# Extracellular Superoxide Dismutase Ameliorates Skeletal Muscle Abnormalities, Cachexia, and Exercise Intolerance in Mice with Congestive Heart Failure

Mitsuharu Okutsu, PhD; Jarrod A. Call, PhD; Vitor A. Lira, PhD; Mei Zhang, BSMed; Jean A. Donet, MD; Brent A. French, PhD; Kyle S. Martin, BS; Shayn M. Peirce-Cottler, PhD; Christopher M. Rembold, MD; Brian H. Annex, MD; Zhen Yan, PhD

***Background***—Congestive heart failure (CHF) is a leading cause of morbidity and mortality, and oxidative stress has been implicated in the pathogenesis of cachexia (muscle wasting) and the hallmark symptom, exercise intolerance. We have previously shown that a nitric oxide–dependent antioxidant defense renders oxidative skeletal muscle resistant to catabolic wasting. Here, we aimed to identify and determine the functional role of nitric oxide–inducible antioxidant enzyme(s) in protection against cardiac cachexia and exercise intolerance in CHF.

***Methods and Results***—We demonstrated that systemic administration of endogenous nitric oxide donor *S*-nitrosoglutathione in mice blocked the reduction of extracellular superoxide dismutase (EcSOD) protein expression, as well as the induction of *MAFbx/Atrogin-1* mRNA expression and muscle atrophy induced by glucocorticoid. We further showed that endogenous EcSOD, expressed primarily by type IId/x and IIa myofibers and enriched at endothelial cells, is induced by exercise training. Muscle-specific overexpression of EcSOD by somatic gene transfer or transgenesis (muscle creatine kinase [MCK]-EcSOD) in mice significantly attenuated muscle atrophy. Importantly, when crossbred into a mouse genetic model of CHF (α-myosin heavy chain–calsequestrin), *MCK-EcSOD* transgenic mice had significant attenuation of cachexia with preserved whole body muscle strength and endurance capacity in the absence of reduced HF. Enhanced EcSOD expression significantly ameliorated CHF-induced oxidative stress, *MAFbx/Atrogin-1* mRNA expression, loss of mitochondria, and vascular rarefaction in skeletal muscle.

***Conclusions***—EcSOD plays an important antioxidant defense function in skeletal muscle against cardiac cachexia and exercise intolerance in CHF. **(*Circ Heart Fail*. 2014;7:519-530.)**

**Key Words:** capillaries ■ exercise ■ mitochondria ■ muscular atrophy ■ oxidative stress



Congestive heart failure (CHF) is a leading cause of morbidity and mortality in industrialized countries. Cachexia, characterized by severe loss of muscle mass, and exercise intolerance, manifested as fatigue and dyspnea during minimal physical activities, are important predictors of mortality in CHF. The progressive decline of exercise capacity results from multiple skeletal muscle abnormalities, including atrophy, impaired excitation–contraction coupling, mitochondrial dysfunction, and vascular rarefaction.[1–3] Oxidative stress due to excessive production of reactive oxygen species (ROS) or reduced antioxidant defenses has emerged as a central player in cardiac cachexia and is mechanistically linked to intracellular signaling, contractile protein function, and degradation.[3–6] Although animal studies have shown the effectiveness of antioxidant supplements in treatment, clinical trials have limited successes,[7,8] possibly because of lack of specificity to the target cells and subcellular environment leading to either poor efficacy or impairment of physiological signaling. It is critical to identify a viable approach to augment the endogenous antioxidant defense system to cope with this complicated clinical syndrome.

## Clinical Perspective on p 530

Our body has very sophisticated, functional antioxidant defense systems for maintaining a delicate redox homeostasis. The first line of antioxidant defense is comprised of superoxide dismutases (SODs), which scavenge superoxide to produce the less reactive $H_2O_2$. Skeletal muscle expresses all 3 isoforms of SOD: the cytosolic copper/zinc-containing SOD (CuZnSOD or SOD1), the mitochondrial manganese-containing SOD (MnSOD or SOD2), and the extracellular SOD (EcSOD or SOD3). EcSOD is a glycoprotein that is

Received May 8, 2013; accepted February 11, 2014.

From the Departments of Medicine (M.O., J.A.C., V.A.L., M.Z., J.A.D., C.M.R., B.H.A., Z.Y.), Pharmacology (Z.Y.), and Molecular Physiology and Biological Physics (Z.Y.), Center for Skeletal Muscle Research (M.O., J.A.C., V.A.L., M.Z., J.A.D., Z.Y.), Robert M. Berne Cardiovascular Research Center, University of Virginia School of Medicine, Charlottesville, VA; and Department of Biomedical Engineering, University of Virginia, Charlottesville, VA (B.A.F., K.S.M., S.M.P.-C.).

**The Data Supplement is available at http://circheartfailure.ahajournals.org/lookup/suppl/doi:10.1161/CIRCHEARTFAILURE.113.000841/-/DC1.**

Correspondence to Zhen Yan, PhD, 409 Lane Rd, MR4-6041A, PO Box 801394, Charlottesville, VA 22908. E-mail zhen.yan@virginia.edu

secreted and has high affinity for sulfated polysaccharides, such as heparin and heparin sulfate, through its haprin-binding domain on the C-terminus. EcSOD may also be internalized by the cells that it binds through endocytosis.[9] Therefore, EcSOD may exert antioxidant function intracellularly and extracellularly in producing cells and target cells.

The expression of this antioxidant enzyme seems to be prone to suppression by pathological conditions, potentially underlying the cause of the diseases. For example, reduced EcSOD expression has been reported in many chronic diseases, including CHF.[10,11] In terms of functionality, genetic deletion of the EcSOD gene exacerbates,[12–14] whereas transgenic overexpression attenuates the pathologies in mouse models of chronic diseases.[14,15] These findings have clearly established the functional importance of EcSOD in protection against diseases caused by oxidative stress; however, its importance of skeletal muscle mass and function is unknown.

We have recently identified a nitric oxide (NO)-dependent antioxidant defense mechanism that underlies the resistance of oxidative muscles to catabolic wasting.[3,16,17] To ascertain the protective function of NO and to identify the antioxidant gene(s) that plays a major role in this protection, we first administered systemically an endogenous NO donor, *S*-nitrosoglutathione (GSNO), in a mouse model of catabolic muscle wasting induced by glucocorticoid, a hormone that is elevated in CHF and involved in muscle catabolism.[18] Our findings underscore the importance of NO-mediated induction of EcSOD expression in protection against catabolic muscle wasting. We then used 2 independent genetic approaches and showed that enhanced EcSOD expression in skeletal muscle ameliorates cardiac cachexia and exercise intolerance along with blunted oxidative stress, atrophy gene expression, mitochondrial loss, and microvascular abnormalities in a mouse model of CHF. We have, therefore, provided convincing evidence to support the effectiveness of enhancing EcSOD expression and antioxidant activity in protecting skeletal muscle structural and functional integrity under the condition of CHF.



## Methods

### Animals

Male wild-type (WT) mice (8 weeks old; C57BL/6J) were obtained commercially (Jackson Laboratory, Bar Harbor, ME) and housed in temperature-controlled (22°C) quarters with a 12/12 hour light/dark cycle and free access to water and normal chow (Harlan). A muscle-specific EcSOD transgenic mouse line was generated at the University of Virginia Gene Targeting and Transgenic Facility. Briefly, RT-PCR was performed using mouse skeletal muscle RNA with forward primer 5′-TGGATCCACCATGTTGGCCTTCTTGTTC-3′ and reverse primer 5′-AGGTACCAGTGGTCTTGCACTCGCTCTC-3′ to obtain EcSOD cDNA (NM_011435). A *Bam H1-Kpn I* fragment (762 bp) of the coding region was then fused to a FLAG sequence followed by a stop codon in *pTarget* (Promega, Madison, WI) to create *pEcSOD*. A 1.3-kb *Hind III-Eco RI* fragment of FLAG-tagged *EcSOD* was then inserted into *pMCKhGHpolyA* between a 4.8-kb mouse muscle creatine kinase (MCK) promoter and the human growth hormone polyadenylation site.[19] A linear *Not I-Xho I* fragment (6.7 kb) was isolated and used for pronuclear injection to generate transgenic mice (C57BL/6.SJL F1 background). We backcrossed the transgene onto pure C57BL/6J background (≥7 generations). To determine if enhanced EcSOD expression prevents CHF-induced muscle atrophy, *MCK-EcSOD* mice were crossbred with α-myosin heavy chain (α-MHC)-calsequestrin mice (CSQ; DBA background)[20] to generate double-transgenic *MCK-EcSOD:α-MHC-CSQ* mice (DTG). The WT littermates or littermates with only *MCK-EcSOD* allele (TG) were used as controls. Therefore, the mice used were of the same genetic background. To prevent negative impact of HF on embryonic development, the *α-MHC-CSQ* breeder mice were treated with β-blocker, metoprolol, in drinking water before and during breeding,[6] and the β-blocker was removed at birth such that none of the mice used in the studies received β-blocker after birth. Voluntary wheel running protocol is as described.[21] All animal protocols were approved by the University of Virginia Animal Care and Use Committee.

Genotyping PCR of tail DNA was performed using *MCK* transgenic forward primer, 5′-GAC TGA GGG CAG GCT GTA AC-3′; *EcSOD* intron forward primer, 5′-GGG AGG TGG GAA GAG TTA GG-3′; and *EcSOD* coding reverse primer, 5′-CAC CTC CAT CGG GTT GTA GT-3′. Exogenous EcSOD expression in adult skeletal muscle was confirmed by immunoblot as described.[6]

### Dexamethasone and GSNO Injections

Mice received daily injection of dexamethasone (25 mg/kg, intraperitoneally; Sigma, St Louis, MO) or normal saline with/without GSNO (2 mg/kg) twice a day for 7 days.[22] At 12 and 24 hours after the last injection of GSNO and dexamethasone, respectively, soleus, plantaris, tibialis anterior, gastrocnemius, extensor digitorum longus (EDL), and white vastus lateralis muscles were harvested and processed for further analyses.

### Semiquantitative RT-PCR and Real-Time PCR

These analyses were performed for *MAFbx/Atrogin-1*, *MuRF1*, and *Gapdh* mRNAs in plantaris muscles as described.[23] Real-time PCR was performed using primers for *MAFbox/Atrogin 1* and *18S* rRNA from Applied Biosystems (Foster City, CA).

### Immunoblot Analysis

Immunoblot analysis was performed as described[6] with the following primary antibodies: CuZnSOD (Abcam), MnSOD (Abcam), EcSOD (R&D, MN), peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α; Chemicon/Millipore), cytochrome oxidase IV (Invitrogen), cytochrome C (Cell Signaling, Danvers, MA), malondialdehyde (Academy Biomedical Company, Inc, Houston, TX), and 4-hydroxynonenal (ab48506; Abcam). Hybridoma for antibodies against MHC I (BA-F8), MHC IIa (SC-71), and MHC IIb (BF-F3) were purchased from German Collection of Microorganisms and Cell Cultures. All the bands for analysis have been validated previously.[3,6,24] OxyBlot Protein Oxidative Detection Kit (Millipore) was used for immunblot detection of carbonylated proteins. Immunoblots were analyzed by Odyssey Infrared Imaging System (LI-COR Biosciences, NE) and quantified by Scion Image software.

### Immunohistochemistry

Fresh frozen muscle sections (5 μm) were stained with rabbit anti-EcSOD (Sigma), and image acquisition and analysis of capillary density were performed as described.[3]

### Somatic Gene Transfer and Measurement of Myofiber Size

We performed electric pulse–mediated gene transfer by injecting DNA (15 μg of *pGFP3* plus 50 μg of *pEcSOD* or *pCI-neo*) in tibialis anterior muscles as described.[24] Confocal microscopy (Fluoview 1000; Olympus) was performed to measure the fiber size of GFP-positive fibers (>100 fibers for each mouse) using ImageJ.[6]

### Treadmill Running Test

The test was performed as described.[3] Blood lactate level was measured before, at 40 minutes, and immediately after the test by Lactate Scout (SensLab, Leipzig, Germany).

### Whole Body Tension Test

Whole body tension test was performed according to an established method[25] in a custom-designed device. Ten trials were performed with 10-second intervals for each mouse, and the mean value of the 5 peak readings was used for statistical analysis.

### Magnetic Resonance Imaging

MRI was used to assess cardiac function as described.[26]

### Electrocardiography

Electrocardiography was performed on conscious mice using the ECGenie (Mouse Specifics, Boston, MA). Mice were placed on the platform for 10 minutes to acclimate. Data with continuous recordings of >40 heart beats were used for analyses by using eMOUSE, a physiological waveform analysis platform (Mouse Specifics).

### Whole-Mount Immunofluorescence

Flexor digitorum brevis muscles were immunolabeled for smooth muscle α-actin (IA4-Cy3; Sigma) and isolectin (IB4-Alexa647 or Alexa488; Invitrogen) as described.[27]

### Statistics

The results are presented as mean±SE with each of the individual data points plotted. Comparisons between 2 groups were analyzed by the Wilcoxon rank-sum test. A 2-way ANOVA was used for comparisons across groups, and when appropriate, a post hoc test was used for detection of differences. When the data failed to meet the normality assumption, logarithmic transformation (base 10) was used before ANOVA. When the data failed to meet the equal variance assumption, a nonparametric test (Kruskal–Wallis) was used and, when appropriate, the Dunn post hoc analysis to detect difference across specific means. A $P$ value <0.05 was required to report significance.

## Results

### Systemic Administration of GSNO Induces EcSOD Expression and Prevents Dexamethasone-Induced Muscle Wasting

We have recently shown that an NO-dependent antioxidant defense system may underlie the resistance of oxidative muscles to catabolic muscle wasting.[3,6,16] To determine whether NO donor is sufficient to provide protection in vivo and to identify the antioxidant gene(s) that is functionally involved, we performed daily injections of dexamethasone (25 mg/kg, intraperitoneally) with/without endogenous NO donor GSNO (2 mg/kg, intraperitoneally twice a day) in mice for 7 days. Dexamethasone injection led to a significant increase in lipid peroxidation in skeletal muscle as assessed by 4-hydroxynonenal immunoblot (Figure SI in the Data Supplement). Dexamethasone injection caused a significant reduction of EcSOD protein expression (−29.7%; $P$<0.001), but not for CuZnSOD, MnSOD, or catalase (Figure 1A). GSNO alone induced a moderate increase of EcSOD ($P$<0.05) and completely prevented the reduction of EcSOD protein expression induced by dexamethasone (Figure 1A). Dexamethasone induced a significant increase of ubiquitin E3 ligase *MAFbx/Atrogin-1* mRNA (1.9-fold; $P$<0.05), which was completely blocked by GSNO (Figure 1B). No significant differences were detected for *MuRF1* mRNA. Importantly, dexamethasone injection induced significant atrophy in fast-twitch, glycolytic EDL (–14.5%; $P$<0.001), tibialis anterior (–4.6%; $P$<0.05), gastrocnemius (–10.8%; $P$<0.001), and plantaris muscles (–9.5%; $P$<0.001), but not in slow-twitch, oxidative soleus muscle. Therefore, endogenous NO donor administration significantly attenuated muscle atrophy in all the fast-twitch, glycolytic muscles (Figure 1C).

### EcSOD Is Highly Expressed in Oxidative Muscle, Induced by Endurance Exercise Training and Accumulates at Capillary Endothelial Cells

To determine which type(s) of muscle fibers expresses EcSOD, we used immunofluorescence. We found that EcSOD was expressed at a moderate level in most of the fibers in fast-twitch, glycolytic plantaris muscle (Figure 2A). In contrast, EcSOD was highly expressed in most of the fibers in slow-twitch, oxidative soleus muscle with clear evidence of enrichment at capillary endothelial cells (Figure 2A, indicated by arrows). The specificity of immunofluorescence was confirmed both by negative staining without primary antibody (not shown) and by positive staining of aortic smooth muscle and bronchioles of the lung (Figure 2A). Fiber type analysis revealed that EcSOD was primarily expressed by type IIa and IId/x myofibers (Figure 2B), whereas neither type IIb nor type I fibers expressed detectable levels of EcSOD. Furthermore, immunoblot analysis provided clear evidence that EcSOD is expressed more abundantly in slow-twitch, oxidative soleus muscle than fast-twitch, glycolytic white vastus lateralis muscle ($P$<0.05; Figure 2C). Finally, voluntary running (4 weeks) induced EcSOD protein expression in plantaris muscle (+79.9%; $P$<0.01; Figure 2D). Therefore, EcSOD expression in skeletal muscle is enhanced by contractile activities, which may underlie the protective effects of exercise training and oxidative phenotype.[3,6]

### Somatic Gene Transfer–Mediated EcSOD Overexpression in Adult Skeletal Muscle Protects Myofibers From Atrophy

Our finding that dexamethasone injection decreases EcSOD expression in atrophying muscle suggests that reduced EcSOD expression plays a role in catabolic wasting. Blocking the reduction of EcSOD expression along with the prevention of muscle atrophy by GSNO administration further supports this notion. To determine if enhanced EcSOD expression is sufficient to protect myofibers from catabolic wasting, we performed electric pulse–mediated gene transfer in fast-twitch, glycolytic tibialis anterior muscle. High levels of exogenous EcSOD expression were confirmed when compared with the contralateral control muscle (*pCI-neo*; Figure 3A). Systemic administration of dexamethasone resulted in a leftward shift (reduction) of the fiber size histogram with a significant reduction of the mean cross-sectional area in *pCI-neo*–transfected myofibers (−14.5%; $P$<0.01), whereas myofibers transfected with EcSOD were protected from atrophy (Figure 3B and 3C).

### Muscle-Specific EcSOD Expression in Transgenic Mice Significantly Attenuates Muscle Wasting

To further ascertain the protective role of EcSOD in catabolic muscle wasting, we generated a transgenic mouse line with muscle-specific overexpression of EcSOD under the

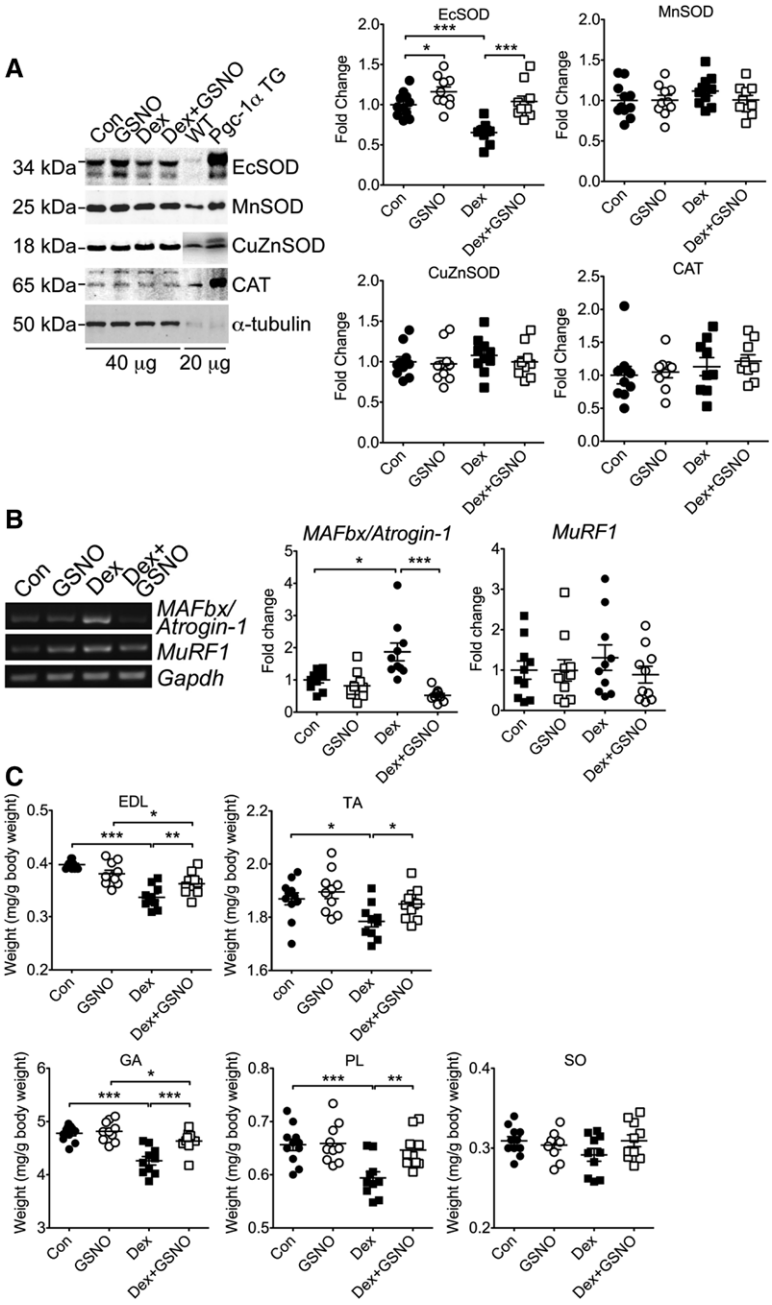


**Figure 1.** Systemic administration of endogenous nitric oxide (NO) donor *S*-nitrosoglutathione (GSNO) induces extracellular superoxide dismutase expression and prevents dexamethasone (Dex)-induced muscle atrophy. Wild-type mice were subjected to daily injections of Dex or saline as control (Con) with/without endogenous NO donor GSNO for 7 d. Various muscles were harvested for muscle weight measurements, and plantaris muscles were processed for immunoblot and semiquantitative PCR analyses. **A**, Immunoblot images of antioxidant enzymes in plantaris muscle (40 μg total protein) with samples from a MCK-Pgc-1α transgenic mouse (Pgc-1α TG) and a wild-type littermate (WT) as positive and negative controls (20 μg), respectively. α-Tubulin was probed as loading control. Quantitative and statistical analyses are presented on the right (n=9–11 per group). **B**, Images of semiquantitative RT-PCR analysis for *MAFbx/Atrogin-1* and *MuRF1* mRNA with quantitative and statistical analyses presented on the right (n=9–11 per group). **C**, Quantification of muscle weight of extensor digitorum longus, tibialis anterior (TA), gastrocnemius (GA), plantaris (PL), and soleus (SO) muscles from mice. Data are presented as normalized muscle weight by body weight (n=9–11 per group). *,**,***$P<0.05$, $P<0.01$, $P<0.001$, respectively.

control of the *MCK* promoter (Figure 4A, top left panel). We detected significantly greater EcSOD protein expression in skeletal muscles in *MCK-EcSOD* mice than in WT littermates (Figure 4A, top right panel). The increases seemed to be more profound in plantaris and white vastus lateralis muscles than in soleus muscle. Immunofluorescence of EcSOD along with fiber typing analysis showed that exogenous EcSOD was highly expressed in type IIb fibers (Figure 4A, bottom panel). These fiber type–specific expression patterns are consistent with the notion that the *MCK* promoter is more active in fast-twitch, glycolytic fibers. EcSOD also appeared to be enriched at endothelial cells (Figure 4A, bottom panel by arrows). Consistent with findings presented in Figure 1, dexamethasone injection induced muscle mass loss in WT mice by −27.5% for EDL ($P<0.001$), −19.5% for tibialis anterior ($P<0.01$), 19.0% for gastrocnemius ($P<0.001$), and 14.7% for plantaris ($P<0.001$) muscles, but not in soleus muscle (Figure 4B). More importantly, dexamethasone injection did not cause significant reduction in muscle mass in any of these muscles in *MCK-EcSOD* mice, suggesting that enhanced EcSOD expression is sufficient to protect fast-twitch, glycolytic fibers from catabolic wasting (Figure 4B). Protein expression of antioxidant enzymes (CuZnSOD, MnSOD, and catalase) and mitochondrial (cytochrome oxidase IV and cytochrome C) and contractile proteins (MHC I, IIa, and IIb) were not significantly different between *MCK-EcSOD* mice and WT littermates in these muscles (Figure 4C), suggesting that the protection in *MCK-EcSOD* mice was most likely because of enhanced antioxidant function, not adaptive responses induced by transgene overexpression.

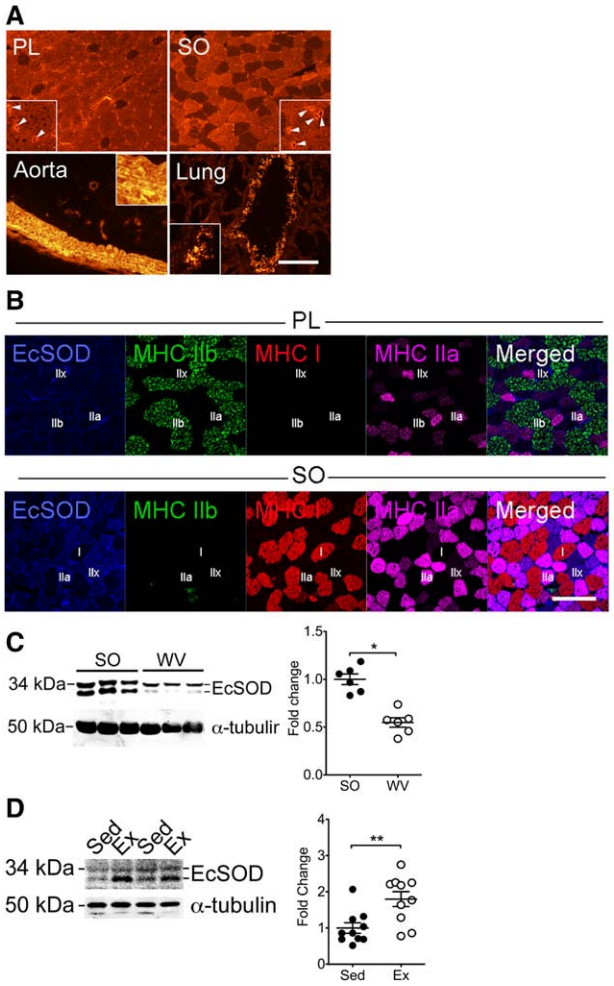


**Figure 2.** Extracellular superoxide dismutase (EcSOD) is highly expressed in oxidative muscle and is induced by endurance exercise training. Various tissues were harvested from wild-type mice and from sedentary mice and exercise-trained mice after 4 weeks of voluntary running and processed for immunoblot analysis and immunohistochemistry. **A**, Immunofluorescence staining showing EcSOD expression in plantaris (PL) and soleus (SO) muscle sections in comparison with the aorta and lung sections. High-magnification images were shown in the brackets with arrows pointing to the capillary endothelial cells. Scale bar, 100 μm. **B**, Immunofluorescence staining of PL and SO muscle sections showing EcSOD (blue) is mainly expressed by type IIa (pink) and IId/x (fibers with no staining) myofibers. **C**, Immunoblot images of EcSOD protein in SO and white vastus lateralis (WV) muscles (40 μg total protein) with α-tubulin as loading control. Quantitative and statistical analyses are presented on the right (n=6 per group). **D**, Immunoblot images of EcSOD protein in plantaris muscle (40 μg total protein) from sedentary (Sed) and exercise-trained (Ex) mice after 4 weeks of voluntary running with α-tubulin as loading control. Quantitative and statistical analyses are presented on the right (n=10 per group). *,**$P$<0.05, $P$<0.01, respectively.

## Muscle-Specific EcSOD Transgenic Mice Are Resistant to Cachexia and Exercise Intolerance Induced by CHF

Our previous studies[6,16] and current findings (Figures 1, 3, and 4) suggest that enhanced EcSOD expression is sufficient to preserve skeletal muscle mass and function under conditions of cachexia. We expected that *MCK-EcSOD* mice would be resistant to CHF in developing cachexia and exercise intolerance. To this end, we generated *MCK-EcSOD:α-MHC-CSQ* double-transgenic mice by crossbreeding. Although *α-MHC-CSQ* and *MCK-EcSOD:α-MHC-CSQ* double-transgenic mice had similar mortality, probably because of severe dilated cardiomyopathy (Figure SII in the Data Supplement), a significant attenuation of cachexia (assessed by body weight) was observed in *MCK-EcSOD:α-MHC-CSQ* double-transgenic mice at 7 weeks of age compared with *α-MHC-CSQ* mice ($P$<0.01; Figure 5A). Importantly, CHF-induced exercise intolerance as indicated by a dramatic reduction in treadmill running distance ($P$<0.001) was blunted in *MCK-EcSOD:α-MHC-CSQ* mice ($P$<0.001 versus CSQ; Figure 5B). This functional protection was confirmed by blood lactate levels; all 4 groups of mice displayed increases in blood lactate, but the highest levels independent of time points were observed in *α-MHC-CSQ* mice ($P$<0.05 versus WT, TG, DTG; Figure 5C). The area under curve of blood lactate for the first 40 minutes of the running test was significantly higher in *α-MHC-CSQ* mice compared with WT mice ($P$<0.01), which was blunted in *MCK-EcSOD:α-MHC-CSQ* mice ($P$<0.05 versus CSQ). Noninvasive whole body tension test showed that *α-MHC-CSQ* mice have a significantly reduced force production (39.9%; $P$<0.01) compared with WT mice (Figure 5D), which was also blunted in *MCK-EcSOD:α-MHC-CSQ* mice ($P$<0.05 versus CSQ).

Improved skeletal muscle function in *MCK-EcSOD:α-MHC-CSQ* mice could theoretically be a result of improved cardiac function by protection from overexpressed EcSOD redistributing to the heart. To test this possibility, we first performed immunoblot and semiquantitative RT-PCR and found significantly increased EcSOD protein in the heart without increased EcSOD mRNA (Figure SIII in the Data Supplement) along with elevated EcSOD in the serum in *MCK-EcSOD* mice, suggesting that muscle EcSOD redistributed to the heart through the circulation. *MCK-EcSOD:α-MHC-CSQ* mice, however, developed the same degree of cardiac hypertrophy (Figure 5E) and reduction of ejection fraction as *α-MHC-CSQ* mice (Figure 5F). Electrocardiography showed significantly increased QRS intervals (Table) and decreased heart rate ($P$<0.01) in these mice to the same degree (Figure 5G). Therefore, reduced cachexia and improved muscle function in *MCK-EcSOD:α-MHC-CSQ* mice did not seem to be a result of improved cardiac function. We also crossbred *MCK-EcSOD* mice with a genetic model of amyotrophic lateral sclerosis, *SOD1-G93A* mice. *SOD1-G93A* mice harbor transgene expressing autosomal dominant fashion of a mutant form of CuZnSOD that results in amyotrophic lateral sclerosis–like motor neuron disease. *SOD1-G93A* and *SOD1-93A:MCK-EcSOD* mice had similar survival curve (Figure SV in the Data Supplement) with significant reduction of body weight at 14 weeks of age and reduced muscle force production at 8 weeks of age (Figure SV in the Data Supplement). Therefore, EcSOD did not provide protection for skeletal muscle when the insults were of motor neuron origin.

## Muscle-Specific EcSOD Transgenic Mice Are Resistant to CHF in Developing Cachexia and Multiple Abnormalities

To obtain structural and biochemical evidence of EcSOD-mediated protection, we measured muscle mass and

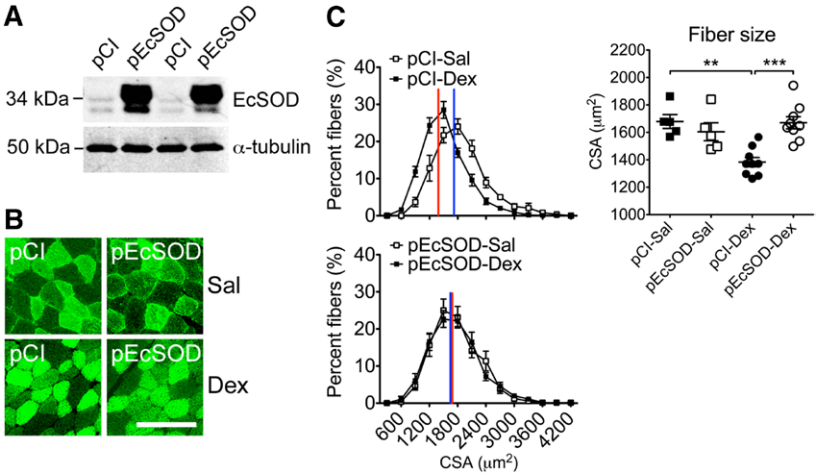


**Figure 3.** Somatic gene transfer–mediated extracellular superoxide dismutase (EcSOD) overexpression in adult skeletal muscle is sufficient to prevent dexamethasone (Dex)-induced catabolic muscle wasting. Ten days after electric pulse–-mediated gene transfer in tibialis anterior muscles (see Methods section), mice received daily injections of Dex or saline (Sal) as control for 7 days, and the tibialis anterior muscles were harvested and processed for protein and muscle fiber size analyses by immunoblot and fluorescent microscopy. **A**, Immunoblot images of EcSOD protein expression in left tibialis anterior muscle transfected with *pEcSOD* and the contralateral control muscle transfected with empty vector *pCI-neo* (pCI) with α-tubulin as loading control (40 μg total protein). **B**, Fluorescent microscopy images of muscle sections with cotransfection of *pGFP3*. **C**, Histograms of fiber size distribution according to cross-sectional area (CSA; **left**, **top**, and **bottom**) and comparison of the mean CSA across treatment conditions (**right;** n=5–9 per group). **,***$P$<0.01 and $P$<0.001, respectively.

normalized by tibia length to minimize the effect of body weight reduction. CHF led to a significant loss of muscle mass in EDL (–38.4%; $P$<0.01), tibialis anterior (–37.3%; $P$<0.01), gastrocnemius (–42.3%; $P$<0.001), plantaris (–43.3%; $P$<0.01) but to a less degree in soleus (–28.7%; $P$<0.001) muscles in *α-MHC-CSQ* mice compared with WT littermates, which were significantly attenuated in *MCK-EcSOD:α-MHC-CSQ* mice (Figure 6A). Fiber size analyses showed that *α-MHC-CSQ* mice had significantly reduced IId/x/IIb fiber size (–42.8%; $P$<0.05; Figure 6B), which was blunted by EcSOD overexpression ($P$<0.05 versus CSQ). Consistent with our previous finding,[3] *α-MHC-CSQ* mice had significantly decreased capillary contacts in glycolytic type IId/x/IIb fibers ($P$<0.01), but not in oxidative type IIa fibers. The loss of capillarity was significantly attenuated in *MCK-EcSOD:α-MHC-CSQ* mice ($P$<0.05 versus CSQ; Figure 6C). We also performed whole-mount immunofluorescence staining for CD31 and smooth muscle α-actin in flexor digitorum brevis muscle and measured the fractal dimension of microvessels (arterioles and venules), a measure of the number of branches and microvessels. There were no significant differences in fractal dimension among different groups

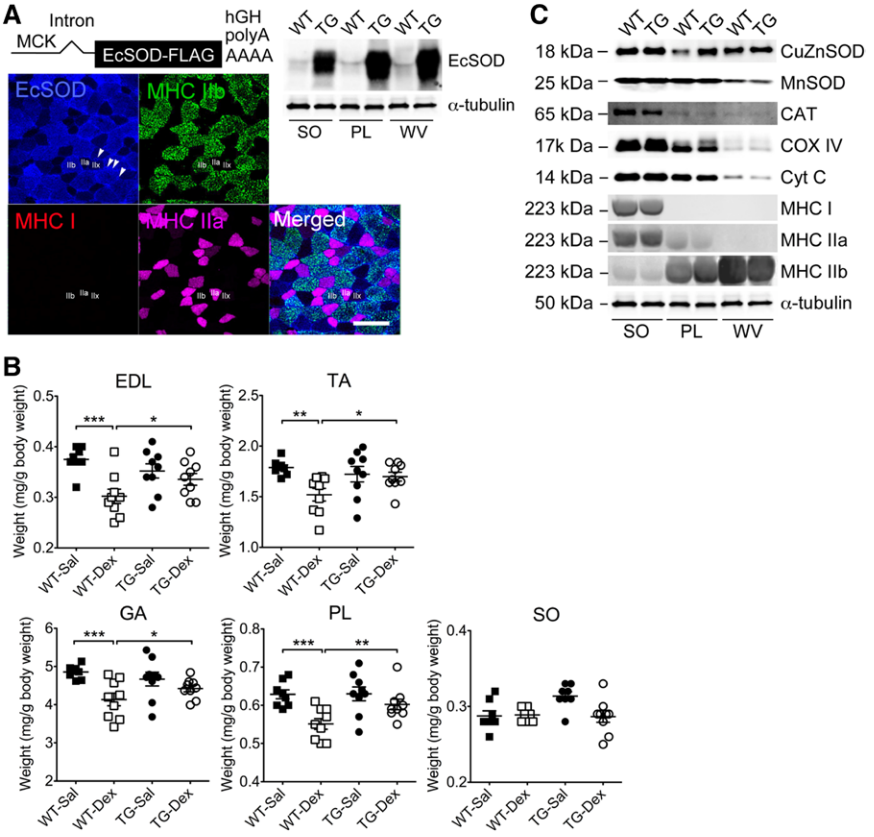


**Figure 4.** Muscle-specific extracellular superoxide dismutase (EcSOD) transgenic mice are resistant to dexamethasone (Dex)-induced catabolic muscle wasting. Skeletal muscles were harvested from *MCK-EcSOD* mice (TG) and wild-type littermates (WT) and processed for immunofluorescence and immunoblot analyses. Muscles were also harvested for muscle weight from TG and WT mice after 7 days of Dex injections with saline (Sal) injections as control. **A**, EcSOD transgene construct is illustrated at the top. Immunoblot images of EcSOD expression in soleus (SO), plantaris (PL), and white vastus lateralis (WV) muscles from TG and WT mice (40 μg total protein) with α-tubulin as loading control are presented on the right. Immunofluorescence images are presented at the bottom showing EcSOD overexpression mainly in fast-twitch, glycolytic type IIb fibers (green) with enrichment around endothelial cells (indicated by arrows). **B**, Quantification of muscle weight in extensor digitorum longus, tibialis anterior (TA), gastrocnemius (GA), PL, and SO muscles as normalized muscle weight by body weight (n=9–12 per group). **C**, Immunoblot images of other antioxidant enzymes, mitochondrial and muscle contractile proteins in SO, PL, and WV muscle in TG and WT mice. *,**,***$P$<0.05, $P$<0.01, $P$<0.001, respectively.

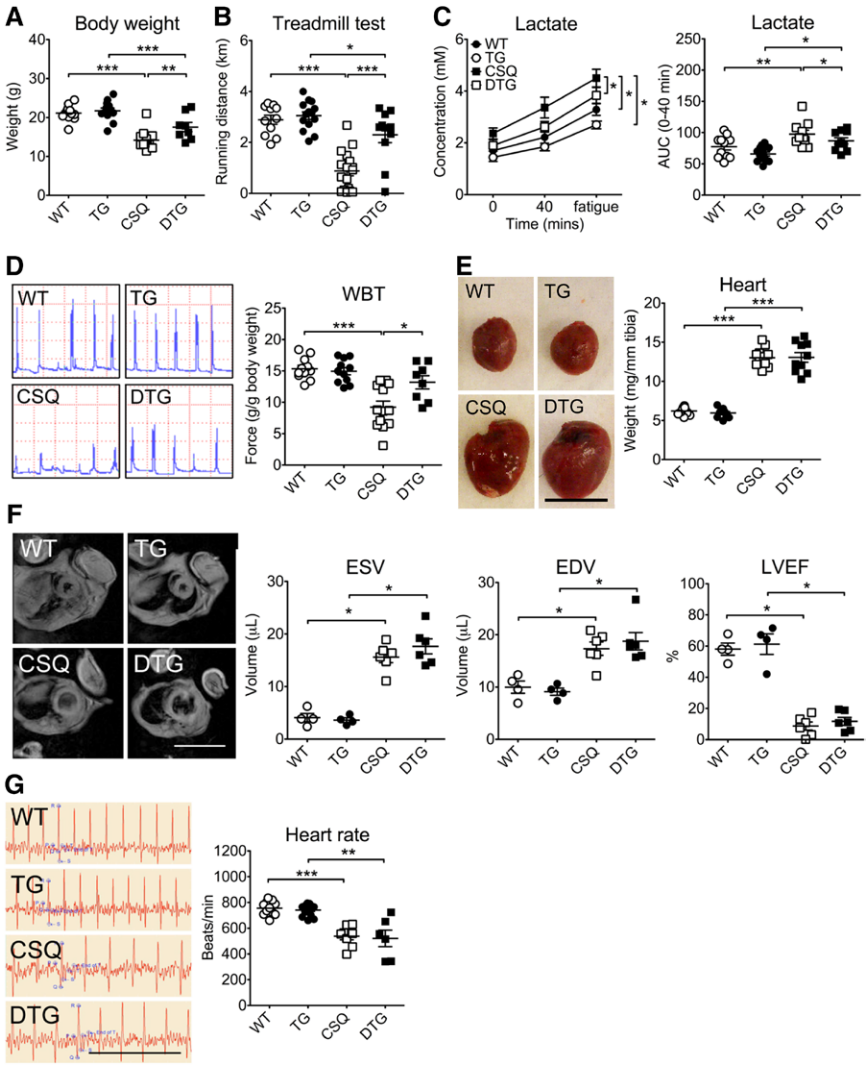


**Figure 5.** Muscle-specific extracellular superoxide dismutase (EcSOD) transgenic mice are resistant to cardiac cachexia and exercise intolerance. *MCK-EcSOD* mice (TG) were crossbred with *α-MHC-CSQ* mice (CSQ) to generate *MCK-EcSOD:α-MHC-CSQ* double-transgenic mice (DTG). These mice were analyzed for body weight, running capacity, whole body tension, electrocardiography, and MRI with wild-type littermates (WT) as control. **A**, Body weight at 7 wk of age (n=8–14 per group). **B**, Treadmill running test at 5 wk of age. **C**, Blood lactate levels (n=11–17 per group) during treadmill running test (**left**) and the area under the curve (AUC) during the first 40 min of the test (**right**). **D**, Representative force production records of whole body tension test at 7 wk of age (**left**) with quantitative and statistical analyses presented on the right (n=8–13 per group). **E**, Representative heart images (**left**; bar, 1 cm) with quantitative and statistical analyses presented on the right (n=10–12 per group). **F**, Representative MRI (**left**; scale bar, 2 cm) of the thoracic chambers with quantification of end-systolic volume (ESV), end-diastolic volume (EDV), and left ventricle ejection fraction (LVEF) of the heart at 6 wk of age (n=4–6 per group). **G**, Representative electrocardiography recordings (**left**; scale bar, 0.5 s) and quantification of the heart rate (**right**) at 7 wk of age (n=6–16 per group). *,**,***$P<0.05$, $P<0.01$, $P<0.001$, respectively.

(Figure 6D), suggesting that vascular rarefaction did not occur at the arteriole and venule levels in *α-MHC-CSQ* mice. *α-MHC-CSQ* mice are known to have reduced mitochondrial (cytochrome C, cytochrome oxidase IV) and PGC-1α protein expression indicative of mitochondrial dysfunction in fast-twitch, glycolytic muscles.[3,6] Here, we showed that a preferentially reduced expression of these proteins in fast-twitch, glycolytic white vastus lateralis muscle was absent in *MCK-EcSOD:α-MHC-CSQ* mice (Figure 6D), suggesting that the overexpression of EcSOD is sufficient to block CHF-induced mitochondrial dysfunction in skeletal muscle. Consistently, CHF condition in *α-MHC-CSQ* mice did not cause any significant changes in myosin heavy chain protein isoforms, and EcSOD overexpression alone in *MCK-EcSOD* mice did not cause compensatory changes either. Real-time PCR analysis showed that *α-MHC-CSQ* mice had significantly elevated *MAFbx/Atrogin-1* mRNA ($P<0.001$), which was significantly reduced in *MCK-EcSOD:α-MHC-CSQ* mice ($P<0.05$ versus CSQ; Figure 6F). We did not detect significant differences of apoptosis assessed by TUNEL staining (Figure SIV in the Data Supplement). Most importantly, we performed 3 different analyses to determine whether EcSOD overexpression had any significant impact on oxidative stress induced by CHF. We found that CHF (in *α-MHC-CSQ* mice) led to significantly elevated levels of oxidative stress markers, malondialdehyde and protein carbonyls, in fast-twitch, glycolytic white vastus lateralis muscle, which were significantly reduced in *MCK-EcSOD:α-MHC-CSQ* mice (Figure 6G; Figure SVI in the Data Supplement). A similar trend, although not statistically significant, was found for another oxidative stress marker of lipid peroxidation, 4-hydroxynonenal

**Table. Muscle-Specific Extracellular Superoxide Dismutase (EcSOD) Expression Does Not Provide Significant Protection Against Congestive Heart Failure in *α-MHC-CSQ* Mice as Assessed by Electrocardiography**

| | WT (n=11) | TG (n=16) | CSQ (n=8) | DTG (n=6) |
|---|---|---|---|---|
| PR, ms | 25.1±1.3 | 26.1±0.7 | 29.9±3.1* | 38.9±6.8‡ |
| QRS, ms | 10.9±0.2 | 10.8±0.2 | 16.5±1.8† | 17.4±2.0§ |
| QT, ms | 39.6±0.8 | 40.4±0.7 | 57.6±4.1† | 64.4±8.6§ |

Data were obtained from *MCK-EcSOD* (TG), *α-MHC-CSQ* (CSQ), and *MCK-EcSOD:α-MHC-CSQ* double-transgenic mice (DTG) and their wild-type (WT) littermates and presented as mean±SE.

*, †$P<0.01$ and $P<0.001$, respectively, vs WT.

‡$P<0.05$ vs CSQ.

§$P<0.001$ vs TG.

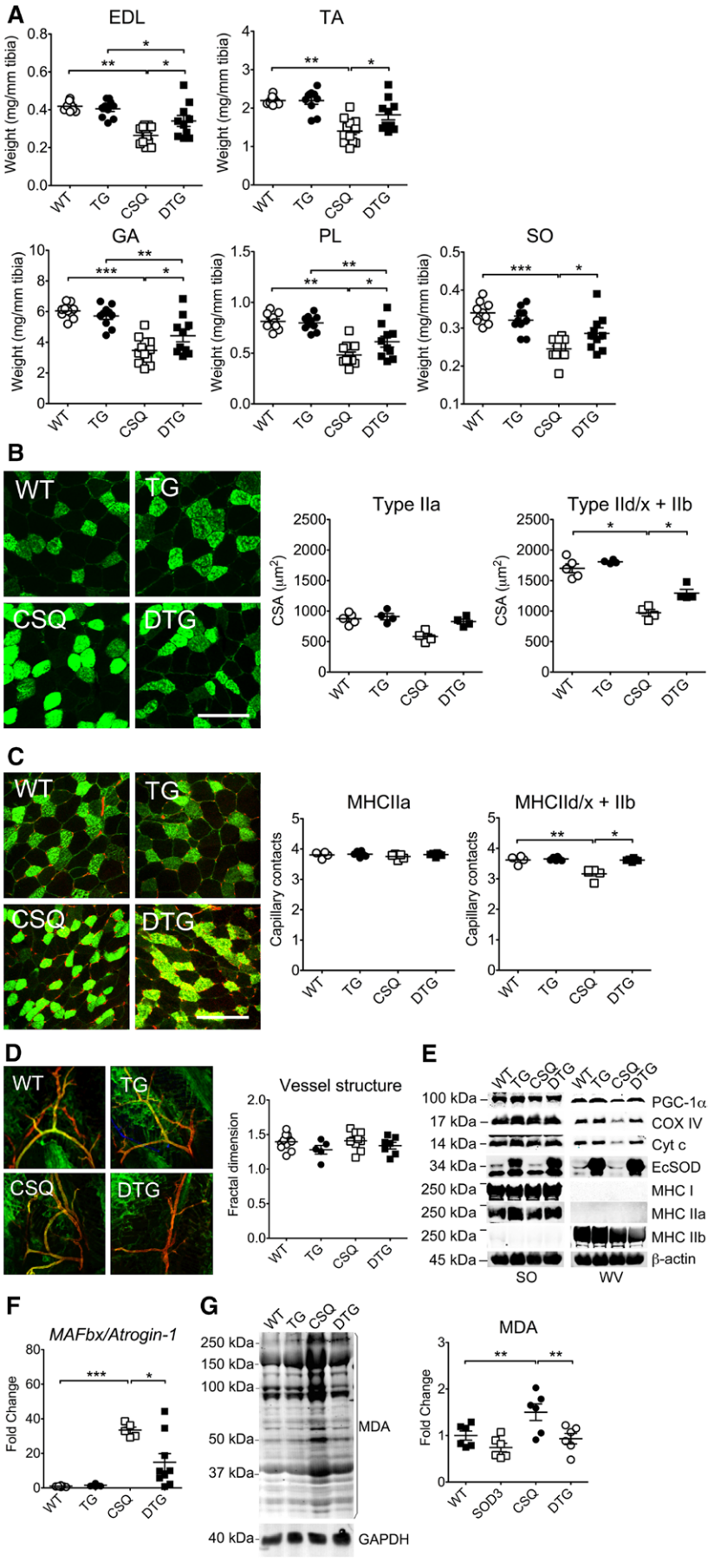


**Figure 6.** Muscle-specific overexpression of extracellular superoxide dismutase (EcSOD) prevents congestive heart failure–induced catabolic muscle wasting, loss of mitochondria, vascular rarefaction, and oxidative stress. Skeletal muscles were harvested from *MCK-EcSOD* (TG), *α-MHC-CSQ* (CSQ), and *MCK-EcSOD:α-MHC-CSQ* double-transgenic mice (DTG) and their wild-type littermates (WT) at 7 wk of age and processed for measurements of muscle weight, muscle fiber size, contractile and mitochondrial protein expression, atrogene mRNA expression, and oxidative stress. **A**, Quantification of muscle weight normalized by tibia length in extensor digitorum longus (EDL), tibialis anterior (TA), gastrocnemius (GA), plantaris (PL), and soleus (SO) muscles (n=9–12 per group). **B**, Immunofluorescence images of type IIa (green) and type IId/x/IIb fibers (negative staining) with quantification of fiber size in plantaris muscle (n=4–5 per group). **C**, Immunofluorescence staining for CD31 (red) and MHC IIa (green) with quantification of fiber type–specific capillary contacts in plantaris muscle (n=4–5 per group). **D**, Whole-mount immunofluorescence staining of CD31 (green) and smooth muscle α-actin (red) in flexor digitorum brevis muscle (**left**) with quantification of the fractional dimension of microvessels (**right**; n=5–12 per group). **E**, Representative immunoblot images of PGC-1α, cytochrome oxidase IV, cytochrome C, EcSOD, MHC I, MHC IIa, and MHC IIb proteins in SO and white vastus lateralis muscles (WV) with β-actin as loading control. **F**, Real-time PCR analysis for *MAFbx/Atrogin-1* mRNA expression in PL muscle (n=7–9 per group). **G**, Representative immunoblot images of protein carbonylation in WV with GAPDH as loading control. *,**,***$P<0.05$, $P<0.01$, $P<0.001$, respectively.

(Figure SVI in the Data Supplement). These findings provide evidence for EcSOD function in protecting skeletal muscle from cardiac cachexia at molecular, biochemical, structural, and functional levels likely through its ectopic antioxidant function.

## Discussion

Studies with genetic and pharmacological interventions in cultured myocytes and intact skeletal muscles have provided ample evidence that intracellular antioxidant defenses are critical in maintaining normal redox state, muscle mass, and function.[28,29] Here, we provide clear in vivo evidence that EcSOD expression in skeletal muscle is reduced under glucocorticoidism, a condition of many chronic diseases including CHF that is associated with increased oxidative stress,[30] muscle wasting, and loss of muscle function.[18] We showed that systemic administration of endogenous NO donor could attenuate skeletal muscle atrophy induced by dexamethasone in mice concurrent with a restoration of EcSOD expression. These findings suggest that NO-dependent EcSOD expression is important for muscle maintenance in vivo. We then used 2 independent genetic approaches and showed that myofibers with acquired EcSOD expression do not undergo significant atrophy on glucocorticoidism. Muscle-specific EcSOD transgenic mice are resistant to CHF, displaying significant protection against cachexia, exercise intolerance, and loss of mitochondria, rarefaction of microvasculature, and increased atrophy gene expression in skeletal muscle. Most importantly, we have shown evidence of reduced oxidative stress when ectopic EcSOD is overexpressed in a mouse model of cardiac cachexia. These findings provide, for the first time, unequivocal evidence that induced EcSOD expression protects myofibers from catabolic muscle wasting in vivo, suggesting that promoting EcSOD expression by augmenting NO bioavailability in skeletal muscle could be an effective intervention for CHF patients.

NO plays an important signaling role in physiological and pathological processes. We have reported that injection of lipopolysaccharide, a major mediator of sepsis, induces greater NO production in slow-twitch, oxidative muscle than in fast-twitch, glycolytic muscle in mice.[3,16] Because these oxidative muscles are resistant to catabolic muscle wasting under many disease conditions, the findings suggest that elevated NO production is protective. Our previous studies in cultured myocytes provided further evidence for a functional link between NO and antioxidant gene expression.[6,16] Consistently, the data presented in this and previous studies[16,31] showed robust induction of EcSOD expression when there is increased NO bioavailability, suggesting that NO-induced EcSOD expression in skeletal muscle may contribute significantly to the protection associated with oxidative phenotype.

The functional importance of EcSOD has been shown by its reduced expression associated with various chronic diseases[10,11] and by the fact that genetic deletion and overexpression of EcSOD leads to exacerbation and protection against pathological changes, respectively, in animal models of diseases.[12–15] Glucocorticoids are used as therapeutic agents because of their potent anti-inflammatory and immunosuppressive functions; however, high dose and long-term use of glucocorticoids causes muscle atrophy.[22,32] Previous studies and our current data support an increased oxidative stress induced by dexamethasone in skeletal muscle with direct evidence of increased free radicals.[22,33] Oxidative stress may promote catabolic wasting and loss of muscle function through cell signaling, impairment of contractile protein, and promotion of protein degradation.[4,5,34,35] Here, we provide new evidence for reduced EcSOD protein expression in catabolic muscle wasting and the functional importance of NO-mediated EcSOD expression in protection against catabolic wasting.

A key question is how EcSOD protects skeletal muscle from atrophy. Because EcSOD is the only isoform of SOD that is secreted to the extracellular space, one straightforward explanation is that elevated extracellular ROS, which could be scavenged by EcSOD, plays an important role in cachexia and exercise intolerance. Several pathological steps might be relevant here. First, extracellular ROS could cause endothelial cell apoptosis,[36] leading to vascular rarefaction, an important feature of cardiac cachexia. Vascular rarefaction not only impairs microcirculation and tissue perfusion but also exacerbates muscle wasting due to hypoxia. Second, superoxide reacts readily with NO to reduce NO bioavailability,[37] impairing exercise-induced increase of blood flow and contributing to exercise intolerance. Finally, extracellular ROS-mediated intracellular ROS production and signaling[38] may play an important role in catabolic muscle wasting. Enhanced EcSOD expression could provide protection through ≥1 of these mechanisms. Studies showing protective effects of antioxidants when applied directly to cultured cells seem to be consistent with EcSOD function in scavenging extracellular superoxide; however, in light of the findings that EcSOD can be internalized by cells that it binds,[9] elevated intracellular EcSOD in myofibers or other cells in skeletal muscle may also contribute to the protection.

The protective role of EcSOD is likely mediated by its antioxidant properties. Based on the evidence of reduced oxidative stress markers and atrogene expression in glycolytic muscles (Figure 6F and 6G), we think that transgenic overexpression of EcSOD in skeletal muscle exerts its protective function by counteracting oxidative stress induced by CHF. The findings of muscle mass, mitochondrial protein, and vascular rarefaction are all extremely consistent with this notion. Future studies should focus on elucidating the necessity of EcSOD expression in skeletal muscle in maintaining muscle mass and function under catabolic wasting conditions.

It has been shown that EcSOD expression by smooth muscle can be enhanced by exercise training in an NO-dependent manner,[31] and EcSOD mRNA is increased by acute exercise in skeletal muscle.[39] It has been shown recently that exercise training protects oxidative stress and proteasome-dependent protein degradation in CHF-induced skeletal muscle atrophy.[40] An unanswered question is which effecter gene(s) is responsible for the enhanced antioxidant function induced by exercise training. Our findings suggest EcSOD could be an important player. We show here that exercise training promotes EcSOD expression in skeletal muscle (Figure 2), and that enhanced EcSOD expression is sufficient to provide protection against cardiac cachexia and exercise intolerance.

Another issue is whether enhanced expression of EcSOD from skeletal muscle redistributes to the extracellular space of

the heart leading to improved cardiac function and preventing cachexia. EcSOD-mediated protection against cardiac toxicity has been shown by Kliment et al[41] where EcSOD global knockout mice showed exacerbated cardiac dysfunction, myocardial apoptosis, and fibrosis after doxorubicin injection. We have previously shown that adenovirus-mediated gene transfer of EcSOD in the heart elicits protective effects on cardiac function after myocardial infarction in rabbits.[42] In this study, although forced expression of EcSOD from skeletal muscle redistributed to the heart (Figure SIII in the Data Supplement), it did not improve cardiac function and prevent premature death. These findings allowed us to focus on the impact of ectopic EcSOD expression in skeletal muscle on catabolic muscle wasting without confounding factors of EcSOD overexpression in impeding HF. Because abnormal calcium and G-protein coupling signaling are caused by calsequestrin overexpression from within cardiac myocytes,[43] the negative findings of EcSOD effects in the heart suggest that elevated EcSOD in the heart of *α-MHC-CSQ* mouse is not enough to deal with such a severe CHF condition or with pathogenesis of CHF of an intracellular origin.

Previous findings support the view that exercise intolerance in CHF is a result of skeletal muscle abnormalities although the failing heart is the primary cause.[44–46] Consistent with this scenario, our findings suggest that the improved skeletal muscle outcomes in *MKC-EcSOD:α-MHC-CSQ* mice were not because of improved cardiac function. Our measurements of baseline heart function, heart weight, skeletal muscle weight, and survival curve collectively suggest that EcSOD overexpression had a negligible impact on the progression of HF induced by cardiac overexpression of calsequestrin in our model. On the contrary, skeletal muscle abnormalities are significantly blocked/attenuated along with improved contractile function and exercise capacity. We, therefore, think that the effects of elevated EcSOD in the heart on cardiac function, if present, likely has a minor impact on exercise capacity. It would be interesting to ascertain whether muscle overexpression of EcSOD is protective against other types of CHF.

The concept that exercise intolerance in CHF results from multiple skeletal muscle abnormalities will be instrumental to developing effective interventions. We have previously shown that CHF leads to preferential vascular rarefaction in fast-twitch, glycolytic muscle fibers in CHF,[3] and that skeletal muscles with overexpression of PGC- 1α have significantly elevated EcSOD expression, less muscle atrophy, and vascular rarefaction under the condition of CHF.[6] Here, we again detected vascular rarefaction in fast-twitch, glycolytic fibers in *α-MHC-CSQ* mice, which was significantly attenuated by EcSOD overexpression. Because EcSOD accumulates at the endothelium, EcSOD may also protect endothelial cells against oxidative stress–induced apoptosis. We have also previously shown that skeletal muscles undergoing catabolic wasting lose mitochondria with reduced expression of PGC-1α and increased oxidative stress,[3,6] which may contribute directly to exercise intolerance. The fact that EcSOD overexpression prevents the reduction of mitochondrial and PGC-1α proteins strongly suggests that ROS induced by CHF induces pathological events in muscle. This could explain why enhanced EcSOD expression in skeletal muscle can provide potent protection and lead to preserved exercise capacity in *MCK-EcSOD:α-MHC-CSQ* mice. Previous studies have also linked oxidative stress to catabolic muscle wasting with evidence for enhanced ubiquitin–proteasome[16,47–49] and autophagy–lysosome systems.[22,50] The link of oxidative stress to autophagy, particularly mitochondrial autophagy (mitophagy), may underlie the loss of mitochondria in CHF. A vicious cycle between oxidative stress and mitochondrial dysfunction could be critical for the development of cardiac cachexia and exercise intolerance.

Finally, we have previously shown that overexpression of PGC-1α in skeletal muscle leads to increased inducible NO synthase, endothelial NO synthase, EcSOD, MnSOD, CuZnSOD, and catalase expression in skeletal muscle along with increased FOXO and Akt phosphorylation.[6] We postulated that PGC-1α enhances FOXO and Akt phosphorylation in parallel with enhanced NO–antioxidant defense axis. Our current findings, consistent with this postulation, suggest that increased NO can directly stimulate EcSOD in skeletal muscle providing protection. It is of note that increased NO alone without overexpression of PGC-1α only stimulates EcSOD, but not other antioxidant enzymes. This NO inducibility of EcSOD expression helped us to focus on the function of this important, yet understudied, antioxidant enzyme in protection against muscle wasting.

In summary, we obtained novel findings to support that NO-mediated protection against catabolic muscle wasting is associated with enhanced EcSOD protein expression. Genetic interventions with forced expression of EcSOD in skeletal muscles significantly attenuate muscle atrophy. Importantly, muscle-specific EcSOD transgenic mice are resistant to CHF in developing cachexia and exercise intolerance with significant protection against oxidative stress, atrophic gene expression, and loss of mitochondria and microvasculature. We now propose a model where enhanced skeletal muscle EcSOD expression prevents endothelial and muscular abnormalities in cachexia and exercise intolerance likely because of the maintenance of redox homeostasis (Figure 7). The findings pave

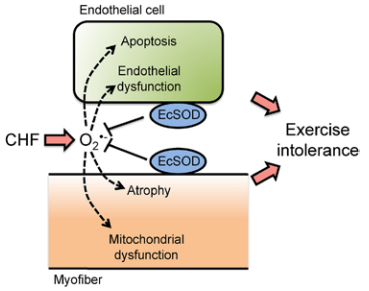


**Figure 7.** Schematic presentation of the working hypothesis for extracellular superoxide dismutase (EcSOD)-mediated protection against congestive heart failure (CHF)-induced catabolic muscle wasting and exercise intolerance. CHF and the resulting systemic factors exacerbate the production of extracellular free radical superoxide ion $O_2^{\cdot-}$, which in turn cause abnormalities in muscle vasculature (endothelial dysfunction and apoptosis) and muscle fibers (atrophy and mitochondrial dysfunction). These abnormalities in skeletal muscle underscore the development of exercise intolerance presumably by impairing blood supply and muscle contractile and metabolic functions. EcSOD in extracellular space in skeletal muscle may provide the first line of defense against the excessive production of extracellular free radicals under the condition of CHF, ultimately preserving muscle function.

the way for targeting NO-dependent EcSOD expression for the prevention and treatment of cardiac cachexia.

## Acknowledgments

We thank John Sanders and R. Jack Roy for their excellent technical support in immunofluorescence and MRI, respectively.

## Sources of Funding

This project was supported by grant AR060444 (to Z.Y.). The research was also supported by Nakatomi Foundation (to M.O.), Postdoctoral Fellowship in Physiological Genomics by the American Physiological Society (to V.A.L.), National Institutes of Health T32-HL007284 (to J.A.C.), and American Heart Association 12POST12030231 (to J.A.C.). This research was supported in part by a grant to S.M.P. (HL082838).

## Disclosures

None.

## References

1. Sullivan MJ, Duscha BD, Klitgaard H, Kraus WE, Cobb FR, Saltin B. Altered expression of myosin heavy chain in human skeletal muscle in chronic heart failure. *Med Sci Sports Exerc*. 1997;29:860–866.
2. Schaufelberger M, Eriksson BO, Grimby G, Held P, Swedberg K. Skeletal muscle fiber composition and capillarization in patients with chronic heart failure: relation to exercise capacity and central hemodynamics. *J Card Fail*. 1995;1:267–272.
3. Li P, Waters RE, Redfern SI, Zhang M, Mao L, Annex BH, Yan Z. Oxidative phenotype protects myofibers from pathological insults induced by chronic heart failure in mice. *Am J Pathol*. 2007;170:599–608.
4. Reid MB, Moylan JS. Beyond atrophy: redox mechanisms of muscle dysfunction in chronic inflammatory disease. *J Physiol.* 2011;589(Pt 9):2171–2179.
5. Powers SK, Smuder AJ, Judge AR. Oxidative stress and disuse muscle atrophy: cause or consequence? *Curr Opin Clin Nutr Metab Care*. 2012;15:240–245.
6. Geng T, Li P, Yin X, Yan Z. PGC-1α promotes nitric oxide antioxidant defenses and inhibits FOXO signaling against cardiac cachexia in mice. *Am J Pathol*. 2011;178:1738–1748.
7. Hauer K, Hildebrandt W, Sehl Y, Edler L, Oster P, Dröge W. Improvement in muscular performance and decrease in tumor necrosis factor level in old age after antioxidant treatment. *J Mol Med (Berl)*. 2003;81:118–125.
8. Mantovani G, Macciò A, Madeddu C, Gramignano G, Lusso MR, Serpe R, Massa E, Astara G, Deiana L. A phase II study with antioxidants, both in the diet and supplemented, pharmaconutritional support, progestagen, and anti-cyclooxygenase-2 showing efficacy and safety in patients with cancer-related anorexia/cachexia and oxidative stress. *Cancer Epidemiol Biomarkers Prev*. 2006;15:1030–1034.
9. Chu Y, Piper R, Richardson S, Watanabe Y, Patel P, Heistad DD. Endocytosis of extracellular superoxide dismutase into endothelial cells: role of the heparin-binding domain. *Arterioscler Thromb Vasc Biol*. 2006;26:1985–1990.
10. Dahl M, Bowler RP, Juul K, Crapo JD, Levy S, Nordestgaard BG. Superoxide dismutase 3 polymorphism associated with reduced lung function in two large populations. *Am J Respir Crit Care Med*. 2008;178:906–912.
11. Chen Y, Hou M, Li Y, Traverse JH, Zhang P, Salvemini D, Fukai T, Bache RJ. Increased superoxide production causes coronary endothelial dysfunction and depressed oxygen consumption in the failing heart. *Am J Physiol Heart Circ Physiol*. 2005;288:H133–H141.
12. Lu Z, Xu X, Hu X, Zhu G, Zhang P, van Deel ED, French JP, Fassett JT, Oury TD, Bache RJ, Chen Y. Extracellular superoxide dismutase deficiency exacerbates pressure overload-induced left ventricular hypertrophy and dysfunction. *Hypertension*. 2008;51:19–25.
13. Carlsson LM, Jonsson J, Edlund T, Marklund SL. Mice lacking extracellular superoxide dismutase are more sensitive to hyperoxia. *Proc Natl Acad Sci U S A*. 1995;92:6264–6268.
14. Yao H, Arunachalam G, Hwang JW, Chung S, Sundar IK, Kinnula VL, Crapo JD, Rahman I. Extracellular superoxide dismutase protects against pulmonary emphysema by attenuating oxidative fragmentation of ECM. *Proc Natl Acad Sci U S A*. 2010;107:15571–15576.
15. Prasad KM, Smith RS, Xu Y, French BA. A single direct injection into the left ventricular wall of an adeno-associated virus 9 (AAV9) vector expressing extracellular superoxide dismutase from the cardiac troponin-T promoter protects mice against myocardial infarction. *J Gene Med*. 2011;13:333–341.
16. Yu Z, Li P, Zhang M, Hannink M, Stamler JS, Yan Z. Fiber type-specific nitric oxide protects oxidative myofibers against cachectic stimuli. *PLoS One*. 2008;3:e2086.
17. Geng T, Li P, Okutsu M, Yin X, Kwek J, Zhang M, Yan Z. PGC-1alpha plays a functional role in exercise-induced mitochondrial biogenesis and angiogenesis but not fiber-type transformation in mouse skeletal muscle. *Am J Physiol Cell Physiol*. 2010;298:C572–C579.
18. Hasselgren PO. Glucocorticoids and muscle catabolism. *Curr Opin Clin Nutr Metab Care*. 1999;2:201–205.
19. Naya FJ, Mercer B, Shelton J, Richardson JA, Williams RS, Olson EN. Stimulation of slow skeletal muscle fiber gene expression by calcineurin in vivo. *J Biol Chem*. 2000;275:4545–4548.
20. Jones LR, Suzuki YJ, Wang W, Kobayashi YM, Ramesh V, Franzini-Armstrong C, Cleemann L, Morad M. Regulation of Ca2+ signaling in transgenic mouse cardiac myocytes overexpressing calsequestrin. *J Clin Invest*. 1998;101:1385–1393.
21. Akimoto T, Ribar TJ, Williams RS, Yan Z. Skeletal muscle adaptation in response to voluntary running in Ca2+/calmodulin-dependent protein kinase IV-deficient mice. *Am J Physiol Cell Physiol*. 2004;287:C1311–C1319.
22. McClung JM, Judge AR, Powers SK, Yan Z. p38 MAPK links oxidative stress to autophagy-related gene expression in cachectic muscle wasting. *Am J Physiol Cell Physiol*. 2010;298:C542–C549.
23. Yan Z, Choi S, Liu X, Zhang M, Schageman JJ, Lee SY, Hart R, Lin L, Thurmond FA, Williams RS. Highly coordinated gene regulation in mouse skeletal muscle regeneration. *J Biol Chem*. 2003;278:8826–8836.
24. Pogozelski AR, Geng T, Li P, Yin X, Lira VA, Zhang M, Chi JT, Yan Z. p38gamma mitogen-activated protein kinase is a key regulator in skeletal muscle metabolic adaptation in mice. *PLoS One*. 2009;4:e7934.
25. Carlson CG, Makiejus RV. A noninvasive procedure to detect muscle weakness in the mdx mouse. *Muscle Nerve*. 1990;13:480–484.
26. Berr SS, Roy RJ, French BA, Yang Z, Gilson W, Kramer CM, Epstein FH. Black blood gradient echo cine magnetic resonance imaging of the mouse heart. *Magn Reson Med*. 2005;53:1074–1079.
27. Bailey AM, O'Neill TJ IV, Morris CE, Peirce SM. Arteriolar remodeling following ischemic injury extends from capillary to large arteriole in the microcirculation. *Microcirculation*. 2008;15:389–404.
28. Muller FL, Song W, Liu Y, Chaudhuri A, Pieke-Dahl S, Strong R, Huang TT, Epstein CJ, Roberts LJ II, Csete M, Faulkner JA, Van Remmen H. Absence of CuZn superoxide dismutase leads to elevated oxidative stress and acceleration of age-dependent skeletal muscle atrophy. *Free Radic Biol Med*. 2006;40:1993–2004.
29. Dobrowolny G, Aucello M, Rizzuto E, Beccafico S, Mammucari C, Boncompagni S, Bonconpagni S, Belia S, Wannenes F, Nicoletti C, Del Prete Z, Rosenthal N, Molinaro M, Protasi F, Fanò G, Sandri M, Musarò A. Skeletal muscle is a primary target of SOD1G93A-mediated toxicity. *Cell Metab*. 2008;8:425–436.
30. Ohtani T, Ohta M, Yamamoto K, Mano T, Sakata Y, Nishio M, Takeda Y, Yoshida J, Miwa T, Okamoto M, Masuyama T, Nonaka Y, Hori M. Elevated cardiac tissue level of aldosterone and mineralocorticoid receptor in diastolic heart failure: Beneficial effects of mineralocorticoid receptor blocker. *Am J Physiol Regul Integr Comp Physiol*. 2007;292:R946–R954.
31. Fukai T, Siegfried MR, Ushio-Fukai M, Cheng Y, Kojda G, Harrison DG. Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. *J Clin Invest*. 2000;105:1631–1639.
32. Waddell DS, Baehr LM, van den Brandt J, Johnsen SA, Reichardt HM, Furlow JD, Bodine SC. The glucocorticoid receptor and FOXO1 synergistically activate the skeletal muscle atrophy-associated MuRF1 gene. *Am J Physiol Endocrinol Metab*. 2008;295:E785–E797.
33. Konno S. Hydroxyl radical formation in skeletal muscle of rats with glucocorticoid-induced myopathy. *Neurochem Res*. 2005;30:669–675.
34. Smuder AJ, Kavazis AN, Hudson MB, Nelson WB, Powers SK. Oxidation enhances myofibrillar protein degradation via calpain and caspase-3. *Free Radic Biol Med*. 2010;49:1152–1160.
35. Clavel S, Siffroi-Fernandez S, Coldefy AS, Boulukos K, Pisani DF, Dérijard B. Regulation of the intracellular localization of Foxo3a by stress-activated protein kinase signaling pathways in skeletal muscle cells. *Mol Cell Biol*. 2010;30:470–480.

36. Cho KS, Lee EH, Choi JS, Joo CK. Reactive oxygen species-induced apoptosis and necrosis in bovine corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 1999;40:911–919.
37. Jung O, Marklund SL, Geiger H, Pedrazzini T, Busse R, Brandes RP. Extracellular superoxide dismutase is a major determinant of nitric oxide bioavailability: *in vivo* and ex vivo evidence from ecSOD-deficient mice. *Circ Res*. 2003;93:622–629.
38. Daiber A. Redox signaling (cross-talk) from and to mitochondria involves mitochondrial pores and reactive oxygen species. *Biochim Biophys Acta*. 2010;1797:897–906.
39. Hitomi Y, Watanabe S, Kizaki T, Sakurai T, Takemasa T, Haga S, Ookawara T, Suzuki K, Ohno H. Acute exercise increases expression of extracellular superoxide dismutase in skeletal muscle and the aorta. *Redox Rep*. 2008;13:213–216.
40. Cunha TF, Bacurau AV, Moreira JB, Paixão NA, Campos JC, Ferreira JC, Leal ML, Negrão CE, Moriscot AS, Wisløff U, Brum PC. Exercise training prevents oxidative stress and ubiquitin-proteasome system overactivity and reverse skeletal muscle atrophy in heart failure. *PLoS One*. 2012;7:e41701.
41. Kliment CR, Suliman HB, Tobolewski JM, Reynolds CM, Day BJ, Zhu X, McTiernan CF, McGaffin KR, Piantadosi CA, Oury TD. Extracellular superoxide dismutase regulates cardiac function and fibrosis. *J Mol Cell Cardiol*. 2009;47:730–742.
42. Li Q, Bolli R, Qiu Y, Tang XL, Guo Y, French BA. Gene therapy with extracellular superoxide dismutase protects conscious rabbits against myocardial infarction. *Circulation*. 2001;103:1893–1898.
43. Cho MC, Rapacciuolo A, Koch WJ, Kobayashi Y, Jones LR, Rockman HA. Defective beta-adrenergic receptor signaling precedes the development of dilated cardiomyopathy in transgenic mice with calsequestrin overexpression. *J Biol Chem*. 1999;274:22251–22256.
44. Sullivan MJ, Green HJ, Cobb FR. Skeletal muscle biochemistry and histology in ambulatory patients with long-term heart failure. *Circulation*. 1990;81:518–527.
45. Massie BM. Exercise tolerance in congestive heart failure. Role of cardiac function, peripheral blood flow, and muscle metabolism and effect of treatment. *Am J Med*. 1988;84(3A):75–82.
46. Jondeau G, Katz SD, Zohman L, Goldberger M, McCarthy M, Bourdarias JP, LeJemtel TH. Active skeletal muscle mass and cardiopulmonary reserve. Failure to attain peak aerobic capacity during maximal bicycle exercise in patients with severe congestive heart failure. *Circulation*. 1992;86:1351–1356.
47. Jin B, Li YP. Curcumin prevents lipopolysaccharide-induced atrogin-1/MAFbx upregulation and muscle mass loss. *J Cell Biochem*. 2007;100:960–969.
48. Li YP, Chen Y, John J, Moylan J, Jin B, Mann DL, Reid MB. TNF-alpha acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle. *FASEB J*. 2005;19:362–370.
49. McClung JM, Kavazis AN, Whidden MA, DeRuisseau KC, Falk DJ, Criswell DS, Powers SK. Antioxidant administration attenuates mechanical ventilation-induced rat diaphragm muscle atrophy independent of protein kinase B (PKB Akt) signalling. *J Physiol.* 2007;585(Pt 1):203–215.
50. Zhao J, Brault JJ, Schild A, Cao P, Sandri M, Schiaffino S, Lecker SH, Goldberg AL. FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. *Cell Metab*. 2007;6:472–483.


## CLINICAL PERSPECTIVE

Congestive heart failure (CHF), a major cause of morbidity and mortality, often leads to cachexia (loss of muscle mass) and exercise intolerance (inability to exercise). These clinical syndromes predict mortality. We have recently shown in mice that an intrinsic antioxidant defense mechanism can protect muscle from cachexia. In this study, we used a genetic approach to enhance this defense system by promoting the expression of extracellular superoxide dismutase (EcSOD) in mice and showed profound protection with preserved muscle mass and function under the conditions of CHF. Enhanced EcSOD expression significantly ameliorated CHF-induced abnormal changes in skeletal muscle. These findings pave the way for interventions that augment EcSOD expression, which may significantly reduce morbidity and mortality and improve quality of life for CHF patients.

# Extracellular Superoxide Dismutase Ameliorates Skeletal Muscle Abnormalities, Cachexia, and Exercise Intolerance in Mice with Congestive Heart Failure

Mitsuharu Okutsu, Jarrod A. Call, Vitor A. Lira, Mei Zhang, Jean A. Donet, Brent A. French, Kyle S. Martin, Shayn M. Peirce-Cottler, Christopher M. Rembold, Brian H. Annex and Zhen Yan

*Circ Heart Fail.* 2014;7:519-530; originally published online February 12, 2014;
doi: 10.1161/CIRCHEARTFAILURE.113.000841
*Circulation: Heart Failure* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1941-3289. Online ISSN: 1941-3297

# Supplemental data

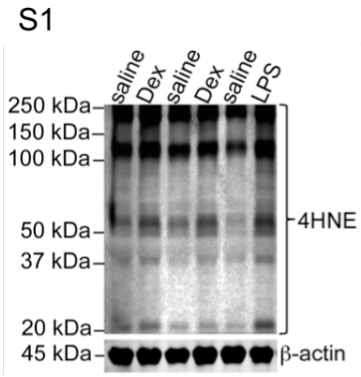


Fig. S1. Dexamethasone injection induces oxidative stress in white vastus muscles in mice. Western blot was performed for 4-HNE in muscle homogenates in mice i.p. injection of Dex (25 mg/kg) for 7 days. A sample from a mouse with lipopolysaccharide (LPS: 1 mg/kg, 12 hrs) injection were used as positive control.

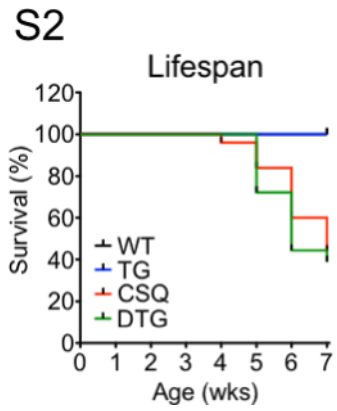


Fig. S2. Muscle-specific overexpression of EcSOD does not improve survival in mice with CHF. Kaplan–Meier survival curves for MCK-EcSOD (TG), α-MHC-CSQ (CSQ) and MCK-EcSOD:α-MHC-CSQ double transgenic mice (DTG) and their wild-type littermates (WT). Death occurred between 5-7 wks of age and showed no significant differences between CSQ and DTG mice (n=12-25/group).

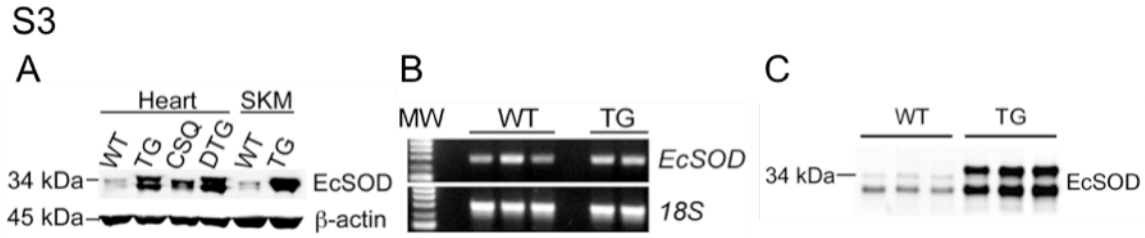


Fig. S3. Elevated EcSOD levels in the heart of muscle-specific EcSOD transgenic mice due to redistribution through circulation. A) Western blot image showing significantly increased EcSOD protein expression in the hearts of MCK-EcSOD mice (TG) compared with wild-type littermates (WT). WT and TG plantaris skeletal muscle (SKM) were used as controls; B) Semi-quantitative RT-PCR showing similar EcSOD mRNA expression in both TG and WT mice; and C) Western blot image showing significantly elevated EcSOD levels in the serum of TG mice compared with WT mice.

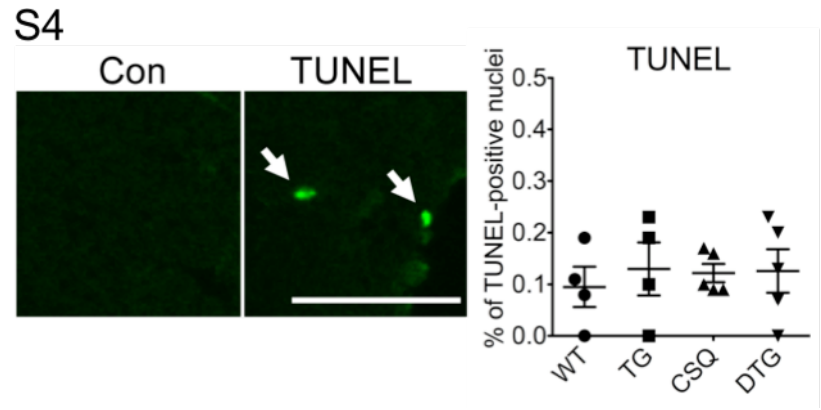


Fig. S4. Measurement of apoptosis in skeletal muscle. Fresh frozen plantaris sections were used to detect apoptosis by TUNEL staining with DAPI staining for nuclei staining. The number of TUNEL-positive nuclei was counted from at least 1000 nuclei. Representative TUNEL staining (left, scale bar = 50 μm) and quantification of the apoptosis (right) at 7 wks of age (n=4-6/group).

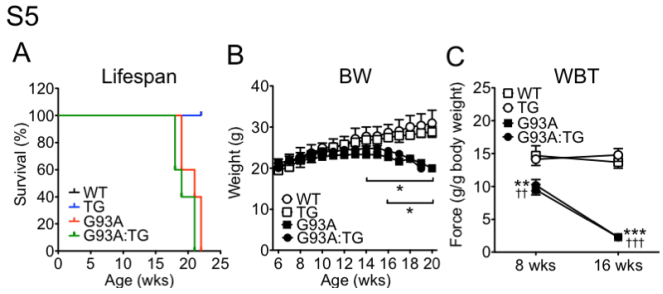


Fig. S5. Muscle-specific overexpression of EcSOD does not reduce mortality, loss of body weight and muscle function in ALS mice. MCK-EcSOD mice (TG) were crossbred with SOD1-G93A mice

(G93A) to generate G93A:TG double transgenic mice and measured for life span, body weight (BW) and whole body tension test (WBT). A) Kaplan–Meier survival curves for TG, G93A, G93A:TG and WT mice. Death occurred between 20 wks of age and showed no significant differences between G93A and G93A:TG mice (n=4-5/group); B) Body weight in G93A and G93A:TG mice were significantly decreased starting at 14 wks of age. * denotes $p<0.05$; C) Muscle force production measured by WBT (n=4-5/group). ** and *** denote $p<0.01$ and $p<0.001$, respectively, between G93A and WT at either 8 or 16 wks. †† and ††† denote $p<0.01$ and $p<0.001$, respectively, between G93A:TG and TG at either 8 or 16 wks.

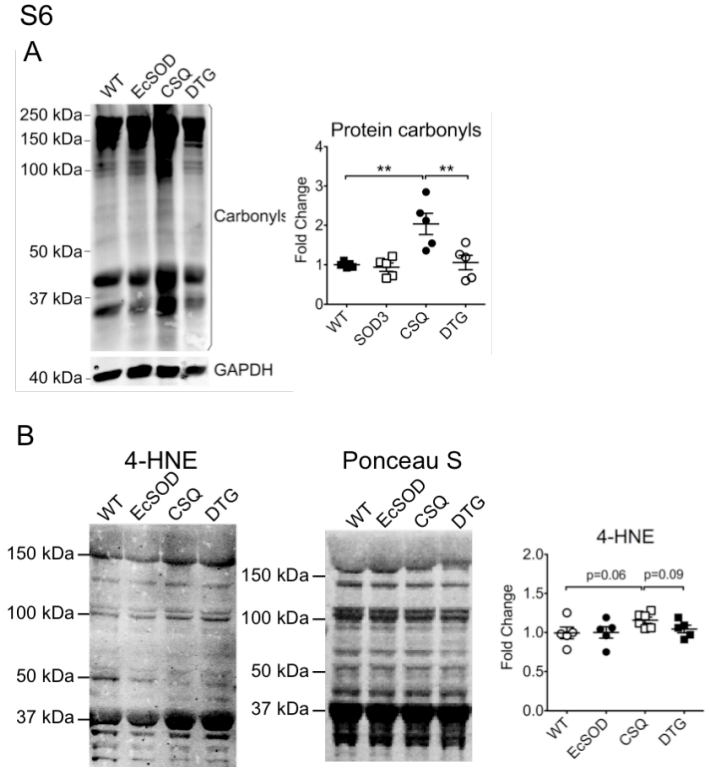


Fig. S6. Muscle-specific overexpression of EcSOD prevents CHF-induced oxidative stress in skeletal muscle. Glycolytic white vastus lateralis muscles (WV) were harvested from MCK-EcSOD (TG), α-MHC-CSQ (CSQ) and MCK-EcSOD:α-MHC-CSQ double transgenic mice (DTG) and their wild-type littermates (WT) at 7 wks of age and processed for measurements of oxidative stress. A) Representative Western blot images of protein carbonylation in WV muscle with GAPDH as loading control. Quantification with statistical analysis were shown on the right; and B) Representative Western blot images of 4-HNE in WV muscle with Ponceau S staining as loading control. Quantification with statistical analysis were shown on the right. ** denotes $p<0.01$.