-galactosidase (AdCMVβgal) and 2) extracellular SOD (AdCMVEC-SOD) generated by standard methods at the University of Iowa Gene Transfer Vector Core Laboratory (37, 38). Human EC-SOD was cloned by blunt-end ligation into plasmid (p)AdCMV4. The resulting plasmid and adenovirus backbone sequences restricted of E1 were transfected into human embryonic kidney-293 (HEK-293) cells, and plaques were isolated and amplified for analysis of EC-SOD expression. Recombinant adenoviruses were triple plaque purified to assure that viral suspensions were free of wild-type virus, and virus titers were determined by plaque assay on HEK-293 cells. After purification, the virus was suspended in PBS with 3% sucrose and kept at −70°C until use.

**Oxidative fluorescent microphotography.** Hydroethidine (Molecular Probes), an oxidative fluorescent dye, was used to evaluate $O_2^-$ levels in situ, as described previously (36, 39). Hydroethidine is freely permeable to cells and, in the presence of $O_2^-$, is oxidized to red fluorescent ethidium bromide (EtBr), where it is trapped by intercalation with DNA. EtBr is excited at 488 nm and has an emission spectrum of 610 nm. In cell-free assays, $H_2O_2$ and NO do not react with hydroethidine to increase EtBr fluorescence. This method provides sensitive detection of $O_2^-$ levels in situ. Penises were removed from the young and aged animals and embedded in optimum cutting temperature (OCT) compound (Sakura Fine-tek), and 40-μm-thick sections were placed on a glass slide. Hydroethidine (2 × 10⁶ mol/l) was topically applied to each tissue section and coveredslipped. Slides were incubated in a light-protected, humidified chamber at 37°C for 30 min. Images were obtained with a Bio-Rad MRC-1024 laser scanning confocal microscope. Laser settings were maintained constant for acquisition of images from both the young and aged animals. Photomicrographs of both young and aged animals were obtained, and the intensity and localization of the oxidized hydroethidine, which reflect superoxide formation, could be observed and compared between the two groups.

**In vivo gene delivery to corpora cavernosa.** Four groups of rats were utilized in the following study: 1) young rats (12 wk), 2) aged rats (80 wk) transfected with AdCMVβgal, 3) aged rats (80 wk) transfected with AdCMVEC-SOD, and 4) young rats (12 wk) transfected with AdCMVEC-SOD. Twelve- and eighty-week-old male Brown Norway rats (young, 200–300 g; aged, 400–525 g) were purchased from the National Institutes of Health (NIH)/National Institutes of Aging (NIA) colony (Harlan Sprague-Dawley), maintained under controlled temperature and lighting, and treated according to NIH regulations. Brown Norway rats were chosen for this study because this species exhibits a combination of primary and secondary testicular failure that more closely resembles human reproductive aging and erectile function (27). The aged rats were anesthetized with pentobarbital sodium (30 mg/kg ip) and placed in a supine position on a temperature-regulated surgical table. With the use of a sterile technique, the penis was exposed. Twenty microliters of recombinant (3%, sucrose in PBS)), AdCMVβgal (1 × 10⁸ parts/ml), or AdCMVEC-SOD (1 × 10⁸ parts/ml) were injected into the corpus cavernosum with a 30-gauge needle attached to a microliter syringe, as previously described (3, 6, 14). Immediately before instillation, blood drainage via the dorsal veins was halted by circumferential compression at the base of the penis with an elastic band. Compression was released 2–5 min after injection of 20 μl of the vehicle/virus. Rats did not show any overt signs of systemic (fever, dyspnea, and tachycardia) or local (purulent discharge, erythema, and edema) infection when observed any day after transfection.

**Detection of $O_2^-$ and SOD activity.** $O_2^-$ levels were measured using lucigenin chemiluminescence, as previously described (37, 39). Rat penises were homogenized in a buffer containing 50 mM Tris, 50 mM NaCl, 1 mM EDTA, 25% sucrose, and p-chloromercuribenzoate (1 mM PMSF, 1 μg/ml of aprotinin, bestatin, and leupeptin). Penile extracts were subjected to 100,000 g ultracentrifugation for 45 min to separate the membrane and cytosolic fractions. The supernatant or the particulate fraction, which had been resuspended in 250 μl of buffer, was used to examine oxidase activity of these subcellular fractions. Scintillation vials containing 2 ml of Krebs-HEPES buffer with 5 μM lucigenin were placed into a scintillation counter switched to the out-of-coincidence mode. After dark adaptation, background counts were recorded and aliquots of the cytosolic and membrane fractions were added to the vial. The chemiluminescence that occurred over the ensuing 5 min in response to the addition of either NADH or NADPH (100 μM) was recorded. The homogenates alone, without addition of NADH or NADPH, gave only minimal signals. Neither NADH nor NADPH evoked lucigenin chemiluminescence in the absence of homogenate. Scintillation counts were recorded every 2 min for 15 min, and the respective background counts were subtracted. Values were standardized to the amount of protein present, and lucigenin counts were expressed as counts per minute per milligram of protein. To measure total SOD activity, rat cavernosal homogenate was studied as previously described with the use of a commercially available technique (Calbiochem), and results were compared per milligram of protein (46). This assay is based on the SOD-mediated increase in the rate of auto-oxidation of a chromogenic reagent (R1) in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. A second proprietary reagent, R2, eliminates major interferences normally caused by mercaptans such as glutathione in samples via a rapid alkylation reaction (46).

**Expression of β-galactosidase in cavernosal tissue.** One to ten days after adenovirus administration of vehicle and AdCMVβgal, the aged rats were euthanized with an overdose of pentobarbital sodium (80 mg/kg ip), and the penile shafts were removed. Expression of β-galactosidase was evaluated by measurement of β-galactosidase activity in cavernosal tissue samples using a β-galactosidase reporter gene assay (Galacto-Light Plus Tropix; Bedford, MA) and X-Gal staining, as previously described (3, 6, 14). Briefly, corpus cavernosal tissue was minced into small pieces with a scalpel and placed in lysis buffer for 15 min (75 μl per sample; 0.2% Triton X-100 and 100 nmol potassium phosphate; pH 7.8). The samples were centrifuged (12,000 revolutions/min for 10 min), and the supernatant was removed. Originals and dilu-
tions (1:10 and 1:100) were prepared in duplicate for each tissue lysate. Aliquots of tissue lysate were assayed for β-galactosidase activity with 3-(4-methoxyphenyl)-2,4-dioxetane-3,2′-tricyclo[3.3.1^3,7]decane-1-yl)phenyl-β-D-galactopyranoside. Light emission was measured with a luminometer (Luminoskan RS, Labsystems) and calibrated with a standard curve generated with the use of purified Escherichia coli β-galactosidase. Protein concentrations of the samples were determined (Pierce Protein Assay, Pierce Endogen), and normalized β-galactosidase activity was expressed as relative light units of β-galactosidase per milligram of protein. For histochemical analysis of β-galactosidase localization, vehicle- and AdCMVβgal-transfected animals were euthanized, and the penile shafts were cut in 2-mm sagittal sections, which were then incubated in X-Gal (Sigma) stain (PBS) composed of 20 mmol/l K₄Fe(CN)₆·3 H₂O, 20 mmol/l K₃Fe(CN)₆, 2 mmol/l MgCl₂, and 1 mg/ml in DMSO of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for 24 h at room temperature, rinsed in PBS, and postfixed in 7% buffered formalin for 6 h, overlaid with OCT compound, and frozen in liquid nitrogen. Cryostat sections (30 μm) were mounted on poly-L-lysine-coated slides and counterstained with eosin Y. The penile sections were examined for positive β-galactosidase staining (blue nuclei) by light microscopy.

Western blot analysis of EC-SOD. Expression of EC-SOD and α-actin in aged rat cavernosal tissue was assessed one day after intracavernosal injection of AdCMVβgal and AdCMVEC-SOD and a younger cohort of animals. The supernatant was mixed with an equal volume of 2% SDS-1% β-mercaptoethanol and fractionated using 8% SDS-PAGE (70 μg/lane). Proteins were transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Life Sciences) by semidry electroblotting. The membranes were blocked 1 h at room temperature with blotto-Tween (5% nonfat dry milk and 0.1% Tween 20) and incubated with a primary monoclonal rabbit anti-EC-SOD (1:5,000; Johns Hopkins Hospital) and primary monoclonal rabbit anti-α-actin (1:10,000; Santa Cruz Biotechnology). Bound antibody was detected with labeled goat anti-rabbit IgG secondary antibody (1:20,000; Santa Cruz Biotechnology) and visualized with enhanced chemiluminescence. The Western blot technique for determination of EC-SOD has been described earlier (19, 25).

Northern blot analysis of EC-SOD. Total RNA was isolated from cavernosal tissue of young rats and aged rats transfected with AdCMVβgal and AdCMVEC-SOD using Tri-reagent (Molecular Research Center), as previously described (5, 6). For Northern blot analysis, 20 μg of total RNA from each sample were separated on 1% formaldehyde agarose gels, identical loading of the gel lanes was confirmed by comparing the EthBr staining of the GAPDH bands. Blotting, cross linking, and hybridization were performed with cDNA for EC-SOD, as previously described (9).

Immunohistochemical localization of nitrotyrosine. Immunohistochemical localization of nitrotyrosine was performed in the penises of young rats and one day after transfection with AdCMVβgal and AdCMVEC-SOD in the aged rat. The penile shaft was separated from the crura, cut in cross sections, fixed in 10% formalin, and paraffin embedded. Endogenous peroxidases were quenched with 5% H₂O₂, and sections were washed with PBS. Nonspecific binding of IgG was blocked using normal horse serum, 1:50 of 0.1% bovine serum albumin in PBS. The sections were incubated with a rabbit monoclonal antibody for antinitrotyrosine (1:3,000; Upstate Biotechnology), washed, and then incubated for an additional 30 min with a biotinylated secondary antibody. After a 30-min incubation with ABC horseradish peroxidase (DAKO), the substrate (DAB, Vectastain, Vector Laborato-

AJP-Heart Circ Physiol • VOL 284 • APRIL 2003 • www.ajpheart.org

H1410 ERECTILE DYSFUNCTION AND O₂⁻ IN AGING
ensure a stable baseline. Injection of 50 μl of the saline vehicle had no significant effect on intracavernosal pressure.

Statistical analysis. The data are expressed as means ± SE and were analyzed using a one-way analysis of variance with repeated measures and Newman-Keuls post hoc test for multiple group comparisons (Statview, Abacus Concepts). A P value of <0.05 was used as the criterion for statistical significance.

RESULTS

Confocal microscopic examination of $O_2^-$ in young and aged penises. Tissue sections from the penis of young and aged rats were evaluated for the presence of $O_2^-$, and these data are summarized in Fig. 1. After incubation with hydroethidine, the aged rat penis had a markedly higher level of expression of EtBr fluorescence compared with the younger animals, indicating higher concentrations of $O_2^-$ in the aging penis. The fluorescence was localized to the endothelium and corpus cavernosal smooth muscle (Fig. 1).

Cavernosal superoxide levels and SOD activity. To quantify $O_2^-$ levels in the penises of young and aged rats, $O_2^-$ concentration was measured by using the lucigenin-enhanced chemiluminescence assay (39), and these data are presented in Fig. 2. Cavernosal tissue obtained from increasing ages (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 mo; n = 5 for each month) of rats demonstrated a significant age-dependent increase in $O_2^-$ concentration with a regression analysis of $R^2 = 0.7198$ (Fig. 2A). There was no age-dependent change in total SOD activity ($R^2 = 0.0.01292$; Fig. 2B). Cavernosal tissue from aged animals (80 wk) had significantly higher levels of $O_2^-$ generation compared with levels in cavernosal tissues from younger animals (12 wk; Fig. 2C). There was no significant difference in SOD activity in young (12 wk) rats, aged (80 wk) rats treated with vehicle, or aged rats transfected with the reporter gene AdCMVβgal (Fig. 2D). Cavernosal tissue from aged rats transfected with AdCMVEC-SOD showed an approximate fivefold increase in SOD activity compared with activity in the other groups (Fig. 2D).

$\beta$-Galactosidase activity in cavernosal tissue. One day after intracavernosal administration of vehicle or AdCMVβgal, $\beta$-galactosidase localization was determined by light microscopy (Fig. 3A). The time course and magnitude of $\beta$-galactosidase activity using chemiluminescence is shown in Fig. 3B. Cavernosal tissue from aged rats transfected with vehicle showed minimal $\beta$-galactosidase expression, whereas cavernosal tissue of AdCMVβgal-transfected aged rats had significantly higher $\beta$-galactosidase expression and quantitated activity (Fig. 3B). $\beta$-Galactosidase activity was similar in the vehicle and AdCMVEC-SOD-transfected aged rats (data not shown). Expression of $\beta$-galactosidase peaked 1 day after transfection with AdCMVβgal and remained at peak levels for 3–5 days at which time expression began to decay to baseline levels, such that at 14 days posttransfection, there was no measurable expression of $\beta$-galactosidase (Fig. 3B).

Western blot analysis of EC-SOD. EC-SOD protein levels were measured in cavernosal tissue of both young and aged rats 1 day after intracavernosal administration of AdCMVβgal and AdCMVEC-SOD, and these data are summarized in Fig. 4A. Cavernosal EC-SOD protein levels were not significantly different in the aged rats (lane 2, Fig. 4A) compared with levels in young rats (lane 1, Fig. 4A). One day after transfection with AdCMVEC-SOD, EC-SOD protein levels (32 kDa) in cavernosal tissue were significantly higher in aged rats (lane 3, Fig. 4A) compared with levels in aged rats transfected with AdCMVβgal (lane 2; Fig. 4A). When EC-SOD levels were analyzed by densitometry and expressed per milligram of protein, EC-SOD protein levels were significantly higher in aged rats after transfection with AdCMVEC-SOD compared with EC-SOD protein levels in aged rats treated with AdCMVβgal and when compared with EC-SOD levels in young animals (Fig. 4A).

Northern blot analysis of EC-SOD. EC-SOD RNA levels were measured in cavernosal tissue of young...
rats and 1 day after intracavernosal administration of AdCMVβgal and AdCMVEC-SOD in aged rats. These data are summarized in Fig. 4B. Cavernosal EC-SOD RNA levels were not significantly different in aged rats transfected with AdCMVβgal (lane 2, Fig. 4B) compared with the young rats (lane 1, Fig. 4B). One day after transfection with AdCMVEC-SOD, EC-SOD RNA levels in cavernosal tissue were significantly higher (lane 3, Fig. 4B) when compared with levels in aged rats transfected with AdCMVβgal (lane 2, Fig. 4B). When EC-SOD levels were analyzed by densitometry and expressed as a ratio of SOD signal divided by GAPDH signal, EC-SOD levels were significantly higher in aged rats after transfection with AdCMVEC-SOD compared with EC-SOD RNA levels in aged rats treated with AdCMVβgal and compared with EC-SOD levels in the young animals (Fig. 4B).

**Cavernosal cGMP levels.** Cavernosal tissue cGMP concentrations were measured in young and in aged rats treated with vehicle, AdCMVβgal, and AdCMVEC-SOD, and these data are summarized in Fig. 5. Cavernosal cGMP levels were significantly lower in the aged rats compared with levels in young rats (Fig. 5). Gene transfer of EC-SOD in the aged rat penis resulted in cavernosal cGMP concentrations that were significantly higher compared with cGMP levels in aged rats transfected with AdCMVβgal (Fig. 5). There was an approximate twofold increase in cGMP levels in cavernosal tissue from animals transfected with AdCMVEC-SOD that was similar to levels found in younger rats. cGMP levels were similar in aged rat cavernosal tissue transfected with vehicle and with AdCMVβgal (data not shown).

**Immunohistochemical localization of nitrotyrosine.** To measure the expression of nitrotyrosine, a specific marker of peroxynitrite formation, in young and aged rats after adenoviral transfection of the penis, immunohistochemical localization of nitrotyrosine was performed 1 day after transfection with AdCMVβgal and AdCMVEC-SOD and these data are summarized in
Fig. 6. Immunohistochemical staining of nitrotyrosine was markedly higher in the endothelium and cavernosal smooth muscle of aged rats transfected with AdCMVβgal compared with the staining in young rats (Fig. 6). There was no difference in penile nitrotyrosine staining in aged animals transfected with AdCMVβgal and vehicle (data not shown). However, animals receiving intracavernosal injection of AdCMVEC-SOD had lower nitrotyrosine expression in the endothelium and smooth muscle of the corpus cavernosum, suggesting that EC-SOD gene transfer reduced peroxynitrite formation (Fig. 6).

Influence of age on erectile response in rat. The effect of cavernosal nerve stimulation and intracavernous injection of ACh on erectile function in vivo was determined in three separate ages (12, 80, and 110 wk) of Brown Norway rats. There was an age-dependent decrease ($P < 0.05$) in erectile function to both cavernosal nerve stimulation and intracavernous injection of ACh (Fig. 7).

Effect of AdCMVEC-SOD on erectile responses in aged rat. The effect of cavernosal nerve stimulation and pharmacological agents on erectile function in vivo was measured to evaluate the physiological consequence of overexpression of the EC-SOD gene transferred to the corpus cavernosum of aged rats. There was a significantly lower voltage-dependent cavernosal nerve-induced erectile response in aged animals when compared with responses in younger rats (Fig. 8). The magnitude of the increase in cavernosal pressure in response to cavernosal nerve stimulation in aged rats transfected with AdCMVβgal was significantly lower than the younger rats, whereas rats transfected with the gene encoding EC-SOD had a larger response to cavernosal nerve stimulation that was similar to the response obtained in young rats (Fig. 8A). The magni-
tude of erectile responses to cavernosal nerve stimulation in vehicle-treated aged rats and those treated with AdCMVβgal was similar (data not shown). The increase in cavernosal pressure in the AdCMVEC-SOD-transfected group was similar to the response in younger control rats at all voltage settings (2.5, 5, and 7.5) (Fig. 8B). Responses were reproducible 30 min after the initial stimulation (data not shown).

The influence of AdCMVEC-SOD transfection on erectile responses to the endothelium-dependent vasodilator ACh and the selective PDE5 inhibitor zaprinast was investigated, and these data are summarized in Fig. 9. When injected intracavernosally, ACh (10 and 30 μg/ml) and zaprinast (30 and 100 μg/ml) induced dose-related increases in cavernosal pressure (Fig. 9). There was a significantly smaller response to both ACh and zaprinast in aged animals transfected with AdCMVβgal compared with responses in the young rats (Fig. 9). Increases in cavernosal pressure were similar in animals transfected with vehicle and AdCMVβgal (data not shown). In aged rats transfected with AdCMVEC-SOD the increase in cavernosal pressure in response to intracavernosal injections of ACh (10 and 30 μg/ml) and zaprinast (30 and 100 μg/ml) were significantly greater in magnitude than responses in aged rats treated with AdCMVβgal and were similar to responses seen in younger animals (Fig. 9).

Effect of AdCMVEC-SOD on erectile responses in young rat. The effect of overexpression of EC-SOD in the corpus cavernosum of young rats was investigated to determine whether EC-SOD transfection had a specific effect on diminished erectile responses in aged rats or if it enhanced normal erectile function in young rats. One day after AdCMVEC-SOD transfection to the young rat, no change in erectile function, as determined by cavernosal nerve stimulation and intracavernosal administration of ACh, was observed compared with responses in age-matched control animals (Fig. 10).

There was no significant difference in resting mean intracavernosal pressure and systemic arterial pressure among the four experimental groups of rats (Table 1).
DISCUSSION

The results of the present study demonstrate an increase in $O_2^•$ in the endothelium and smooth muscle of the corpus cavernosum in the aged rats and were age dependent. This increase in $O_2^•$ generation is associated with decreased NO synthesis in the penis, thus impairing endothelial-dependent smooth muscle relaxation, resulting in reduced erectile function. These data indicate a physiological role for $O_2^•$ in modulating erectile function by reducing bioavailability of NO, thus decreasing cGMP levels. Moreover, the results of the present study demonstrate for the first time that direct injection of an adenovirus encoding the EC-SOD gene to the aged rats penis decreases $O_2^•$ and nitrotyrosine immunostaining. Gene transfer of EC-SOD was associated with increased cavernosal EC-SOD protein, EC-SOD RNA, SOD activity, and cGMP concentrations, as well as physiologically measurable improvement in erectile function. These data suggest that the EC-SOD transgene has biological activity in the rat penis and reverses the erectile and endothelial dysfunction seen in the aged rat.

The process of biological aging is multifactorial in nature and is dependent on several factors, including but not limited to, metabolic rate, genetics, lifestyle, and environmental issues (41). The concept that free radical damage plays a role in mediating degenerative changes of aging was originally postulated by Harman (31) in 1956, in which it was hypothesized that progressive aging is associated with increased amounts of oxidatively modified biomolecules resulting from free radical reactions. Since the development of this theory, several lines of evidence provide support for the concept that free radical damage is an important component of the aging process. More recently, oxidative damage to the vasculature has been postulated to be a caused by $O_2^•$ leading to oxidative stress, which plays

Fig. 5. Bar graph showing cGMP levels in cavernosal tissue of young and aged rats transfected with AdCMVβgal or AdCMVEC-SOD (n = no. of experiments). *P < 0.05, cGMP levels are significantly different compared with young rats. **P < 0.05, cGMP levels are significantly different compared with aged rats transfected with AdCMVβgal.

Fig. 6. Representative immunohistochemical localization of nitrotyrosine, a marker of peroxynitrite formation, in rat corpus cavernosum of young and aged rats transfected with AdCMVβgal or AdCMVEC-SOD. Nitrotyrosine staining (dark brown staining) was localized in the endothelium and corpus cavernosum smooth muscle cells of aged rats but was markedly lower in aged rats transfected with AdCMVEC-SOD. These results are typical of five independent observations. Magnification ×100.
in role in the natural aging process (2, 30). With regard to the cardiovascular system, it has been shown that the myocardium of older animals is more susceptible to oxidant damage when compared with young animals (1, 20). Although O$_2^\cdot$/H$_2$O$_2$/H$_8$S formation has been implicated in decreased tolerance of the cardiovascular system to free radical damage, little if anything is known about the role of O$_2^\cdot$/H$_2$O$_2$/H$_8$S in mediating the natural decrease in erectile function with advancing age.

As men age, a reduction in libido and a significant decline in erectile function occur. The constitutive forms of NOS, eNOS and nNOS, are the principal mediators of cavernosal smooth muscle relaxation (10, 32). Most experts believe this reduction in erectile function can be attributed to endogenous decreases in the formation of NO in the corpus cavernosum. We (6, 14) have shown that overexpression of eNOS by adenoviral gene transfer to the penis of aged rats restores erectile function, suggesting that decreased production or bioavailability of NO and a resulting reduction in cGMP formation in the penis play a significant role in mediating age-associated erectile dysfunction. O$_2^\cdot$ reacts with NO to form peroxynitrite, which is more toxic than its precursors. This reaction does not allow NO to perform its role in vasodilation and penile erection. The results of the present study show that O$_2^\cdot$ formation is higher in aged rat penises. Although there is an increase in O$_2^\cdot$ formation, there is no change in total SOD activity and EC-SOD mRNA or protein, as measured by Northern and Western analysis, such that there is an imbalance of O$_2^\cdot$ generation and inactivation in the penile vasculature of the aged rat. It is because of this imbalance in O$_2^\cdot$ formation and EC-SOD expression that has led to the hypothesis that increasing EC-SOD expression might be beneficial in limiting O$_2^\cdot$ formation. In rats transfected with AdCMVEC-SOD, expression of EC-SOD mRNA and protein was significantly higher compared with values in rats transfected with the reporter gene. Transfection with AdCMVEC-SOD resulted in a significant reduction in O$_2^\cdot$ formation in the aged rat penis, suggesting that the transgene was biologically active.

O$_2^\cdot$/H$_2$O$_2$/H$_8$S and NO contain unpaired electrons in their outer orbitals and undergo an extremely rapid, diffusion-limited, radical/radical interaction, leading to the formation of peroxynitrite. Although the half-life of peroxynitrite is short, it is a potent stimulator of tyrosine nitrosylation (42, 44). Tyrosine nitrosylation has been implicated in a number of disease states, including diabetes, atherosclerosis, and neurodegenerative disorders (45). Immunohistochemical localization of nitrotyrosine, a marker of peroxynitrite formation, showed a marked increase in the aged rat penis compared with younger rats and is consistent with previous observations in the aged rat penis (21). The observation that nitrotyrosine staining is markedly reduced in aged rats after EC-SOD transfection may be interpreted to suggest that less peroxynitrite is formed in the penis after EC-SOD gene transfer may be interpreted to suggest that less peroxynitrite is formed in the penis after EC-SOD transfection. These data imply that reduced endothelium-dependent relaxation and erectile dysfunction associated with aging is in part due to increased NO inactivation by O$_2^\cdot$ decreasing NO bioavailability. This finding is consistent with the knowledge that NO is inactivated by superoxide. The evidence for a direct association between peroxynitrite formation and age-associated penile vascular dysfunc-
Fig. 8. A: representative time course of changes in intracavernosal pressure during cavernosal nerve stimulation (5 V) for young rats (left), aged rats transfected with AdCMVβgal (middle), and aged rats transfected with AdCMVEC-SOD (right). B: bar graph showing the increase in intracavernosal pressure in response to cavernosal nerve stimulation in young rats and aged rats transfected with AdCMVβgal or AdCMVEC-SOD. In vivo erection experiments were conducted 1 day after transfection with adenoviruses (n = no. of experiments); *P < 0.05, response significantly different compared with young rats; **P < 0.05, response significantly different compared with aged rats transfected with AdCMVβgal.
tion is further supported by the observation that overexpression of EC-SOD corrects age-related erectile dysfunction. This may have long-term implications in that an age-related reduction in NO released from the cavernosal nerve and peroxynitrite-mediated nitrosylation of cavernosal nervous tissue may play a role in reducing erectile function in aging. SOD limits the formation of peroxynitrite by reducing $O_2^-/H_2O_2$ levels decreasing the amount available to react with NO, thus increasing NO, which can exert its relaxing effect in the corpus cavernosum by increasing cGMP levels through its interaction with soluble guanylate cyclase to produce an erectile response. Additionally, the limiting of superoxide formation prevents damage by peroxynitrite or its conversion to hydroxyl radical. It is also possible that limiting tyrosine nitrosylation may improve cavernosal nerve NO release, thus enhancing erectile function. It is important to note that the direct effect of peroxynitrite and tyrosine nitrosylation on cavernosal nerve function has not been studied at this time and therefore remains speculative.

Superoxide radical production is increased in the cavernosal tissue of hypercholesterolemic rabbits and leads to a functional impairment of endothelium-dependent cavernosal smooth muscle relaxation in vitro (34). Additionally, NO-mediated cavernosal smooth muscle relaxation is impaired in organ bath studies using diabetic rabbit cavernosal tissue; an effect that was reversed with SOD treatment (33). These data provide evidence that in certain disease, such as hypercholesterolemia and diabetes, superoxide radicals are increased, possibly by the upregulation of NADPH oxidase. Collectively, these studies document a potential role for superoxide radicals in the pathophysiology of male ED. Our results support previous studies by providing evidence that overexpression of $O_2^-$, as evidenced by the increase in penile lucigenin-enhanced chemiluminescence and hydroethidine staining, can limit erectile function in vivo. The results of the present study extend these findings by suggesting that there is a normal, age-related increase in $O_2^-$ formation presumably caused by an upregulation of NADPH oxidase. Moreover, these data demonstrate that gene transfer of EC-SOD augments erectile responses to both cavernosal nerve stimulation, as well as to the direct injection of ACh and zaprinast in the aged rat. The observation that cGMP levels are increased in rats transfected with EC-SOD implies greater NO availability and that instead of forming peroxynitrite, NO is available to stimulate soluble guanylate cyclase (28). Of note, the beneficial effect of adenoviral transfection of EC-SOD was age specific and was not observed in young animals with normal erectile function, suggesting that this therapy is specific for pathophysiological situations in which $O_2^-$ levels are overexpressed.

ACh interacts with muscarinic receptors on the cavernosal endothelium to primarily release NO, and the results of the present study show that with aging, the erectile response to ACh is reduced. This reduction may be multifactorial in nature and may reflect changes in NOS expression, availability of NO substrate, the scavenging of NO, or changes in guanylate cyclase activity and downstream mechanisms. Our results may be interpreted to suggest that bioavailability of NO, once released, may be an important factor in aging because transfection with EC-SOD resulted in erectile responses that were similar to those observed in young animals. Alternatively, because tyrosine nitrosylation is markedly reduced by AdCMVEC-SOD, the beneficial effect of EC-SOD transfection may result from a reduction in effector system function caused by
peroxynitrite. Similar reductions in response to the PDE5 inhibitor zaprinast were observed in aged animals and may reflect alterations in enzyme activity caused by tyrosine nitrosylation. Alternatively, because cGMP levels were reduced in aged rats, the inhibition of PDE5 was not optimal because the enzyme did not have sufficient substrate, and inhibition of the enzyme did not increase cGMP levels high enough to induce significant erectile responses in the aged rat.

Sildenafil citrate (Viagra), a selective PDE5 inhibitor, inhibits the hydrolysis of cGMP in the corpus cavernosum, thereby increasing cavernosal smooth muscle relaxation and prolonging penile erection (8). Despite the general overall success of oral agents, such as sildenafil, there are still patients who do not respond to this pharmacological agent. Therefore, there are still cases of incomplete response. There are also side effects and contraindications for the use of oral PDE5 inhibitors for the treatment of ED. Gene therapy for ED offers a distinct advantage in that the anatomy of the penis and its own external circulation, allows a gene to be transferred and localized in the organ and thereby lessening the risk of systemic side effects (16). By identifying specific genes crucial to normal erectile physiology, a specific gene therapy approach may be useful for the treatment of erectile dysfunction.

In conclusion, the enhanced generation of O$_2^{-}$ and peroxynitrite may play an important role in the pathophysiology of age-related ED. Our results demonstrate that adenoviral-mediated gene transfer of the EC-SOD gene can increase EC-SOD protein, mRNA, cGMP formation, and SOD activity and thus reduce the formation of O$_2^{-}$ and peroxynitrite in cavernosal tissue of the aged rat. Moreover, overexpression of EC-SOD enhances erectile responses to cavernosal nerve stimulation and the endothelium-dependent vasodilator ACh in the aged rat to values similar to responses in young animals. These results support the hypothesis that in vivo gene transfer of targeted genes improve erectile function when administered intracavernosally in aged rats. These data suggest that adenoviral-mediated transfer of the EC-SOD gene or other SOD genes may represent an exciting new form of therapy for the treatment of male ED in the aging population.

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Table 1. **MSAP and MICP in young and aged animals transfected with AdCMVβgal and AdCMVEC-SOD**

<table>
<thead>
<tr>
<th></th>
<th>MSAP, mmHg</th>
<th>MICP, mmHg</th>
</tr>
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<tbody>
<tr>
<td>Young</td>
<td>118.2 ± 5.2</td>
<td>10.1 ± 0.6</td>
</tr>
<tr>
<td>Aged + AdCMVβgal</td>
<td>115.7 ± 4.6</td>
<td>12.9 ± 1.1</td>
</tr>
<tr>
<td>Aged + AdCMVEC-SOD</td>
<td>112.6 ± 2.3</td>
<td>11.5 ± 0.8</td>
</tr>
<tr>
<td>Young + AdCMVEC-SOD</td>
<td>116.5 ± 7.8</td>
<td>9.8 ± 0.9</td>
</tr>
</tbody>
</table>

Values are means ± SE. MSAP, mean systemic arterial pressure; MICP, mean intracavernosal pressure; AdCMVβgal, replication-deficient nuclear-targeted β-galactosidase adenovirus; AdCMVEC-SOD, extracellular superoxide dismutase adenovirus; CMV, cytomegalovirus. The adenoviruses were directly injected into the corpus cavernosum of the rats and MSAP and MICP were measured 1 day after transfection.
REFERENCES


