Original Article

Enhanced Skeletal Muscle Expression of Extracellular Superoxide Dismutase Mitigates Streptozotocin-Induced Diabetic Cardiomyopathy by Reducing Oxidative Stress and Aberrant Cell Signaling

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Background—Exercise training enhances extracellular superoxide dismutase (EcSOD) expression in skeletal muscle and elicits positive health outcomes in individuals with diabetes mellitus. The goal of this study was to determine if enhanced skeletal muscle expression of EcSOD is sufficient to mitigate streptozotocin-induced diabetic cardiomyopathy.

Methods and Results—Exercise training promotes EcSOD expression in skeletal muscle and provides protection against diabetic cardiomyopathy; however, it is not known if enhanced expression of EcSOD in skeletal muscle plays a functional role in this protection. Here, we show that skeletal muscle–specific EcSOD transgenic mice are protected from cardiac hypertrophy, fibrosis, and dysfunction under the condition of type 1 diabetes mellitus induced by streptozotocin injection. We also show that both exercise training and muscle-specific transgenic expression of EcSOD result in elevated EcSOD protein in the blood and heart without increased transcription in the heart, suggesting that enhanced expression of EcSOD from skeletal muscle redistributes to the heart. Importantly, cardiac tissue in transgenic mice displayed significantly reduced oxidative stress, aberrant cell signaling, and inflammatory cytokine expression compared with wild-type mice under the same diabetic condition.

Conclusions—Enhanced expression of EcSOD in skeletal muscle is sufficient to mitigate streptozotocin-induced diabetic cardiomyopathy through attenuation of oxidative stress, aberrant cell signaling, and inflammation, suggesting a cross-organ mechanism by which exercise training improves cardiac function in diabetes mellitus. (*Circ Heart Fail*. 2015;8:188-197. DOI: 10.1161/CIRCHEARTFAILURE.114.001540.)

There are ≈26 million individuals with diabetes mellitus in the United States, and the disease is becoming more prevalent as predicted by global projections that by year 2030 every 4 of 100 people will be diabetic.[1] Individuals with diabetes mellitus have a greater propensity (3–7-fold increase) in developing cardiovascular disease than nondiabetic population,[2] and the adverse cardiovascular events are the leading cause of diabetes mellitus–related death and disease.[3] Diabetic cardiomyopathy (DCM) was first described in 1972 as chronic heart failure in diabetic patients and has since been diagnosed as left ventricular (LV) failure in the absence of atherosclerosis and hypertension.[4] Ventricular failure is the phenotypic end point preempted by calcium irregularities,[5] apoptosis,[6] fibrosis,[7] and oxidative stress.[8,9] Because there is no cure for diabetes mellitus, finding effective modalities to prevent the development of or treat DCM is urgently needed to reduce the morbidity and mortality of the diabetic population.

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Oxidative stress in the myocardium plays an important role in the pathogenesis of DCM.[10] Both diabetic patients and animal models of diabetes mellitus display signs of oxidative damage in the heart tissue, such as lipid peroxidation, protein nitrosylation, and altered endogenous antioxidant enzyme levels.[11] Oxidative damage in the heart is often compounded by oxidative stress–induced signaling for apoptosis and fibrosis,[12] which induce maladaptation leading to impaired structure and function. Although the primary source of oxidative stress in DCM is not fully understood (ie, intracellular versus extracellular), there is a clear link to hyperglycemia. More research is needed to identify ways to

stymie oxidative stress in the heart to preserve the structural and functional integrity.

Because DCM is strongly associated with reduced endogenous antioxidant pools and activities,[13] and increased oxidative stress in the myocardium,[11] supplementing diabetic subjects with antioxidants was favorably considered as a therapy. This concept is supported by studies demonstrating that antioxidant therapy can improve certain features of cardiomyopathies in animal models of diabetes mellitus. Of note, overexpression of antioxidant enzymes in cardiomyocytes improves cardiac morphology and contractility, compared with wild-type (WT) control mice under the condition of diabetes mellitus.[14,15] However, randomized clinical trials in humans failed to demonstrate the effectiveness of antioxidants as therapy for heart failure,[16] including diabetic patients.[17] Despite the species differences between rodents and human patients, several biological reasons could potentially explain the failure of the antioxidant treatment in humans. In particular, a combination of lack of target specificity and continuous supply of the antioxidant with the supplementation approach may explain why genetic augmentation worked in animal models, but pharmacological treatment failed in humans. In addition, antioxidant supplementation in a pulsatile manner will not probably mimic the endogenous antioxidant defense under the condition of DCM, but may impair normal oxidant-mediated cell signaling that is important for cell physiology.[18]

Recent development in antioxidant research, in particular, with regard to extracellular superoxide dismutase (EcSOD), prompted us to speculate that EcSOD expression in skeletal muscle provides protection to the heart against disease conditions, such as DCM. First, EcSOD is a glycoprotein that is secreted and has high affinity for sulfated polysaccharides, such as heparin and heparin sulfate, through its heparin-binding domain on the C terminus.[19] Second, EcSOD is expressed and secreted by skeletal muscle and redistributed into the circulation,[20] and can be taken up by tissue cells (ie, endothelial cells and cardiomyocytes).[21] Third, EcSOD is potent in protection against skeletal muscle wasting in cardiac cachexia by reducing oxidative stress and cellular damage.[20] Fourth, exercise training and increased contractile activity promotes EcSOD expression in skeletal muscle.[20] Finally and most importantly, exercise training improves cardiac phenotypes in both the human patients and the animal models of diabetes mellitus.[22–24] It is, therefore, important to ascertain if enhanced expression of EcSOD in skeletal muscle, such as under the condition of exercise training, would provide protection to the heart against DCM. Answering this question will improve our understanding of the biological importance of exercise training–induced expression of EcSOD in skeletal muscle and foster effective therapeutics for DCM.

Herein, we tested the hypothesis that enhanced expression of EcSOD in skeletal muscle is sufficient to prevent DCM. We took advantage of the model of streptozotocin (STZ)–induced diabetes mellitus in our recently generated skeletal muscle–specific EcSOD transgenic mice (TG). We show that EcSOD TG mice have elevated EcSOD in the heart and other organs because of EcSOD redistribution through the circulation and are protected from oxidative stress, aberrant cell signaling, cardiac hypertrophy and fibrosis, and cardiac dysfunction.

Methods

Animals

Male WT, C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). In addition, EcSOD TG mouse line was generated at the University of Virginia Gene Targeting and Transgenic Facility as described.[20] All animals were housed 3 to 4 mice per cage on a 12:12-hour light–dark cycle. Food and water were provided ad labitum. Voluntary wheel running protocol was performed as described, but briefly involved 24/7 access to a running wheel for 4 weeks.[25] All protocols were approved by the University of Virginia Animal Care and Use Committee.

EcSOD TG and WT littermate control mice received a low-dose injection (50 mg/kg body weight) of STZ on 5 consecutive days (TG-STZ [n=11] and WT-STZ [n=12]). Briefly, mice were fasted for 6 hours at 0900-hour each morning, and received an STZ injection at 1500-hour each afternoon. Nondiabetic controls (WT-CON [n=6] and TG-CON [n=6]) received an equal volume injection of citrate buffer each day. Blood glucose levels were assessed for all mice 72 hours after the final injection of STZ or citrate buffer. Briefly, tail vein blood glucose was assessed using a glucose meter (Ascensia, Bayer). The low-dose regimen of STZ had an equivalent success rate of inducing a diabetic phenotype (blood glucose, >250 mg/dL) in TG-STZ and WT-STZ mice.

Ten weeks after the 5-day treatment with STZ and confirmation of hyperglycemia, all mice were assessed for cardiac function via electrocardiography and echocardiography. One week later, mice were euthanized by carbon dioxide followed by cervical dislocation. The heart was removed, and a small section of the LV was placed in 10% formalin for histology. The remaining portions of the heart were minced, mixed, and separated into fractions for protein and mRNA analyses. The tibialis anterior and soleus skeletal muscles were harvested, weighed, and flash frozen in liquid nitrogen.

Electrocardiography

Electrocardiography was performed on fully conscious mice using the ECGenie (Mouse Specifics, Boston, MA) as described.[20]

Echocardiography

Mice were anesthetized in an induction chamber using isoflurane, and maintained anesthetized via an inhalation mask using 1.0% isoflurane mixed with oxygen at a flow rate of 200 mL/min in a supine position on a warm plate maintained at 37°C by a circulating water bath. Depilatory cream, 70% ethanol, and betadine were used to clear and clean the chest area. Warmed echo gel was applied, and the heart imaged with a 13 MHz linear transducer. Several 2-dimensional–guided M-mode recordings of the short axis of the heart were obtained for determination of LV end-diastolic diameter, end-systolic diameter, end-diastolic wall thickness, and end-systolic wall thickness by a blinded operator. Fractional shortening and ejection fraction were calculated as described.[22]

Immunofluorescence

Fresh frozen sections were stained with anti-EcSOD (1:500), antilectin (1:1000), and antiα smooth muscle (1:1000) to visualize vasculature smooth muscle. Confocal images were acquired and analysis was performed,[20] using ImageJ software.

Western Blot Analysis

For protein analysis, fractions of the LV were immediately homogenized with glass homogenizers in protein loading buffer containing 50 mmol/L Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate, 10% glycerol, 20 mmol/L dithiothreitol, 127 mmol/L 2-mercaptoethanol, and 0.01% bromophenol blue, supplemented with protease inhibitors

(Roche) and phosphatase inhibitors (Sigma–Aldrich). Homogenates were boiled for 5 minutes and centrifuged for 5 minutes at 13 000 rpm. The following antibodies were used to probe polyvinylidene fluoride membranes: SOD3/EcSOD (1:1000), phosph-p38 mitogen-activated protein kinase (MAPK; Thr180/Tyr182; 1:1000), total p38 (1:1000), calcineurin (1:1000), myostatin (1:1000), and GAPDH (1:1000). Membranes were analyzed and quantified using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).[25] Immunoblot analyses for oxidative stress markers was performed as follows: 4-hydroxinonenal resulted in a definitive band at ≈43 kDa and the density of this band alone was analyzed for each sample; carbonylation was assessed according to the manufacturer's instructions (OxyBlot, Millipore), and at the end resulted in a multiple band pattern across many molecular weights. The density of each band alone and the density of the entire column were assessed for each sample, and no significant difference was found between the 2 analysis methods.

Semiquantitative Reverse Transcription Polymerase Chain Reaction

The analysis was performed as described,[26] using primers for atrial natriuretic peptide (F:5′-TGAAAAGCAAACTGAGGGC-3′; R:5′-CAGAGTGGGGAGAGGCAAGAC-3′), brain natriuretic peptide (F:5′-CTGAAGGTGCTGTCCCAGAT; R:5′-ACTTCAGTGCG TTACAGCCC-3′), EcSOD (F:5′-TGATCCTGTCCCATACTAC; R:5′-AAGCCACACACATGCACATT), tumor necrosis factor-α (; F:5′-AGT CCGGGCAGGTCTACTTT; R:5′-GCACCTCAGGGAAGAGTCTG), interleukin-1β (F:5′-CTCACAAGAGCACAAGC; R:5′-CTCAGTG CAGGCTATGACCA), and 18s (F:5′-AAACGGCTACCACATCCAAG; R:5′-CCCTCTTAATCATGGCCTCA).

Histology

Cardiomyocyte cross-sectional area was determined from paraffin-embedded sections stained with hematoxylin and eosin. Cardiac fibrosis was determined using a picosirius red staining. Multiple images were acquired from each stained heart section. Images were assessed using ImageJ software.

Statistical Analysis

Data are represented as mean±SEM. Differences among individual groups of mice are only reported when a significant interaction was observed by a 2-way ANOVA (genotype×treatment) using JMP (SAS, Cary, NC) statistical software. Data were required to pass normality (Shapiro–Wilk) and equal variance tests (Brown–Forsythe *F* test) before proceeding with the ANOVA. All data passed the equal variance tests. When a significant interaction was observed, a Tukey honest significant difference post hoc analysis was performed. An α=0.05 was used for all analyses.

Results

Skeletal Muscle–Specific EcSOD Transgenic Overexpression Leads to Elevated EcSOD Level in the Heart With Redistribution Through the Circulation

Previous studies have shown that exercise training provides protection against DCM,[22,23] and ectopic expression of EcSOD is effective in counteracting oxidative stress in various tissues/organs.[27,28] To ascertain the potential protective function of EcSOD in the heart and its origin under the condition of exercise training, we first tested the extent to which EcSOD protein level in the heart and serum was promoted by endurance exercise training in mice. Four weeks of voluntary wheel running significantly increased EcSOD

protein level in the heart and serum in reference to the sedentary control mice. The increased EcSOD protein level in the heart did not seem to result from increased EcSOD gene transcription in the heart, as we did not detect significant increases in EcSOD mRNA (Figure 1A). These findings suggest that elevated EcSOD protein in the heart is a result of enhanced expression and secretion of EcSOD from other tissue(s) in the periphery, in this case skeletal muscle.

Because voluntary wheel running in mice enhances EcSOD protein expression in skeletal muscles,[20] we speculated that enhanced expression of EcSOD in skeletal muscle could redistribute through the circulation to the heart. EcSOD TG had greater EcSOD levels in the serum and heart compared with WT mice (Figure 1B). To determine if the elevated EcSOD level in the heart was because of leaky
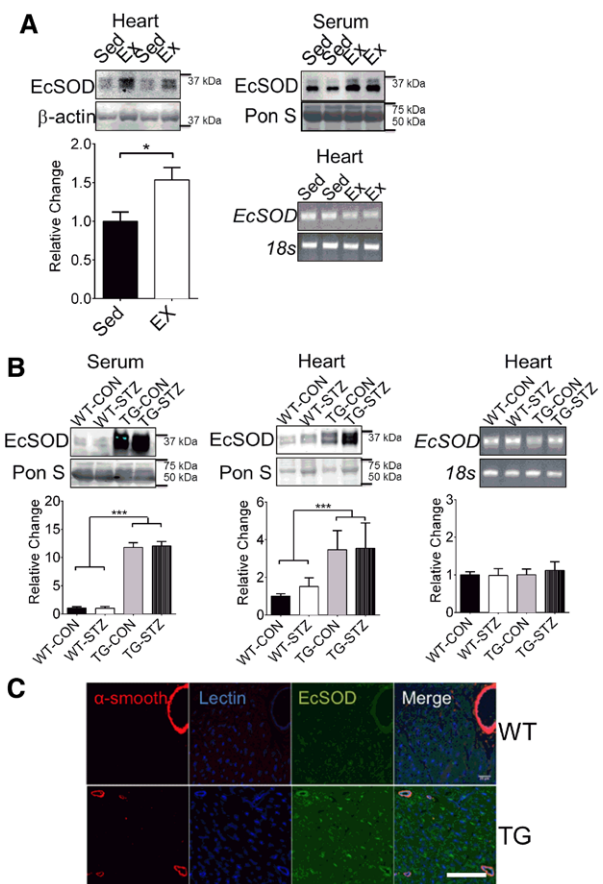

Figure 1. Enhanced expression of extracellular superoxide dismutase (EcSOD) in skeletal muscle redistributes to the heart. **A**, Four weeks of voluntary wheel running affected EcSOD expression in the hearts and serum of exercise trained wild-type (WT; Ex; n=9) mice compared with WT sedentary (Sed; n=9) controls, and EcSOD levels in the heart were independent of mRNA expression. Student *t* test, *P<0.05. **B**, EcSOD levels were 11-fold greater in the serum and 4-fold greater in the hearts of EcSOD transgenic mice (TG) mice, independent of streptozotocin (STZ; n=12) compared with WT littermate controls, independent of STZ (n=12), and EcSOD levels in the heart were independent of mRNA expression. ANOVA, group effect for genotype, ***P<0.001. **C**, EcSOD protein was enriched near the endothelial cells in the heart, and had greater presence within individual cardiomyocytes in EcSOD TG mice compared with WT littermate controls. Scale bar is 25 μm. Graphical bars are mean±SE.

expression of the transgene by the muscle creatine kinase promoter, we examined the mRNA expression of EcSOD in the heart. There was no significant difference in EcSOD mRNA expression between EcSOD TG and WT mice (Figure 1B). Because the muscle creatine kinase promoter is not active in nonmuscle tissues, these findings collectively suggest that elevated level of EcSOD protein in the heart of TG mice is mainly coming from skeletal muscle. We next examined the localization of EcSOD within the heart of EcSOD TG and WT mice (Figure 1C). Endogenous EcSOD seems to be enriched at the endothelium as evidenced by the signals from immunofluorescence staining of the ventricules in WT mice (Figure 1C). In EcSOD TG heart, significantly more EcSOD signals were detected near the endothelium and within the cardiomyocytes. These findings are consistent with the notion that EcSOD from the circulation can adhere to endothelial cells and can also be taken up by cardiac myocytes in the heart.

Enhanced Expression of EcSOD in Skeletal Muscle Prevents DCM Induced by STZ Injection

To determine if EcSOD TG mice are protected from DCM, we subjected EcSOD TG and WT mice to a low-dose regimen of STZ injections. EcSOD and WT mice had equal severity in hyperglycemia after STZ injections (374 ± 32 versus 362 ± 32 mg/dL; $P=0.459$). LV end-diastolic diameter, a marker of diastolic dysfunction, showed an increased trend without statistical significance (Table 1) along with a significant increased LV end-systolic diameter, a marker of systolic dysfunction, in WT-STZ mice compared with WT-CON and TG-STZ mice 10 weeks after the 5-day injection treatment (Table 1). We used these 2 parameters to calculate ejection fraction and fractional shortening and showed that they were both significantly lower in WT-STZ mice than in WT-CON, which were completely prevented in TG-STZ mice (Figure 2A–2C). Fully conscious ECG analysis, a sensitive measurement of resting heart rate, showed that WT-STZ mice had significantly slower resting heart rate (bradycardia) than WT-CON, which was significantly attenuated in TG-STZ (Figure 2D). Consistently, electrocardiography waveform analysis showed that QRS-interval was lengthened in WT-STZ mice than in WT-CON (Table 1),
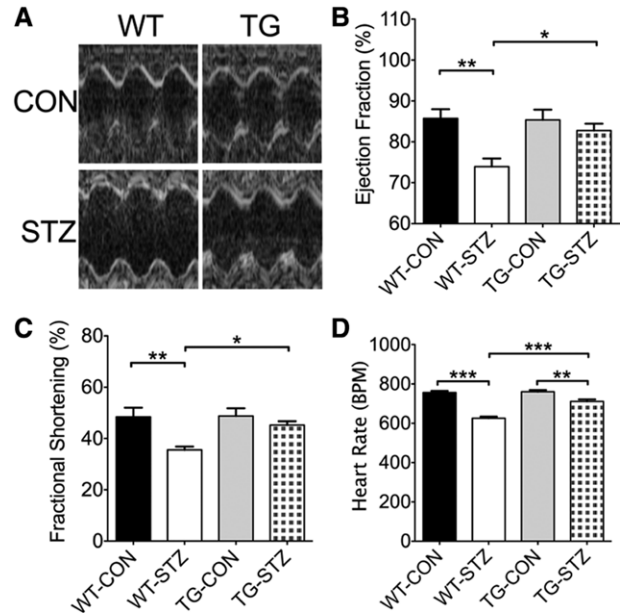
Figure 2. Enhanced expression of extracellular superoxide dismutase in skeletal muscle prevents streptozotocin (STZ)–induced impairment in cardiac function. **A,** Representative M-mode echocardiograph heart images. **B** and **C,** Graphical representation showing ejection fraction and fractional shortening calculated from echocardiograph analysis. **D,** Graphical representation of in vivo, fully conscious heart rate from ECG analysis. WT-CON, n=6; WT-STZ, n=11; TG-CON, n=6; and TG-STZ, n=12. ANOVA, significant interaction, followed by Tukey HSD post hoc test, *$P<0.05$, **$P<0.01$, and ***$P<0.001$. Bars are mean±SE. TG indicates transgenic mice; and WT, wild-type.

which was mitigated in TG-STZ mice. Together, these in vivo data strongly suggest that STZ treatment produced a phenotype of cardiac dysfunction, which was significantly attenuated in mice with skeletal muscle–specific EcSOD overexpression.

Enhanced Expression of EcSOD in Skeletal Muscle Prevents Cardiac Hypertrophy and Fibrosis Induced by STZ Injection

Cardiac dysfunction is often caused by abnormal morphological changes in the heart, such as hypertrophy and deposition of noncontractile, fibrotic components. To assess

Table 1. In Vivo Heart Function of WT-CON, WT-STZ, TG-CON, and TG-STZ Mice

	WT-CON (n=6)	WT-STZ (n=11)	TG-CON (n=6)	TG-STZ (n=12)
PR interval, µs	23.8±0.7	27.5±0.8	23.0±0.5	24.5±0.5
QRS interval, µs	10.3±0.3	11.7±0.4*	10.5±0.1	10.4±0.3
QT interval, µs	41.7±0.7	46.0±1.1	39.4±0.7	41.3±0.6
LVEDD, mm	261±12	335±15	289±19	295±20
LVESD, mm	135±14	212±7*	147±10	164±13
LVEDwt, mm	99±19	104±10	115±15	106±10
LVESwt, mm	151±14	160±5	170±13	170±9

Values are mean±SEM. LVEDD indicates left ventricular end-diastolic diameter; LVEDwt, LV end-diastolic wall thickness; LVESD, left ventricular end-systolic diameter; and LVESwt, LV end-systolic wall thickness.

*$P<0.05$ vs WT-CON, TG-CON, and TG-STZ mice.

Table 2. Body Mass, Heart, Tibialis Anterior, Soleus Muscle Mass of WT-CON, WT-STZ, TG-CON, and TG-STZ Mice

	WT-CON (n=6)	WT-STZ (n=11)	TG-CON (n=6)	TG-STZ (n=12)
Body mass, g	24.1±0.9	23.6±1.2	24.8±0.9	25.7±1.0
Heart mass, mg	118.9±4.3	149.9±5.6	121.3±1.9	130.0±8.1
H/BM, mg/g	4.9±0.1	6.3±0.3*	4.9±0.2	5.0±0.1
TA mass, mg	38.4±1.9	27.7±3.7	42.3±2.4	40.1±3.0
TA/BM, mg/g	1.59±0.04	1.14±0.11*	1.71±0.08	1.55±0.09
Sol mass, mg	7.3±0.2	7.5±0.5	7.5±0.4	8.2±0.4
Sol/BM, mg/g	0.31±0.02	0.32±0.02	0.30±0.01	0.32±0.01

Values are mean±SEM. BM indicates body mass; H, heart; Sol, soleus muscle; and TA, tibialis anterior.

*$P<0.05$ vs WT-CON, TG-CON, and TG-STZ mice.

cardiac hypertrophy, we measured heart mass relative to body mass and cardiomyocyte cross-sectional area in all mice 11 weeks after STZ or citric acid injections and 1 week after in vivo functional analysis. Relative heart mass was significantly greater in WT-STZ mice than in WT-CON mice, and TG-STZ mice did not show significant increase (versus TG-CON; Table 2). Kruskal–Wallis analysis of hematoxylin and eosin–stained ventricular muscle sections (Figure 3A) showed that the median cross-sectional area of individual cardiomyocytes was greater in WT-STZ mice compared with WT-CON (254 versus 200 μm^2), which was completely blocked in TG-STZ mice (206 μm^2; Figure 3B). χ^2 analysis revealed that STZ treatment in littermate controls resulted in more large fibers, whereas STZ treatment in EcSOD TG mice did not (Figure 3C). Overall, STZ resulted in a rightward distribution shift in cardiomyocyte cross-sectional area for WT-STZ mice, which was significantly attenuated in TG-STZ mice. Picosirius red staining showed that there was enhanced deposition of collagen in interstitial space of the ventricular muscle sections in WT-STZ (versus WT-CON), which was significantly attenuated in TG-STZ (versus WT-STZ) consistent with the protection against cardiac fibrosis (Figure 3D–3E). These data indicate that STZ-induced hyperglycemia is associated with DCM with pathological hypertrophy and cardiac fibrosis, and skeletal muscle–specific EcSOD overexpression mitigates these pathological processes.

DCM Cachexia is Prevented With Overexpression of EcSOD

An important clinical outcome of cardiomyopathy is skeletal muscle catabolic wasting and cachexia. We have recently reported that enhanced expression of EcSOD in skeletal muscle is sufficient to prevent chronic heart failure–induced catabolic wasting.[20] Hyperglycemia induced by diabetes mellitus may also directly impair skeletal muscle function. To determine the effects of skeletal muscle–specific EcSOD overexpression on DCM-induced catabolic muscle wasting, we examined the masses of the fast-twitch tibialis anterior muscle, and slow-twitch soleus muscle. After 11 weeks of hyperglycemia, relative tibialis anterior muscle masses were less in WT-STZ mice compared with WT-CON, which was attenuated in TG-STZ mice (versus WT-STZ; Table 2). This catabolic muscle wasting was not observed in the slow-twitch soleus muscles (Table 2). These findings are completely consistent with previous findings in other models of catabolic muscle wasting.[29]

Enhanced Skeletal Muscle Expression of EcSOD Results in Reduced Oxidative Stress in the Heart Under the Condition of Diabetes Mellitus Induced by STZ Injections

Oxidative stress is clearly involved in the pathogenesis of DCM. To determine if enhanced skeletal muscle expression of EcSOD may result in reduced oxidative stress in the heart under the diabetic condition, we examined oxidative stress markers in the heart. 4-hydroxynoneal, a by-product of lipid peroxidation, was greater in WT-STZ compared with WT-CON, which was mitigated in TG-STZ mice (versus WT-STZ; Figure 4A). Protein carbonylation, which generally defines the post-translation modification of amino acid side chains to aldehyde and ketone derivatives (ie, carbonyls), was greater in WT-STZ compared with WT-CON (Figure 4B), but not different among other groups. These findings collectively are consistent with the notion that enhanced expression of EcSOD in skeletal muscle is sufficient to protect the heart from oxidative stress and damage induced by diabetic hyperglycemia.

Enhanced Skeletal Muscle Expression of EcSOD Prevents Aberrant Cell Signaling and Inflammation in the Heart

Cell signaling in response to hyperglycemia and oxidative stress is clearly an important part of maladaptive remodeling in the heart during the development of DCM. To examine the mechanism of EcSOD-mediated protection in the heart further, we probed for proteins associated with signaling in cardiac hypertrophy (Figure 5A). Activation
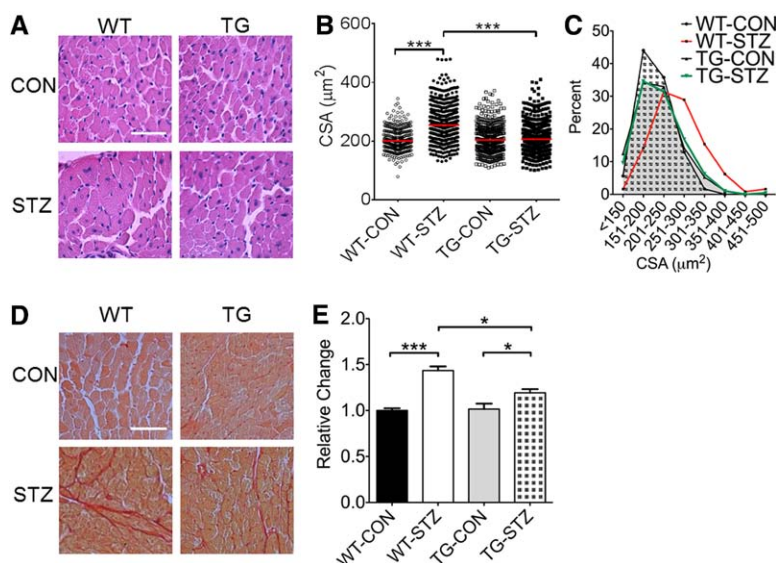

Figure 3. Enhanced expression of extracellular superoxide dismutase in skeletal muscle mitigates streptozotocin (STZ)–induced cardiac hypertrophy and fibrosis. **A**, Representative heart images stained with hemotoxylin and eosin to identify individual cardiomyocytes. **B**, Graphic representation of individual cardiomyocyte cross-sectional area. **C**, Cardiomyocyte size frequency distributions based on cross-sectional area. WT-CON, black bar with dotted area; WT-STZ, red bar; TG-CON, gray area; and TG-STZ, green bar. Note the rightward distribution shift of the WT-STZ fibers relative to all other groups. Among these mice, there was an abnormally large amount of large fibers (>300 μm). **D**, Representative heart images stained for fibrotic tissue deposition by sirius red. **E**, Graphical representation of collagen staining. WT-CON, n=6; WT-STZ, n=11; TG-CON, n=6; and TG-STZ, n=12. ANOVA, significant interaction, followed by Tukey HSD post hoc test, ** $P<0.01$ and ***$P<0.001$. TG indicates transgenic mice; and WT, wild-type.
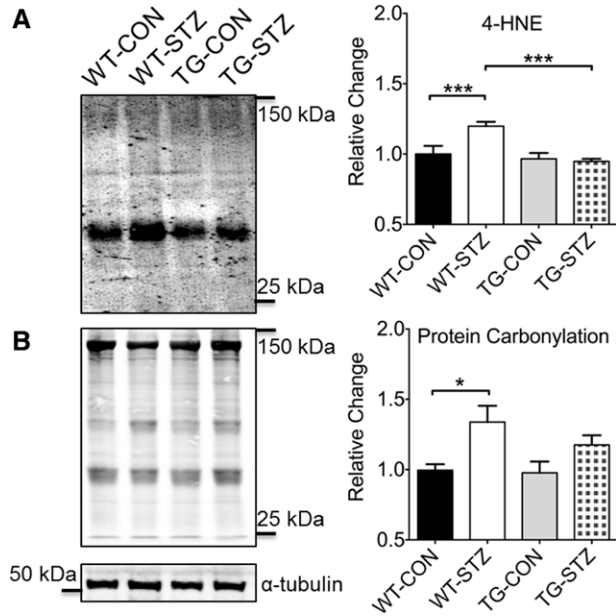
Figure 4. Enhanced expression of extracellular superoxide dismutase in skeletal muscle protects against streptozotocin (STZ)–induced oxidative damage and aberrant signaling in the heart. Representative immunoblots and graphical representation of statistical analysis is shown for 4-hydroxinonenal (4-HNE; **A**) and protein carbonylation (**B**). Staining was normalized by tubulin and is shown relative to WT-CON. WT-CON, n=6; WT-STZ, n=11; TG-CON, n=6; and TG-STZ, n=12. ANOVA, significant interaction, followed by Tukey HSD post hoc test, *P<0.05 and **P<0.001. Bars are mean±SE.

Figure 5. Enhanced expression of extracellular superoxide dismutase (EcSOD) in skeletal muscle protects against streptozotocin (STZ)–induced aberrant signaling in the heart. **A**, STZ-induced activation of calcineurin and p38 mitogen-activated protein kinase in hearts is mitigated in EcSOD TG mice. **B**, EcSOD alleviates STZ-induced tumor necrosis factor-α (TNF-α) and atrial natriuretic peptide (ANP) mRNA expression. WT-CON, n=6; WT-STZ, n=11; TG-CON, n=6; and TG-STZ, n=12. ANOVA, significant interaction, followed by Tukey HSD post hoc test, *P<0.05. Bars are mean±SE. BNP indicates brain natriuretic peptide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; and IL, interleukin.

of calcineurin is involved in cardiac hypertrophy in both the animals and the humans.[30,31] Here, we observed a modest but significant increase in calcineurin expression in the heart of WT-STZ mice compared with WT-CON mice (Figure 5A). We did not observe any significant difference in myostatin expression across groups (Figure 5A). We observed an activation (phosphorylation) of p38 MAPK, a stress pathway that is critical for the inflammatory process in maladaptive remodeling of the heart, in WT-STZ compared with WT-CON mice (Figure 5A), which coincided with greater expression of the inflammatory cytokine tumor necrosis factor-α (Figure 5B). Finally, we confirmed in DCM in our hands was associated with the more tradition expression of hypertrophy molecules, atrial natriuretic peptide and brain natriuretic peptide. We observed a significant elevation of atrial natriuretic peptide gene expression in the heart of WT-STZ mice compared with WT-CON mice (Figure 5B). We did not observe any significant difference in brain natriuretic peptide gene expression across groups. These findings suggest a complex mechanism of DCM, but support that premise of EcSOD derived from skeletal muscle as having some protective function in this disease model of type 1 diabetes mellitus.

Discussion

In this study, we showed that STZ-induced diabetes mellitus elicited DCM with clear evidence of ventricular dysfunction and pathological remodeling. Cardiac dysfunction, hypertrophy, fibrosis, and oxidative stress
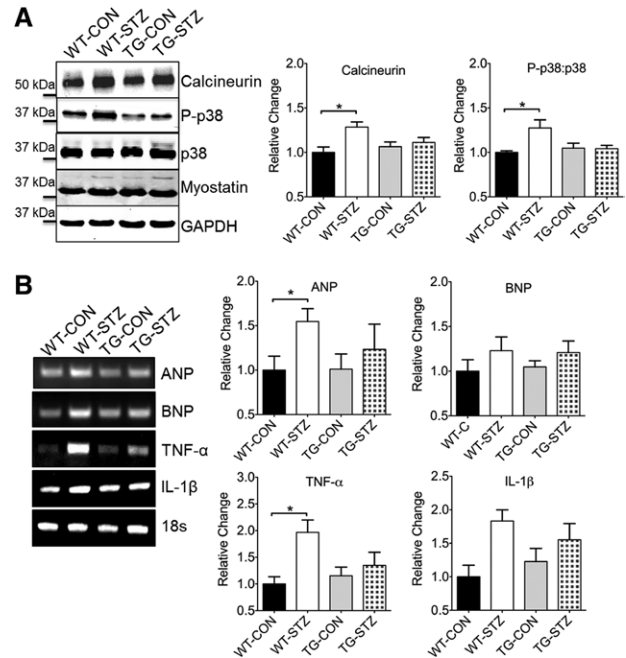
were all effectively mitigated in skeletal muscle–specific EcSOD TG. This, to our knowledge, is the first report of genetically enhanced gene expression in skeletal muscle showing protection against DCM. Because elevated EcSOD protein level in the heart in these TG is because of redistribution of EcSOD from skeletal muscle via the circulation, these findings support that enhanced skeletal muscle expression of EcSOD is sufficient to mitigate oxidative stress and aberrant cell signaling and preserves cardiac function in vivo. Because exercise training improves cardiac function in both the human patients and the animal models of diabetes mellitus,[22,23] and exercise training leads to elevated EcSOD levels in the heart in the absence of enhanced ectopic mRNA expression, we speculate that induced EcSOD expression in skeletal muscle underlies at least in part the beneficial effects of exercise training in diabetic population.

The most exciting aspect of our findings is the protection of the heart from enhanced expression of EcSOD in skeletal muscle. This presents an interesting feature of the integrated biological system in our body and raises the possibility of using this feature to circumvent disease conditions of other tissues/organs. In fact, organ–organ cross-talk is emerging as an important area of research, which will probably advance biomedical science with profound clinical relevance. For example, interleukin-6 has

been shown mediate brown adipose tissue–facilitated glucose sensitivity in the heart,[32] and skeletal muscle–derived interleukin-6 can significantly influence lipid metabolism in the liver.[33] Myostatin, a potent regulator of muscle mass, secreted from the failing heart, is involved in heart failure–induced skeletal muscle wasting.[34] More recently, irisin secreted from skeletal muscles has been shown to promote browning of adipose tissue.[35] Up to date, there has been no evidence for an antioxidant functioning as a mediator of organ–organ cross-talk. Because skeletal muscle is the largest internal organ in the body, it represents a potentially important, continuous source of EcSOD to mitigate oxidative stress in peripheral tissues/organs (eg, the heart) under disease conditions. Our findings support the notion that enhanced expression of EcSOD in skeletal muscle induced by exercise training contributes to the improved cardiac phenotypes in diabetes mellitus.[22,23] The findings also support augmenting EcSOD expression in skeletal muscle or other tissue/organs as therapeutic intervention for DCM. Continued research efforts in this exciting new field will undoubtedly lead to the discovery of other potential factors.

DCM features structural remodeling of the heart (ie, hypertrophy and fibrosis) and eventual contractile dysfunction. Preempting the development of DCM is the elevated blood glucose levels (hyperglycemia), which is positively correlated with cardiovascular cell death.[36] Myocardial hypertrophy initially occurs as an adaptive response to mitigate the negative effects of the pathological insults. When the pathological insults persist, excessive extracellular matrix deposition occurs as a result of increased production or reduced degradation of extracellular matrix components. In fact, hyperglycemia per se is sufficient to increase cardiac fibroblast proliferation leading to interstitial and perivascular fibrosis.[37] As a consequence, the myocardium increases its stiffness and reduces its compliance, which together with apoptotic loss of cardiomyocytes leads to systolic and diastolic dysfunctions. Our findings that EcSOD TG mice are resistant to both cardiac hypertrophy and fibrosis independent of improved glycemic control strongly suggest that elevated EcSOD protein in the heart mitigates the pathological signal that is upstream of the hypertrophy and fibrosis cascades in the myocardium under the diabetic condition. In future, we will determine if enhanced skeletal muscle expression of EcSOD would reverse the pathological changes and cardiac dysfunction in DCM.

The development of ventricular dysfunction in diabetes mellitus is a complex process with many contributing factors. LV diastolic dysfunction often occurs in isolation, or before LV systolic dysfunction in DCM in humans,[38] although some studies also showed prevalence of LV systolic dysfunction.[39] Here, we showed clear evidence of LV systolic dysfunction concurrent with a trend of LV diastolic dysfunction in STZ-injected WT mice, which were significantly ameliorated in EcSOD TG mice (Figure 2). Because hyperglycemia-induced impairment of calcium handling in cardiomyocytes is a major contributor to LV systolic dysfunction,[5] our findings suggest that elevated EcSOD in the heart may mitigate hyperglycemia-induced impairment of calcium handling.

Herein, changes in intracellular calcium handling may have led to aberrant signaling and maladaptation in the diabetic heart. Calcineurin, a calcium-regulated phosphatase that is activated during prolonged increases of intracellular calcium concentrations,[30] has been shown to dephosphorylate nuclear factor of activated T cell and lead to its nuclear translocation and activation of genes involved in cardiac hypertrophy.[40] Here, we observed a significant increase in calcineurin protein expression in DCM, which was blunted in EcSOD TG mice. Elevated calcineurin protein expression in the heart has been observed previously under the condition of human heart failure,[31] suggesting that increased calcineurin expression is involved in cardiac hypertrophy. Because the main function of EcSOD is scavenging superoxide radical, our finding that EcSOD TG mice are resistant to pathological hypertrophy induced by diabetes mellitus suggests that oxidative stress may directly, or indirectly, lead to impaired calcium handling and aberrant calcium–calcineurin signaling in DCM.

Numerous studies have shown that redox-sensitive, stress signaling pathways, such as c-jun N-terminal protein kinase and p38 MAPK, are activated by diabetic conditions.[41] In particular, the p38 MAPK pathway has been shown to regulate the expression of inflammatory cytokines in DCM, and pharmacological or genetic inhibition of p38 MAPK mitigates STZ-induced systolic dysfunction, hypertrophy, fibrosis, oxidative stress, and inflammation.[12] These findings support that p38 MAPK activity is functionally important for the induction of adverse cardiac remodeling and dysfunction under the diabetic condition. We showed that p38 MAPK activation was greater in WT mice compared with EcSOD TG in response to STZ injection. Together with the findings that EcSOD TG diabetic mice were protected from pathological hypertrophy along with significant abrogation of fibrotic tissue deposition, we speculate that p38 MAPK plays an important role in fibrosis as previously described.[12]

Oxidative stress is accepted as an important player in the development of cardiac hypertrophy and fibrosis in DCM.[8] Previous studies have demonstrated that STZ-induced diabetes mellitus are associated with increases in many known markers of oxidative stress in the heart, including superoxide anion production, protein nitrosylation, and lipid peroxidation.[42,43] Here, we examined 4 markers of oxidative damage, of which 2 were significantly elevated in diabetic heart and the other 2 showed increased trends. Although the cause of increased reactive oxygen species in DCM is still being elucidated, mitochondria, xanthine oxidase, nicotinamide adenine dinucleotide phosphate oxidase, monoamine oxidase A, and uncoupled nitric oxide synthases might all play a role in the increased production of reactive oxygen species.[44,45]

An increasing number of studies have shown the efficacy of enhancing antioxidant defense in counteracting the pathophysiology of DCM. For example, Shen et al[15] showed that enhanced expression of mitochondrial manganese superoxide dismutase reduces oxidative stress, improves mitochondrial morphology, and attenuates
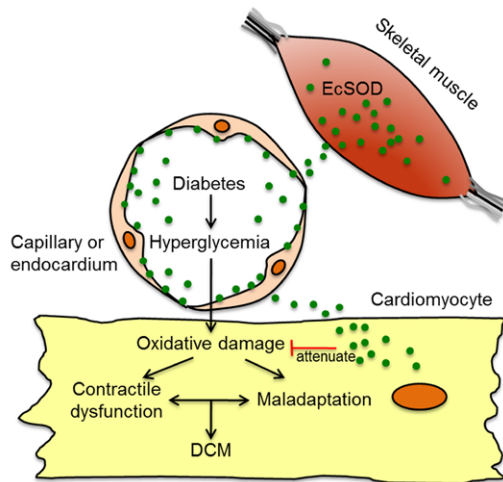
Figure 6. Schematic illustration of the role of enhanced expression of extracellular superoxide dismutase (EcSOD) in skeletal muscle in protection against diabetic cardiomyopathy (DCM). Enhanced expression of EcSOD in skeletal muscle leads to increased distribution of EcSOD via the circulation to cardiomyocytes through either capillary endothelium or endocardial endothelium. Increased EcSOD in the cardiomyocytes mitigates hyperglycemia-induced oxidative stress hence preventing contractile dysfunction and maladaptation and blocking the pathogenesis of DCM.

cardiac contractility in a mouse model of type 1 diabetes mellitus. Overexpression of catalase or GPx1also improves cardiac morphology, mitochondrial structure, and myofibrillar structure, as well as cardiomyocyte contractility.[46,47] However, clinical application of antioxidant therapies have not been successful. Administration of exogenous SOD in vivo is limited by the large size (restricting cell permeability) and short half life. Vitamin C or E supplementation as a pharmacotherapy have failed to show clear cardiovascular benefit.[48] These unfavorable outcomes may be related to one or all of the following limitations, such as low activity of exogenous antioxidants, lack of targeting to the intended action site, and mistargeting that impairs physiological function. Our findings that elevated EcSOD level in EcSOD TG mice is localized at the endothelial cells and within cardiomyocytes in the heart suggest that targeted scavenging of oxidative stress in endothelial cells or cardiomyocytes is a promising intervention against pathogenesis of DCM.

Several studies using genetic, pharmacological, and nutritional approaches to mitigate oxidative stress demonstrate in proof-of-principle that maintaining a healthy redox status in the heart can provide protection against cardiac dysfunction.[9] In this study, we chose to focus on EcSOD because it seems to be critically involved in heart health and because EcSOD knockout mice have exacerbated cardiac hypertrophy.[49] The true novelty of this study is that EcSOD expressed in skeletal muscle could attenuate oxidative stress in the heart, most probably through redistribution of EcSOD through the circulation. Here, we show that enhanced expression of EcSOD in skeletal muscle by exercise training in mice leads to increased EcSOD levels in the blood and heart, without

increased ectopic EcSOD expression in the heart, consistent with previous gene array findings.[50] Collectively, our findings support the notion that enhanced expression of EcSOD in skeletal muscle acts through the circulation to prevent oxidative stress in peripheral tissues; a finding well in line with the concept of organ–organ cross-talk. It is important in the future to determine whether skeletal muscle–derived EcSOD is required for the exercise training–mediated protection against DCM.

Overall, this study provides novel evidence that enhanced expression of EcSOD in skeletal muscle through its redistribution to the heart is sufficient to reduce oxidative stress and aberrant cell signaling and hence ameliorates pathological changes and cardiac dysfunction in STZ-induced DCM (Figure 6). The STZ model of diabetes mellitus in rodents is not without its limitations, as the manifesting condition is severe with a complete loss of insulin control. Indeed, the severity of the phenotype might contribute to the development of DCM, which is absent in other models of diabetes mellitus, such as the Akita mouse. It is worth speculating that the protective effects observed herein may in fact be bolstered in a more appropriate model. Finally, because of EcSOD's antioxidant capabilities and cellular location, our findings raise the possibility of enhancing EcSOD levels systemically by pharmacological, genetic or exercise intervention as therapeutic approaches for diabetic patients with DCM.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Diabetic cardiomyopathy is a major contributor to morbidity and mortality in diabetes mellitus. The American Diabetes Association endorses regular physical activity as a suitable therapy to mitigate the adverse effects of diabetes mellitus, although the mechanisms of this protection are not completely understood. We have previously shown that skeletal muscle extracellular superoxide dismutase (EcSOD), an intrinsic antioxidant defense system, is enhanced with exercise training in mice and protects against skeletal muscle wasting associated with chronic heart failure. Herein, we demonstrate the exercise training also enhances serum and heart levels of EcSOD. We also show greater levels of serum and heart EcSOD in mice with genetic overexpression of skeletal muscle–specific EcSOD. These mice were protected from the development of hyperglycemic-induced maladaptation in the heart. This demonstrated that skeletal muscle–specific EcSOD is sufficient to protect against diabetic cardiomyopathy, and more importantly, indicates that skeletal muscle–derived EcSOD traveling to the heart may be 1 mechanism by which regular physical activity is beneficial in diabetes mellitus.

Enhanced Skeletal Muscle Expression of Extracellular Superoxide Dismutase Mitigates Streptozotocin-Induced Diabetic Cardiomyopathy by Reducing Oxidative Stress and Aberrant Cell Signaling

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