Vascular effects of a common gene variant of extracellular superoxide dismutase in heart failure

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Submitted 19 January 2006; accepted in final form 23 March 2006

Extracellular superoxide dismutase (ecSOD) is a major form of SOD in the vascular wall and is an important vascular antioxidant (8, 26). In patients with chronic heart failure, a decrease in endothelium-bound ecSOD activity may contribute to an increase in oxidative stress and endothelial dysfunction (17). Recently, our group (11) demonstrated that gene transfer of human ecSOD reduces superoxide and improves endothelial function in rats with heart failure. In that model, the heparin-binding domain (HBD) of ecSOD is necessary for improvement of endothelial function with heart failure (11).

A common gene variant in the HBD of human ecSOD (ecSODR213G), in which there is substitution of arginine 213 by glycine resulting in a C to G transversion at the first base of codon 213 in the ecSOD, is associated with increased risk of ischemic heart disease (13) and of reduced survival in diabetic patients receiving hemodialysis (29). The primary goal of this study was to determine whether beneficial vascular effects of gene transfer of ecSOD in rats with heart failure are reduced in the ecSOD variant (ecSODR213G).

Peroxynitrite, which is formed by the combination of NO and superoxide, is a marker of oxidative stress and also has adverse vascular effects (3, 18, 28). Vascular effects of peroxynitrite in heart failure are not known. The second goal of this study was to examine vascular effects of peroxynitrite in rats with heart failure.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (250–300 g) (n = 36) 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.

Heart failure was induced by coronary artery ligation as described previously (9, 11). Sham rats were prepared in the same manner but did not undergo coronary artery ligation.

Echocardiography. Six weeks after coronary ligation, left ventricular function was examined by echocardiography under light anesthesia (ketamine: 25 mg/kg ip) (9, 11). Size of the akinetic zone was assessed by planimetry and expressed as percentage of the whole left ventricle. Left ventricular end-diastolic volume (LVEDV), volume-to-mass ratio, and ejection fraction (LVEF) were computed. Echocardiographic image acquisition and quantitative analysis were performed in blinded fashion without knowledge of the specific gene transfer protocol.

In vivo gene transfer. Seven weeks after coronary ligation, rats with heart failure and sham were divided into four groups based on results of echocardiography, and a virus (0.5 ml of 5 x 10^{11} particles in 3% sucrose in PBS) was injected in the penile vein with the rats under
light anesthesia (ketamine; 25 mg/kg ip). We used three replication-deficient recombinant adenoviruses, encoding either human ecSOD (wild-type ecSOD), ecSOD gene variant (ecSOD R213G), or β-galactosidase (LacZ) as a control virus, each driven by a cytomegalovirus promoter (5). We studied four groups of rats: HF-ecSOD, HF-ecSOD R213G, HF-LacZ, sham-LacZ, where HF is heart failure.

**Table 1. Sequences of primers and probes for SODs and eNOS**

<table>
<thead>
<tr>
<th>Sense (5’-3’)</th>
<th>Antisense (5’-3’)</th>
<th>Probe (5’-FAM-TAMRA-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuZnSOD</td>
<td>TGCTGAAGGGCGACGG</td>
<td>GTCACCGTTTCTCTTCTG</td>
</tr>
<tr>
<td>MnSOD</td>
<td>GGCGCTTCGCTGCTGCTG</td>
<td>CAGAGATCTTCACTGCATTGGCTA</td>
</tr>
<tr>
<td>ecSOD</td>
<td>GGCGCTTCGCTGCTGCTG</td>
<td>CAGAGATCTTCACTGCATTGGCTA</td>
</tr>
<tr>
<td>eNOS</td>
<td>CTTCCCGCTACCCAGGACA</td>
<td>GAGAGATCTTCACTGCATTGGCTA</td>
</tr>
</tbody>
</table>

FAM, 6-carboxy fluorescein; TAMRA, 6-carboxy tetramethylrhodamine; SOD, superoxide dismutase; eNOS, endothelial nitric oxide synthase; ecSOD, extracellular SOD.

**Table 2. Cardiac function in sham rats and rats with heart failure 6 wk after coronary ligation**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Heart Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>27</td>
</tr>
<tr>
<td>LVEDV, μL</td>
<td>415±25</td>
<td>1,363±44*</td>
</tr>
<tr>
<td>Volume/mass, ml/mg</td>
<td>0.48±0.03</td>
<td>1.90±0.10*</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>82±2</td>
<td>28±1*</td>
</tr>
<tr>
<td>Akinetic zone, %</td>
<td>0</td>
<td>53.2±0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = number of rats. LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction, *P < 0.05 for heart failure vs. sham rats.

Fig. 1. Effects of gene transfer on lung-to-body weight ratios (n = 9 in each group). Values are means ± SE. *P < 0.05 heart failure (HF) vs. sham. **P < 0.05 HF-extracellular SOD (ecSOD) vs. HF-LacZ or HF-ecSOD R213G.
RESULTS

Assessment of heart failure. Cardiac function was measured by echocardiography before injection of the virus. There was a large akinetic zone of the left ventricle in rats with heart failure (Table 2). LVEF was low, LVEDV was larger, and volume-to-mass was greater in rats with heart failure than sham.

After in vivo gene transfer, lung-to-body weight ratio was greater in rats with heart failure than in sham rats (Fig. 1). Gene transfer of ecSOD significantly reduced lung-to-body weight ratio in rats with heart failure, whereas gene transfer of ecSODR213G had no effect (Fig. 1). Body weights (410–430 g) are not significantly different among the groups.

Vasomotor responses in aorta. Relaxation to acetylcholine was impaired in rats with heart failure compared with sham rats (Fig. 2A). Gene transfer of ecSOD to rats with heart failure improved relaxation to normal, whereas gene transfer of ecSODR213G had no effect (Fig. 2A). In the presence of L-NNA, relaxation to acetylcholine was essentially abolished in rats with heart failure and sham rats (data not shown).

Relaxation to sodium nitroprusside was similar among the groups (Fig. 2B). In rats with heart failure, relaxation to acetylcholine was not different in the presence and absence of ebselen (Fig. 2C).

Basal NO availability assessed by L-NNA-induced constriction was less in rats with heart failure than in sham rats (Fig. 3A). Gene transfer of ecSOD improved contraction (i.e., basal NO availability) to normal in rats with heart failure, whereas gene transfer of ecSODR213G produced minimal improvement (Fig. 3A).

Contractile responses to phenylephrine were greater in rats with heart failure than in sham rats (Fig. 3B). Gene transfer of ecSOD significantly reduced contraction in rats with heart failure, whereas gene transfer of ecSODR213G had no effect on contraction (Fig. 3B). In the presence of L-NNA, contractile responses to phenylephrine were greater in the aorta from rats with heart failure than from sham rats but were not altered by gene transfer of ecSOD or ecSODR213G (Fig. 3C).

Superoxide in aorta. Levels of superoxide were greater in the aorta from rats with heart failure than sham rats (Fig. 4).
Gene transfer of ecSOD reduced levels of superoxide to normal in rats with heart failure, whereas gene transfer of ecSODR213G had minimal effects (Fig. 4). In rats with heart failure, levels of superoxide were not different in the presence (2.1 ± 0.3 RLU·s⁻¹·mm⁻²; n = 4) and absence of ebselen (2.3 ± 0.2 RLU·s⁻¹·mm⁻²; n = 7).

Peroxynitrite in aorta. Levels of peroxynitrite were greater in the aorta from rats with heart failure than sham rats (Fig. 5A). Gene transfer of ecSOD reduced levels of reactive oxygen species in the aorta from rats with heart failure, whereas gene transfer of ecSODR213G had minimal effects (Fig. 5A).

Levels of peroxynitrite in the aorta from rats with heart failure were significantly reduced in the presence of ebselen or uric acid (Fig. 5B). The uric acid inhibitable signal was lower in rats with heart failure after gene transfer of ecSOD (0.3 ± 0.2 RLU·s⁻¹·mm⁻²; n = 3) and sham rats (0.2 ± 0.2 RLU·s⁻¹·mm⁻²; n = 4) than in rats with heart failure (1.3 ± 0.3 RLU·s⁻¹·mm⁻²; n = 4).

After gene transfer of ecSOD, levels of peroxynitrite in the aorta from rats with heart failure were not different in the absence (0.9 ± 0.1 RLU·s⁻¹·mm⁻²; n = 9) and presence of ebselen (0.7 ± 0.1 RLU·s⁻¹·mm⁻²; n = 3) or uric acid (0.7 ± 0.2 RLU·s⁻¹·mm⁻²; n = 3).

Binding and circulating ecSOD proteins. Binding of human ecSOD proteins to carotid artery after gene transfer of ecSOD is 10-fold higher than that after gene transfer of ecSODR213G (Fig. 6A).

In plasma, levels of human ecSOD proteins after gene transfer of ecSOD are 10-fold lower than those after gene transfer of ecSODR213G (Fig. 6B). Because the antibody is specific for human ecSOD, there was no detectable binding of human ecSOD to the carotid artery or circulating human ecSOD proteins in HF-LacZ and sham-LacZ rats.

SODs and eNOS. Endogenous mRNA for SODs (CuZnSOD, MnSOD, and ecSOD) and eNOS were not different in the aorta from rats with heart failure and sham rats (Fig. 7, A and B).

**DISCUSSION**

The major new findings in this study are 1) gene transfer of a common human gene variant of ecSOD (ecSODR213G), in contrast to ecSOD (wild-type ecSOD), produced minimal reduction of oxidative stress, improvement of endothelial dysfunction, or increase in basal bioavailability of NO in the aorta from rats with heart failure; and 2) levels of peroxynitrite, like superoxide, were increased in heart failure and were reduced by gene transfer of ecSOD. In contrast to superoxide, however, peroxynitrite appears to have minimal role in impairment of endothelial function in the aorta from rats with heart failure. Based on these findings, we speculate that humans who carry the ecSODR213G gene variant may have impaired protection against oxidative stress and endothelial function in heart failure.

Effects of ecSOD vs. ecSODR213G on endothelial dysfunction in heart failure. Endothelial dysfunction in patients with heart failure is associated with increased mortality and is an early indicator of adverse outcome in heart failure (10, 14). Endothelial dysfunction may be produced in part by a decrease in production of NO due to decreased expression of eNOS but appears to be produced largely by decreased bioavailability of NO due to increased levels of superoxide (19). We observed that eNOS mRNA expression was similar among the experimental groups and not reduced by heart failure, which suggests that endothelial dysfunction in this model is not due to attenuated expression of eNOS. Increases (2) or decreases (1) in expression of eNOS have been observed in the aorta from rats...
after coronary ligation, in association with endothelial dysfunction.

Gene transfer of ecSOD\textsubscript{R213G}, in contrast to ecSOD, failed to improve responses to acetylcholine in the aorta from rats with heart failure. Vasomotor responses to acetylcholine were abolished in all groups in the presence of \textit{l}-NNA. This finding indicates that responses to acetylcholine in the aorta were mediated by eNOS, and that compensatory mechanisms (e.g., an endothelium-derived hyperpolarizing factor) were not involved in the aorta in this setting. Responses to sodium nitroprusside, an endothelium-independent NO-mediated relaxation by stimulation, were similar among the groups. Furthermore, gene transfer of ecSOD did not change vasomotor function in the aorta from sham rats (11). These results are compatible with the conclusion that gene transfer of ecSOD improved NO-dependent endothelial function in the aorta from rats with heart failure.

Basal NO release is decreased in vessels from patients (30) and animals (27) with heart failure. We estimated basal NO release by examining effects of \textit{l}-NNA-induced contraction (24) in the aorta from rats with heart failure and sham rats. Basal NO release was blunted in heart failure. Gene transfer of ecSOD, but not ecSOD\textsubscript{R213G}, improved basal NO release in rats with heart failure. Thus gene transfer of ecSOD appears to increase bioavailability of NO, both by increased basal NO release and acetylcholine-stimulated NO release, by reducing superoxide.

We also examined basal NO release during phenylephrine-induced contraction in the absence and presence of \textit{l}-NNA (27). Phenylephrine-induced contraction was increased in rats with heart failure compared with sham rats. Gene transfer of ecSOD, but not ecSOD\textsubscript{R213G}, attenuated phenylephrine-induced contraction in rats with heart failure. In the presence of \textit{l}-NNA, gene transfer of ecSOD did not alter phenylephrine-induced contraction in rats with heart failure. These results also suggest that gene transfer of ecSOD increased basal NO.

The ratio of lung to body weight increases in rats with heart failure after coronary ligation, which suggests that the rats developed heart failure with increases in fluid in the lungs (9). Gene transfer of ecSOD, but not ecSOD\textsubscript{R213G}, reduced lung weight in rats with heart failure. It is possible that reduction of lung weight by ecSOD is the result of a decrease of peripheral resistance and afterload, by increased bioavailability of NO, or through a direct effect of ecSOD on pulmonary vascular filtration (15).

Vascular oxidative stress in heart failure. In patients and animals with heart failure, levels of superoxide increase in blood vessels and produce endothelial dysfunction (2, 11, 12, 17). Gene transfer of ecSOD, in contrast to ecSOD\textsubscript{R213G}, reduced levels of vascular superoxide in heart failure.

Peroxynitrite, which is formed by the reaction of NO and superoxide, is associated with endothelial dysfunction during hypoxia-reoxygenation (28), after lipopolysaccharide (3), and in atherosclerosis (18). We observed that levels of per-

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**Fig. 6.** Binding of ecSOD proteins to carotid artery from rats with HF after gene transfer of ecSOD or ecSOD\textsubscript{R213G} (A) and circulating of ecSOD proteins in plasma from rats with HF after gene transfer of ecSOD or ecSOD\textsubscript{R213G} (B), as quantified by immunoblotting. Values are means ± SE; \( n = 6–7 \) rats for each group. *\( P < 0.05 \) HF-ecSOD vs. HF-ecSOD\textsubscript{R213G}.

**Fig. 7.** Expression of mRNA for SODs (CuZnSOD, MnSOD, and ecSOD) (A) and endothelial nitric oxide synthase (eNOS) (B) in aorta from rats with HF and sham rats. Open bar is sham-LacZ, and filled bar is HF-LacZ. Values are means ± SE; \( n = 4–5 \) rats.
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oxynitrite, estimated by luminol-derived chemiluminescence, are increased in heart failure. Gene transfer of ecSOD, but not ecSODR213G, reduced levels of peroxynitrite. Because luminol-derived chemiluminescence may be evoked by a variety of reactive oxygen species, including O$_2^·$, hydroxyl radical, hydrogen peroxide, and peroxynitrite (21), we determined whether ebselen and uric acid, which are peroxynitrite scavengers, reduced luminol-derived chemiluminescence in the aorta from rats with heart failure (18). Although ebselen reduced luminol-derived chemiluminescence, ebselen did not improve responses to acetylcholine after gene transfer of ecSOD to rats with heart failure (data not shown). These results suggest that increased levels of peroxynitrite have little or no role in endothelial dysfunction in the aorta from rats with heart failure.

Ebselen is not only a peroxynitrite scavenger (3, 18, 28) but also a glutathione peroxidase mimetic (22). Gene transfer of ecSOD increases hydrogen peroxide in vessels from rats with heart failure (11), but we found that ebselen did not reduce luminol-enhanced chemiluminescence, levels of superoxide, or responses to acetylcholine after gene transfer of ecSOD to rats with heart failure (data not shown). These results suggest that the dose of ebselen that we used may reduce levels of peroxynitrite but not other mediators of oxidative stress in this setting (28).

Vascular effects of ecSODR213G in heart failure. ecSOD, in contrast to other SODs, contains a HBD that mediates binding of ecSOD to cells in the vessel wall. We speculated recently that, after gene transfer of ecSOD, human ecSOD protein is secreted from the liver, circulates in the blood, and binds to blood vessels (11). We also reported recently (11) that gene transfer of ecSOD with deletion of HBD fails to bind to vessels in rats with heart failure, whereas levels of ecSOD activity increased in blood.

ecSODR213G, a variant in the HBD of ecSOD, is common because it affects about 5% of humans (20). A recent large study suggests that heterozygous carriers of ecSODR213G have increased risk of ischemic heart disease (13). Hemodialysis patients with diabetes carrying ecSODR213G had an increase in five-year mortality of ischemic heart disease (29).

In rats, the endogenous form of ecSOD exhibits little binding to the arterial wall (8). Expression of the other SODs (CuZnSOD and MnSOD) was similar in rats with heart failure. The expression of the other SODs (CuZnSOD and MnSOD) was similar in rats with heart failure, so that endothelial dysfunction is the result.

Gene transfer produces very high levels of ecSOD and ecSODR213G. Thus it is appropriate to be cautious in interpreting the findings in relation to pathophysiology of endothelial dysfunction in heart failure.

After gene transfer of ecSODR213G, we observed attenuated binding of ecSOD to the vessel wall in rats with heart failure. We have reported previously that in contrast to impaired tissue binding of ecSODR213G, enzymatic activity of SOD is similar in ecSOD and ecSODR213G (5). Other investigators have observed that higher concentrations of ecSODR213G in plasma are due to impaired binding to cells, with decreased affinity for heparin-like cell membrane sites (23). These results suggest that impaired binding of ecSOD to blood vessels may account for failure to reduce oxidative stress and improve endothelial function in rats with heart failure.

In summary, after gene transfer of ecSODR213G, a common gene variant of HBD of ecSOD, there was minimal binding of ecSOD to blood vessels, improvement of endothelial function, or reduction of oxidative stress in rats with heart failure. Superoxide, in contrast to peroxynitrite, appears to account for endothelial dysfunction in rats with heart failure. We speculate that in humans who carry this gene variant, minimal vascular effects of ecSODR213G may contribute to increased risk of cardiovascular disease and endothelial dysfunction, which is a predictor of adverse outcome in patients with heart failure.

ACKNOWLEDGMENTS

We are grateful to Dr. James Crapo 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 Teresa Ruggle for preparation of figures. We acknowledge the University of Iowa Gene Transfer Vector Core.

GRANTS

This work was supported in part by the National Institutes of Health (NIH) and the Veterans Affairs Medical Center, and a Carver Trust Research Program of Excellence.

REFERENCES


