Gene transfer of extracellular superoxide dismutase improves endothelial function in rats with heart failure

Shinichiro Iida, Yi Chu, Joseph Francis, Robert M. Weiss, Carol A. Gunnett, Frank M. Faraci, and Donald D. Heistad

Cardiovascular Center and Departments of Internal Medicine, Pharmacology, Physiology, University of Iowa Roy J. and Lucille A. Carver College of Medicine and Veterans Affairs Medical Center, Iowa City, Iowa

Submitted 2 February 2005; accepted in final form 12 March 2005

Heart failure (HF) is characterized by an increase in peripheral vascular resistance, which contributes importantly to symptoms and mortality (3). Endothelium-dependent responses are impaired in patients (14, 16, 27) and animals (1, 10, 22) with HF and may contribute to increases in peripheral resistance. Indexes of oxidative stress, especially superoxide, are increased in HF and may be a major mechanism of endothelial dysfunction (1, 10, 16, 22) because superoxide decreases bioavailability of endothelium-derived nitric oxide (NO) by rapidly inactivating NO.

One of the major defenses against superoxide is superoxide dismutase (SOD). Three mammalian SODs have been identified: intracellular SOD (CuZn-SOD and Mn-SOD) and extracellular SOD (ecSOD) (4). ecSOD, but not intracellular SODs (CuZn-SOD and Mn-SOD), is released into the extracellular space and binds to glyocalyx of the endothelial cell surface (4, 23, 26). In patients with chronic HF, a decrease in endothelium-bound ecSOD activity and an increase in oxidative stress appear to contribute to endothelial dysfunction (16). Gene transfer of ecSOD protects against myocardial infarction and hypertension (2, 17, 18). The first goal of this study was to determine whether gene transfer of human ecSOD improves endothelial function in the aorta from rats with chronic HF.

In contrast to the rat aorta, in which relaxation to acetylcholine is entirely NO dependent, relaxation to acetylcholine in the mesenteric artery appears to be mediated in part by an endothelium-derived hyperpolarizing factor (EDHF) (25). The second goal of this study was to determine whether non-NO-dependent relaxation in the mesenteric artery is impaired by HF and improved by gene transfer of ecSOD.

ecSOD is the only SOD that contains a heparin-binding domain (HBD) (4, 23). The HBD mediates bindings of ecSOD to cells and interstitium. Studies of ecSOD and its HBD are timely because there is a mutation in the HBD of ecSOD in ~5% of humans, which is associated with ischemic heart disease (11). The third goal of this study was to determine whether the HBD of ecSOD is necessary for improvement of endothelial function by gene transfer of ecSOD in HF.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (~250–300 g) (n = 72) were obtained from Harlan-Sprague-Dawley (Indianapolis, IN) and were housed in the Animal Care Facility at the University of Iowa. The animal protocols were approved by the Animal Care and Use Review Committee of the University of Iowa.

HF was induced by coronary artery ligation as described previously (6). Briefly, rats were anesthetized, intubated, and ventilated. Under sterile conditions, a left thoracotomy was performed, and the left anterior descending coronary artery was ligated. Sham-operated rats were prepared in the same manner but did not undergo coronary artery ligation.

Six weeks after coronary ligation, left ventricular function was examined by echocardiography (6). Light general anesthesia was induced with ketamine (25 mg/kg ip), and two-dimensional images were acquired in parasternal short- and long-axis planes using an Acuson Sequoia 256 unit. Endo- and epicardial borders were manually traced at end diastole and end systole by using the leading edge method. Size of the akinetic zone was assessed by planimetry and expressed as a percentage of the whole left ventricle. Left ventricular ejection fraction was computed. Echocardiographic image acquisition and quantitative analysis were performed in blinded fashion without knowledge of the specific gene transfer protocol.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Gene transfer. Seven weeks after coronary ligation, rats were divided into five groups based on size of akinetic zone and left ventricular ejection fraction determined by echocardiography. Rats were lightly anesthetized with ketamine (25 mg/kg ip) and a virus (0.5 ml of 5 × 10^11 particles in 3% sucore in PBS) was injected into the penile vein. We used three replication-deficient recombinant adenoviruses, encoding either human ecSOD, ecSOD with deletion of HBD (ecSOD∆HBD), or β-galactosidase (β-gal) as a control virus, each driven by a cytomegalovirus promoter (2). We studied five groups of rats: HF-ecSOD, HF-ecSOD∆HBD, HF-β-gal, sham-β-gal, and sham-ecSOD.

Vasomotor function. Four days after injection of the virus, rats were anesthetized with ketamine (50 mg/kg ip) to collect blood samples for ecSOD activity and euthanized with pentobarbital (150 mg/kg ip). The thoracic aorta and first branch of mesenteric artery were removed and immediately placed in cold, oxygenated Krebs buffer (2). Loose connective tissue was removed carefully, vessels were cut into two rings (3–4 mm in length), rings were mounted in an organ bath, and isometric tension was measured. Optimal resting tension was determined, and resting tension was gradually increased to 1.0 g for aorta and 0.5 g for mesenteric artery. Rings were allowed to equilibrate for 60 min.

The concentration-response curve to phenylephrine (10^-9 to 10^-5 M) was examined. Concentration-response curves for acetylcholine (10^-9 to 10^-5 M), ADP (10^-8 to 10^-4 M) (13), and sodium nitroprusside (10^-9 to 10^-5 M) were generated during precontraction with phenylephrine (50% of maximum).

Responses of the aorta and mesenteric artery to acetylcholine were also examined in the presence of Nω-nitro-l-arginine (l-NNA, 10^-4 M) and indomethacin (Indo, 10^-5 M), which were applied 30 min before precontraction with phenylephrine (25). In the mesenteric artery, after 30 min incubation of l-NNA and Indo, responses to acetylcholine were examined after addition of charybdotoxin (10^-7 M) and apamin (10^-6 M), inhibitors of calcium-activated potassium channels (20), or catalase (300 U/ml), which dismutates hydrogen peroxide (15). All drugs were dissolved in distilled water except Indo, which was dissolved in Na2CO3 (0.1 M).

Measurement of superoxide and hydrogen peroxide. Generation of superoxide by vascular rings was assessed by lucigenin-enhanced chemiluminescence as described previously (9). Briefly, segments of vessels (6–8 mm for aorta, 3–4 mm for mesenteric artery) were placed in propylene tubes containing PBS and lucigenin (5 μM), and tubes were read in a luminometer. Levels of superoxide are reported as tissue values of tissue plus lucigenin-containing buffer minus background. Relative light units (RLU) were normalized to surface area of the vessel.

Dihydroethidium (DHE), an oxidative fluorescent dye, was used to localize superoxide in vessels in situ as previously described (9). In brief, segments of aorta from rats with HF or sham-operated rats were frozen in OCT compound until examination for superoxide. Transverse sections (30 μm thick) were cut and incubated in DHE (2 μM) for 30 min. Images were obtained by using a Bio-Rad MRC-1024 laser (krypton/argon) scanning confocal microscope. The fluorescence excitation/emission spectrum for ethidium bromide was used during the imaging process (488 and 610 nm, respectively). Fluorescence was detected with a 585-nm long-pass filter.

Dichlorodihydrofluorescein diacetate (DCF), a peroxide-sensitive fluorescence dye, was used to localize hydrogen peroxide in vessels in situ as previously described (20). Fresh, unfixed segments of the mesenteric artery from rats with HF were frozen in OCT compound and cut in transverse sections (30 μm thick). The sections were incubated with DCF and Indo and/or catalase (300 U/ml) for 20 min and then with DCF (5 μM) for 10 min. The fluorescence excitation/emission spectrum for the scanning confocal microscope used during the imaging process was 498 and 522 nm, respectively.

Immunohistochemistry for ecSOD. Immunohistochemistry for ecSOD was performed in segments of the aorta from rats with HF 4 days after gene transfer of β-gal, ecSOD, or ecSOD∆HBD. Vessels were embedded in OCT unfixed, and immunohistochemistry was performed on 8-μm-thick sections, using rabbit-anti-human ecSOD antisera (1:1,000; 4°C overnight) followed by horseradish peroxidase-conjugated goat-anti-rabbit IgG antibody (1:200) (2).

SOD assay. ecSOD activity in plasma was measured as described previously (28). Briefly, each plasma from rats after gene transfer of ecSOD, ecSOD∆HBD, or β-gal was separated from CuZn-SOD and Mn-SOD with affinity column chromatography on concanavalin A and were eluted by α-methyl-m-mannoside.

Total SOD activity in the aorta was measured by similar methods as described above for ecSOD activity, without separating CuZn-SOD and Mn-SOD. Each transfected aorta was homogenized and centrifuged. The supernatant was used for determination of protein content by Lowry assay and total SOD activity.

Measurement of ecSOD or total SOD activity was based on inhibition of superoxide-mediated reduction of NBT to formazan, the photoabsorbant product (28). ecSOD activity in plasma is expressed as units per milliliter and total SOD activity in aorta is expressed in units per milligram of protein.

Data analysis. Results are expressed as means ± SE. Relaxation is expressed as the percent change from the precontracted tension. Data for vascular function were analyzed with repeated-measures ANOVA. One-way ANOVA followed by Bonferroni-Dunn test was used for multiple comparisons. A probability value of P < 0.05 was considered significant.

RESULTS

Assessment of HF. Size of the akinetic zone of myocardium averaged 60% of the left ventricle in rats with HF (Fig. 1A).

---

*Fig. 1. Effects of coronary ligation in rats with heart failure (HF, n = 22) or sham-operated (Sham, n = 14) measured with echocardiography. A: size of akinetic zone of myocardium; B: left ventricular ejection fraction (LVEF). Values are means ± SE. *P < 0.05 HF vs Sham rats.*
LVEF was much lower in rats with HF than sham rats (Fig. 1B), measured by echocardiography before injection of virus. We observed pleural effusions in all rats with HF but not in sham-operated rats.

**Detection of ecSOD.** Expression of ecSOD was observed in the endothelium of the aorta from rats with HF transfected with ecSOD but not ecSODΔHBD or β-gal (Fig. 2A).

ecSOD activity in plasma from rats that were transfected with ecSOD was significantly higher than in those with β-gal, and ecSOD activity was highest in rats transfected with ecSODΔHBD (Fig. 2B). Plasma ecSOD activity was not different in sham-operated and HF rats that were transfected with β-gal.

Total SOD activity in the aorta from rats transfected with ecSOD was significantly higher than rats transfected with ecSODΔHBD or β-gal (Fig. 2C). Total SOD activity in the aorta from rats transfected with β-gal was not different between sham-operated and HF rats.

**Vasomotor responses in aorta.** Contractile responses to phenylephrine tended to be enhanced in the aorta from rats with HF, but there were no significant differences among the groups (Fig. 3A). Relaxation to acetylcholine (Fig. 3B) and ADP (Fig. 3C) was significantly impaired in rats with HF (HF-β-gal or HF-ecSODΔHBD).

After gene transfer of ecSOD in rats with HF (HF-ecSOD group), relaxation of the aorta to acetylcholine and ADP was normal. In the presence of L-arginine and Indo, relaxation of the aorta to acetylcholine was essentially abolished in both HF and sham-operated rats (Fig. 3B). Relaxation to sodium nitroprusside was similar among all groups (Fig. 3D).

**Superoxide in aorta.** Levels of superoxide were twofold greater in the aorta from rats with HF transfected with β-gal or ecSODΔHBD, than in sham-operated rats. ecSOD significantly reduced superoxide levels in the aorta from rats with HF to levels comparable to sham-operated rats (Fig. 4A).

In aortic sections from rats with HF after gene transfer of ecSOD, DHE staining was reduced. Staining in rats with HF transfected with β-gal or ecSODΔHBD was greater than in sham-operated rats (Fig. 4B).

**Vasomotor responses in mesenteric artery.** Contraction of the mesenteric artery to phenylephrine was similar in all groups (Fig. 5A). Relaxation of the mesenteric artery to acetylcholine was significantly impaired in rats with HF (HF-β-gal or HF-ecSODΔHBD) compared to sham-operated rats. Relaxation to ADP was also significantly impaired in rats with HF (HF-β-gal or HF-ecSODΔHBD) compared to sham-operated rats. In the presence of L-arginine and Indo, relaxation of the mesenteric artery to acetylcholine was essentially abolished in both HF and sham-operated rats (Fig. 5B). Relaxation to sodium nitroprusside was similar among all groups (Fig. 5C).
HF-ecSOD in Heart Failure

Superoxide and hydrogen peroxide in mesenteric artery. Levels of superoxide were twofold greater in the mesenteric artery from rats with HF, transfected with β-gal, or ecSOD ΔHBD than in sham-operated rats (Fig. 7A). ecSOD significantly reduced superoxide levels in the mesenteric artery from rats with HF to levels comparable to sham rats.

In sections of the mesenteric artery from rats with HF transfected with ecSOD, DCF-loaded fluorescence intensity (which is an index of levels of hydrogen peroxide) was significantly greater ($P < 0.05$) than in sections from rats with HF transfected with ecSOD ΔHBD or β-gal: 550 ± 91 in HF-ecSOD vs. 220 ± 42 in HF-ecSOD ΔHBD and 169 ± 54 in HF-β-gal (Fig. 7B, top). Catalase significantly ($P < 0.05$) reduced the fluorescence intensity after gene transfer of ecSOD to 137 ± 50 (Fig. 7B, bottom). There were no differences in the fluorescence intensity between sections transfected with ecSOD ΔHBD or β-gal in the presence or absence of catalase. After catalase, values were 85 ± 29 in HF-ecSOD ΔHBD and 95 ± 29 in HF-β-gal.

**DISCUSSION**

The major new findings in this study are the following. 1) After in vivo gene transfer, ecSOD binds to endothelium, decreases levels of superoxide, and improves endothelial function in the aorta and mesenteric artery from rats with HF. Thus even though total SOD activity in the aorta from rats with HF is normal, overexpression of ecSOD can preserve NO-dependent endothelial dysfunction. 2) In the mesenteric artery in rats with HF, in which responses to acetylcholine are mediated in large part by EDHF, in vivo gene transfer of ecSOD also improves endothelial dysfunction. Improvement of endothelial function by ecSOD is mediated in part by increased levels of hydrogen peroxide. 3) Beneficial effects of ecSOD in HF require the HBD of ecSOD. This finding is timely because recent evidence suggests that a common gene variant in the HBD of humans is associated with increased risk of ischemic heart disease (11). Thus our findings are compatible with a new hypothesis that impairment of function of the HBD of ecSOD may contribute to maladaptive vascular responses to HF.

**Mechanism of endothelial dysfunction in HF.** Endothelial dysfunction is produced by decreased bioavailability of NO,
from a decrease in expression of endothelial NO synthase (7), an increase in superoxide (which inactivates NO) (1, 10, 16, 22), and/or possibly to a decrease in ecSOD (16). Gene transfer of eNOS improves endothelium-dependent relaxation in HF (7). We observed in this study that gene transfer of ecSOD improved endothelial dysfunction (impaired responses to acetylcholine) and attenuated increases in superoxide in the aorta and mesenteric artery from rats with HF.

We also observed effects of gene transfer of ecSODΔHBD, which failed to improve endothelial function even though it increased SOD activity in plasma. In several previous studies (16–18), administration of heparin, after gene transfer of ecSOD, was used as an alternative approach to examine effects of ecSOD. Heparin displaces ecSOD from endothelial cells and inhibits effects of ecSOD.

We also found that gene transfer of ecSOD improved the impaired responses to ADP, which is another receptor-mediated activator of eNOS (13). The finding suggests that effects of ecSOD are not limited to muscarinic receptors.

Responses to sodium nitroprusside, which are not endothelium dependent, were similar between HF and sham-operated groups. The finding suggests that relaxation of smooth muscle cells is preserved in HF.

**Superoxide in vessels during HF.** Superoxide is increased and contributes to endothelial dysfunction in patients (16) and animals (1, 10, 22) with HF. We observed that levels of superoxide were increased in the aorta and mesenteric artery from rats with HF. These data suggest that superoxide inactivates NO within the vascular wall and reduces bioavailability of NO. Superoxide derived from NAD(P)H oxidase is increased in the aorta from rats with HF (1). The renin-angiotensin system is activated in HF (3), which is compatible with the hypothesis that enhanced angiotensin II contributes to generation of vascular superoxide by activation of vascular NAD(P)H oxidase in HF.

**Effect of ecSOD on endothelial function in mesenteric artery.** In the mesenteric artery, endothelium-dependent relaxation is produced by a combination of endothelium-derived NO, a product of cyclooxygenase, and an EDHF, whereas NO accounts for the response to acetylcholine in aorta (25). In the presence of L-NNA (which inhibits eNOS-mediated relaxation) and Indo (which inhibits cyclooxygenase), responses of the mesenteric artery in rats to acetylcholine (non-NO-dependent relaxation) are generally attributed to EDHF (25). There are several EDHFs in different vessels from different species, including epoxyeicosatrienoic acid, gap junctions, potassium ion, and hydrogen peroxide (5). To determine whether a response is mediated by an EDHF, inhibitors of calcium-activated potassium channels are used to block non-NO-dependent relaxation (5, 20). We observed that relaxation of the mesenteric artery in sham or HF rats, in the presence of L-NNA and Indo, was abolished by inhibitors of calcium-activated potassium channels (charybdotoxin and apamin), which suggests that the response is mediated by non-NO-, non-cyclooxygenase-dependent relaxation, presumably an EDHF.

**Endothelium-dependent relaxation in coronary microvessels, which is mediated largely by an EDHF (21), is impaired as well as in large arteries, in patients with HF (27).** We found
that gene transfer of ecSOD improves non-NO-dependent relaxation in the mesenteric artery from rats with HF. Hydrogen peroxide, which is produced from superoxide by SODs, is one candidate for an EDHF (24). Hydrogen peroxide relaxes and hyperpolarizes the mesenteric artery in mice (20) and rats (8), cerebral arteries in rats (15), and coronary microvessels in pigs (19). Catalase, an endogenous peroxidase, inhibits EDHF-mediated relaxation and hyperpolarization in the presence of l-NNa and Indo (15, 19, 20). We observed that catalase decreased levels of hydrogen peroxide (as estimated by DCF-loaded fluorescence intensity) and inhibited the improvement of non-NO-dependent relaxation in the mesenteric artery transfected with ecSOD. These findings suggest that hydrogen peroxide may be increased by gene transfer of ecSOD and contribute to improvement in endothelial function in the mesenteric artery from rats with HF.

Role of HBD of ecSOD. ecSOD, in contrast to the other SODs, contains a HBD that is positively charged and has a high affinity for heparan sulfate (4, 23). The HBD anchors ecSOD primarily to heparan sulfate proteoglycans on cell surfaces, which are negatively charged (4, 23). We examined the role of the HBD of ecSOD in rats with HF.

Rats have an endogenous form of ecSOD with little binding to the arterial wall, although the enzymatic activity of ecSOD

Fig. 5. Effects of gene transfer on response of mesenteric artery to phenylephrine (A), acetylcholine (B), ADP (C), and sodium nitroprusside (D) in rats with HF and Sham. Number of rats studied was the following: HF-ecSOD, n = 8 (6 for ADP); HF-ecSODΔHBD, n = 6; HF-β-gal, n = 8 (6 for ADP); Sham-β-gal, n = 8 (6 for ADP); Sham-ecSOD, n = 6. Values are means ± SE. *P < 0.05 HF-ecSOD vs. HF-β-gal or HF-ecSODΔHBD.

Fig. 6. A: relaxation of mesenteric artery to acetylcholine with HF-ecSOD, HF-ecSODΔHBD, HF-β-gal, Sham-β-gal, and Sham-ecSOD were performed in the presence of l-NNa (10^{-4} M) and Indo (10^{-5} M) (n = 6). In the presence of l-NNa (10^{-4} M) and Indo (10^{-5} M), relaxation to acetylcholine were performed in addition to charybdotoxin (Ctx, 10^{-7} M) and apamin (10^{-6} M) (n = 5, open symbols). Values are means ± SE. *P < 0.05 HF-ecSOD vs. HF-β-gal or HF-ecSODΔHBD. B: in the presence of l-NNa (10^{-4} M) and Indo (10^{-5} M), relaxation to acetylcholine with HF-ecSOD, HF-ecSODΔHBD, HF-β-gal, Sham-β-gal, and Sham-ecSOD were performed in addition to catalase (300 U/ml) (n = 6). Values are means ± SE. *P < 0.05 HF-ecSOD vs. Sham-β-gal or Sham-ecSOD.
We observed that gene transfer of ecSOD, but not ecSOD with deletion of HBD (ecSODΔHBD) or β-gal, produced an increase in total SOD activity in the aorta from rats with HF or sham operation. Total SOD activity in the aorta was not different between rats with HF and sham operation. Because our method was not sufficiently sensitive to measure ecSOD activity in the rat aorta, we measured total SOD activity (CuZn-SOD, Mn-SOD, and ecSOD). Our data indicate that the gene transfer of human ecSOD increased total SOD activity of aorta from rats with HF. We should point out that we have measured ecSOD activity in plasma, but not in blood vessels, and measured total SOD activity in aorta, but not mesenteric arteries. Because of these limitations, we are not able to provide a comprehensive view of alterations in the SODs in HF.

In contrast to the failure of gene transfer of ecSODΔHBD to increase tissue levels of ecSOD, ecSOD activity in plasma was higher after gene transfer of ecSODΔHBD than after ecSOD. These observations suggest that ecSOD protein, which is secreted from liver after intravenous injection of adenovirus and circulates in plasma (2, 17, 18), bound to endothelium to reduce levels of superoxide and improve endothelial function. In contrast, levels of ecSODΔHBD increased in plasma after gene transfer but had no protective effect on endothelial function, presumably because ecSODΔHBD circulated in plasma without binding to endothelium. Our findings in this study, and a previous study in spontaneously hypertensive rats (2), suggest that the HBD is necessary for vascular effects of ecSOD.

In summary, these studies provide direct evidence for an important concept in vascular biology of HF. Previous studies suggest that oxidative stress is a major mechanism of endothelial dysfunction (1, 10, 22), and ecSOD is protective against endothelial dysfunction in HF (16). This study provides direct evidence to support this hypothesis and also provides evidence that the HBD is critically important for the protective effect of ecSOD in HF.

There is a mutation in the HBD of ecSOD in humans, which appears to confer a high risk of ischemic heart disease (11). Thus the finding in this study, that the HBD is essential for protection against vasomotor dysfunction, may be relevant to patients with mutation in the HBD. We speculate therefore that the R213G gene variant, a mutation in the HBD of ecSOD, may prove to be a risk factor for vascular maladaptation and endothelial dysfunction in patients with HF.

ACKNOWLEDGMENTS

We are grateful to Dr. James Crapo of National Jewish Medical and Research Center at Denver, CO, for providing human extracellular SOD cDNA plasmid, from which the recombinant virus was made, and antibodies against human ecSOD. We thank Arlinda A. LaRose for typing the manuscript and Pamela K. Tompkins for immunohistochemistry. We acknowledge the University of Iowa Gene Transfer Vector Core, supported in part by the National Institutes of Health and Roy J. Carver Foundation, for viral vector preparations.
The research was supported by National Institutes of Health Grants HL-16066, HL-62984, NS-24621, DK-54739, DK-15843, DK-52617, HL-55006, funds provided by the Veterans Affairs Medical Service, and a Carver Trust Research Program of Excellence.

REFERENCES


