

Protection of Cardiac Mitochondria by Overexpression of MnSOD Reduces Diabetic Cardiomyopathy

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We previously reported damage and elevated biogenesis in cardiac mitochondria of a type 1 diabetic mouse model and proposed that mitochondria are one of the major targets of oxidative stress. In this study, we targeted overexpression of the mitochondrial antioxidant protein manganese superoxide dismutase (MnSOD) to the heart to protect cardiac mitochondria from oxidative damage. Transgenic hearts had a 10- to 20-fold increase in superoxide dismutase (SOD) activity, and the transgenic SOD was located in mitochondria. The transgene caused a twofold increase in cardiac catalase activity. MnSOD transgenic mice demonstrated normal cardiac morphology, contractility, and mitochondria, and their cardiomyocytes were protected from exogenous oxidants. Crossing MnSOD transgenic mice with our type 1 model tested the benefit of eliminating mitochondrial reactive oxygen species. Overexpression of MnSOD improved respiration and normalized mass in diabetic mitochondria. MnSOD also protected the morphology of diabetic hearts and completely normalized contractility in diabetic cardiomyocytes. These results showed that elevating MnSOD provided extensive protection to diabetic mitochondria and provided overall protection to the diabetic heart. *Diabetes* 55:798–805, 2006

Cardiac failure is a leading cause of death for diabetic patients. Accumulated evidence indicates that heart failure in diabetes is due at least in part to a specific cardiomyopathy, referred to as diabetic cardiomyopathy, which is distinct from coronary arteriosclerosis. This was first proposed by Rubler et al. (1) in 1972 based on postmortem findings of heart failure in diabetic patients free of coronary artery disease. This finding has been confirmed by others in many subsequent clinical studies (2,3).

Excess reactive oxygen species (ROS) production has been widely implicated in both the onset of diabetes and many of its complications (4–6). Mitochondria are known to continuously generate superoxide radical as a by-product of electron transport. The significance of mito-

chondria-generated ROS in diabetes has been proposed by several laboratories (7–11). Brownlee's laboratory provided strong evidence that ROS from mitochondria activate pathological pathways that induce diabetic complications (8,12,13). The normalization of these changes in high glucose-cultured endothelial cells by overexpression of manganese superoxide dismutase (MnSOD), uncoupling protein-1, or inhibitors of mitochondrial electron transport (8) suggests that mitochondrial respiration acts as a major source of oxidative stress in diabetes complications. However, the role of mitochondrial oxidative stress has not been confirmed in diabetic cardiomyopathy.

In a previous study, we observed defects in structure and function of mitochondria from diabetic heart (14) and proposed that mitochondria-derived ROS play an important causal role in mitochondrial damage and compensatory biogenesis. To confirm this hypothesis, we designed and constructed a transgenic line overexpressing the mitochondrial antioxidant MnSOD in a cardiac-specific manner and crossed it with our transgenic type 1 diabetic model. Our results showed that reducing mitochondrial superoxide was effective in protecting both mitochondria and cardiomyocytes from diabetic cardiomyopathy. This indicates that mitochondrial ROS are an essential cause of diabetic heart complications.

RESEARCH DESIGN AND METHODS

We have previously described the development and maintenance of OVE26 diabetic mice (15,16). The development of MySOD transgenic lines is described below. All transgenic and nontransgenic animals were maintained on the inbred FVB background. A U.S. Department of Agriculture-certified institutional animal care committee approved all animal procedures.

Development of MySOD transgenic lines. A 7-kb transgene designated MySOD was constructed for overexpression of MnSOD in cardiac tissue of transgenic mice. This transgene contains all of the coding sequences of human MnSOD (17). Transcription of the transgene is controlled by a fragment of the mouse α -cardiac myosin heavy chain (MHC) gene described by Gulick et al. (18) to produce high-level, cardiac-specific expression of cDNA constructs in transgenic mice. In our laboratory, we have produced >50 transgenic lines using this MHC promoter, and all of the transgenes have been very active and cardiac specific. To produce MySOD, an 830-bp MnSOD fragment that included the MnSOD start and stop codons was ligated behind a 5.7-kb fragment of the MHC gene. The MHC fragment included the promoter, the first two introns, and the first three noncoding exons of the MHC gene. To provide a polyadenylation signal for the MySOD transcript, the MnSOD coding sequence was ligated in front of a 550-bp fragment of the rat insulin II gene containing the insulin polyadenylation sequence (19). Before microinjection, the transgene was purified from the plasmid sequences by restriction digestion and gel electrophoresis.

Seven transgenic founder lines were originally produced by microinjecting MySOD transgene together with the tyrosinase coat color marker (20) into FVB embryos. The presence of transgene was determined by PCRs on mouse tail DNA and the dark gray pigmentation due to the cointegration of tyrosinase. MySOD3 and MySOD4 lines were kept for further studies because of their stable inheritance of the transgene and high superoxide dismutase (SOD) activity.

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DCFDA, 2'-7'-dichlorofluorescein diacetate; GSH, glutathione; MHC, myosin heavy chain; MnSOD, manganese superoxide dismutase; RCR, respiratory control ratio; ROS, reactive oxygen species; SOD, superoxide dismutase.

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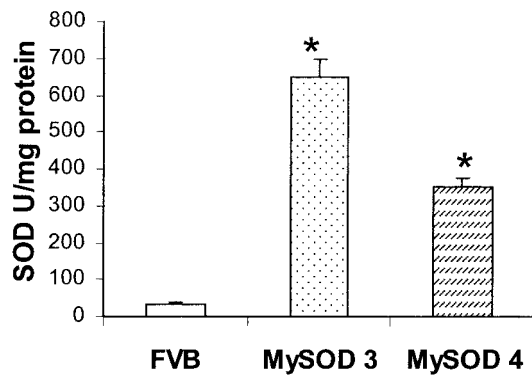


FIG. 1. Elevated SOD activity in MnSOD transgenic mice. SOD activity was determined by measuring the inhibition of pyrogallol autoxidation per milligram of cardiac protein extract. Data are the means obtained from five mice in each group. Vertical bars indicate the SE. * $P < 0.01$ vs. FVB.

SOD and catalase enzyme activity assay. SOD activity was determined by measuring the inhibition of pyrogallol autoxidation (21). Heart samples were homogenized in sample assay buffer (50 mmol/l tri-cacodylic acid and 1 mmol/l diethylenetriamine-pentaacetic acid, pH 8.2), and the homogenate was incubated on ice for 30 min to solubilize SOD from tissue followed by centrifugation. The supernatant was mixed with assay buffer, and the reaction was initiated by adding 8 mmol/l pyrogallol. The increase of absorbance at 405 nm was followed for 2 min by SPECTRA Fluor Plus plate reader (Tecan U.S., Durham, NC). SOD activity was assessed as the degree of inhibition of the pyrogallol autoxidation rate. An inhibition of 50% by standard SOD was defined as 1 unit SOD. Sample protein concentration was measured by bicinchoninic acid protein assay method. The final results were reported as SOD units per milligram protein. Catalase was measured using the catalase assay kit from Cayman Chemicals (Ann Arbor, MI) as described in the manual. The method is based on the peroxidatic function of catalase, which results in oxidation of the chromagen Purpald, which was measured at 540 nm on the SPECTRA Fluor Plus plate reader. Data are presented as units of catalase per minute per milligram tissue, where 1 unit catalase is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol methanol to formaldehyde per minute at 25°C.

Western blot for MnSOD location. Sheep anti-human MnSOD antibody (PEROX, Birmingham, U.K.) and goat anti-sheep IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were used to identify transgenic MySOD by Western blotting. Ten micrograms of cardiac proteins were run on the 18% SDS-PAGE gel and transferred to polyvinylidene fluoride membrane. After blocking, the membrane was incubated with primary antibody (1:500) at 4°C overnight followed by the secondary antibody (1:2,000). Finally, the membrane was developed using ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Isolation of cardiomyocytes. Cardiomyocytes from adult mice hearts were isolated according to the protocol from The Alliance for Cellular Signaling (<http://www.signaling-gateway.org>; protocol ID, PP00000125) with modification. Fresh hearts from 3-month-old FVB or MySOD transgenic mice (three mice in each group) were quickly removed and perfused with oxygenated Krebs-Henseleit calcium-free buffer and then with Krebs-Henseleit buffer with added Liberase Blendzyme 4 (Roche Applied Science, Indianapolis, IN). The left ventricle was removed after perfusion and teased to small pieces with fine forceps. The cell suspension was transferred to a conical tube, and Krebs-Henseleit buffer with 10% serum and 12.5 $\mu\text{mol/l}$ calcium was added to stop the digestion. The heart tissues were further dissociated, and myocytes were allowed to sediment by gravity. After removal of supernatant, the pellet was

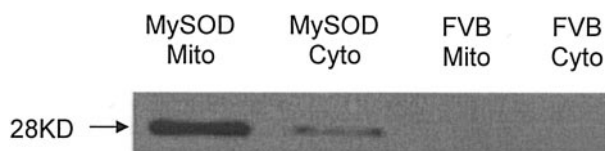


FIG. 2. Mitochondrial localization of overexpressed MnSOD in MySOD transgenic mice. Western blots were performed using 10 μg cardiac proteins extracted from mitochondrial and cytoplasmic fractions of FVB and MnSOD mice. Blots were probed with a sheep anti-human MnSOD antibody. Mito, mitochondrial protein; Cyto, cytoplasmic protein without mitochondria.

carefully suspended in Krebs-Henseleit buffer and filtered through a stainless mesh (170- μm pore size) to ensure the separation of individual myocytes. Usually, 0.5–1 million cardiomyocytes can be harvested from one mouse heart using this method. Calcium was reintroduced into cells to the final concentration of 1.25 mmol/l. Isolated ventricular myocytes were maintained at room temperature in Hank's buffer containing 5.6 mmol/l d-glucose and 1.25 mmol/l calcium. Cardiomyocytes were used immediately for ROS measurement.

Cardiomyocyte ROS measurement. The cardiomyocytes were loaded with 0.5 $\mu\text{mol/l}$ 2'-7'-dichlorofluorescein diacetate (DCFDA) (Molecular Probes, Eugene, OR) at 37°C in the dark for 0.5–1 h, rinsed, and resuspended. The cardiomyocytes from both FVB and MySOD mice were divided into several groups and plated onto a 48-well plate for different treatments: control, hypoxanthine with xanthine oxidase, or hydrogen peroxide of different concentrations. Immediately after addition of above chemicals, cellular fluorescence produced by 2'-7'-dichlorofluorescein was measured with SPECTRA Fluor Plus plate reader (Tecan U.S.). The increase of intracellular fluorescence was followed for 100 min at 492/530 nm.

Analysis of heart morphology. Cardiac ultrastructure in 5-month-old control, diabetic, diabetic with MySOD overexpression, and MySOD transgenic mice was examined by transmission electron microscopy. Mitochondrial area and number were also accomplished using transmission electron microscopy micrographs from random areas as previously described (14). Mitochondrial areas on these micrographs were determined by manually blackening in mitochondrial outlines on photographs. These images were then scanned into Adobe Photoshop to determine the percentage of mitochondrial area over total cardiac area excluding the areas of nucleus or blood vessels. Mitochondrial numbers on these electron microscopy micrographs were manually counted by a blind observer and normalized to unit area.

Mitochondrial function study. Mitochondria were isolated from 4-month-old FVB, OVE26, OVE26-MySOD, and MySOD mice as described before (14). RCR and (P/O) oxygen utilized per unit of ADP were evaluated by measuring oxygen consumption using a Clark-type oxygen electrode (model 1302; Strathkelvin Instruments, Glasgow, U.K.) as described in our last study (14). In brief, the reaction was carried out in respiration buffer at 25°C with 80–100 μg mitochondrial protein added. Pyruvate (20 mmol/l) and 10 mmol/l malate were also added as substrates. State 3 and 4 respiration rates were measured in the presence or after depletion of 1 mmol/l ADP. For measurement of state 4 respirations, 1 $\mu\text{mol/l}$ oligomycin was also added. Respiratory control ratio (RCR) was defined as the ratio of state 4 to state 3 respiratory rate. P/O was calculated as the amount of oxygen used during state 3 respiration per unit added ADP. State 3 and 4 rates were also calculated and normalized to the protein content.

Quantitative real-time PCR for mitochondrial DNA. Mitochondrial DNA content was estimated as described previously (14). Briefly, cardiac DNA was extracted from frozen heart tissues of 4- to 5-month-old control, diabetic, diabetic with MySOD overexpression, and MySOD transgenic hearts using DNeasy tissue kit (Qiagen, Valencia, CA). Total DNA concentration was determined using PicoGreen DNA quantitation kit (Molecular Probes). Two nanograms DNA was mixed with ABS Taqman universal PCR master mix (Applied Biosystems, Foster City, CA), cytochrome b or β -actin probe and primers and run on Matrix 4000 Real Time PCR system (Stratagene, La Jolla, CA). The results were analyzed by Mx4000 real-time quantitative detection software version 3.0. Mitochondrial DNA per nuclear genome was calculated as the ratio of cytochrome b DNA level to β -actin DNA level.

Measurement of mitochondrial reduced glutathione level. Mitochondrial reduced glutathione (GSH) level was measured by Bioxyteck GSH/GSSH 412 kit (OxisResearch, Portland, OR) with modification. Twenty-microliter mitochondrial samples from 4-month-old mice hearts were vortexed in the same amount of 8.75% metaphosphoric acid and centrifuged at $2,000 \times g$ at 4°C for 10 min to extract mitochondrial GSH. Supernatant (10 μl) was mixed with 200 μl assay buffer containing 100 mmol/l Na_2PO_4 and 4 mmol/l EDTA, pH 7.5. Samples or standards were then mixed with 5,5'-dithiobis-(2-nitrobenzoic acid) and GSH reductase, followed by incubation at room temperature for 5 min. The absorbance was read at 412 nm for 3 min after the addition of NADPH.

Cardiac contractility. Mechanical properties of cardiomyocytes from 5-month-old FVB, OVE26, OVE26-MySOD, and MySOD mice were assessed using a video-based edge-detection system (IonOptix, Milton, MA) as described previously (22). Isolated cardiomyocytes prepared with the method above were placed in a Teflon glass coverslip dish (Harvard Apparatus, Hillston, MA) mounted on the stage of an inverted microscope (IX-70; Olympus, Melville, NY). The cells were field stimulated at a frequency of 1.0 Hz for 4 min with a pair of platinum wires placed on opposite sides of the dish chamber connected to the MyoPacer Field Stimulation (IonOptix). The movement of the myocytes was monitored by an IonOptix MyoCam camera, which rapidly scans the image area every 8.3 ms. The soft-edge software (IonOptix) was used to capture changes in cell length during shortening and

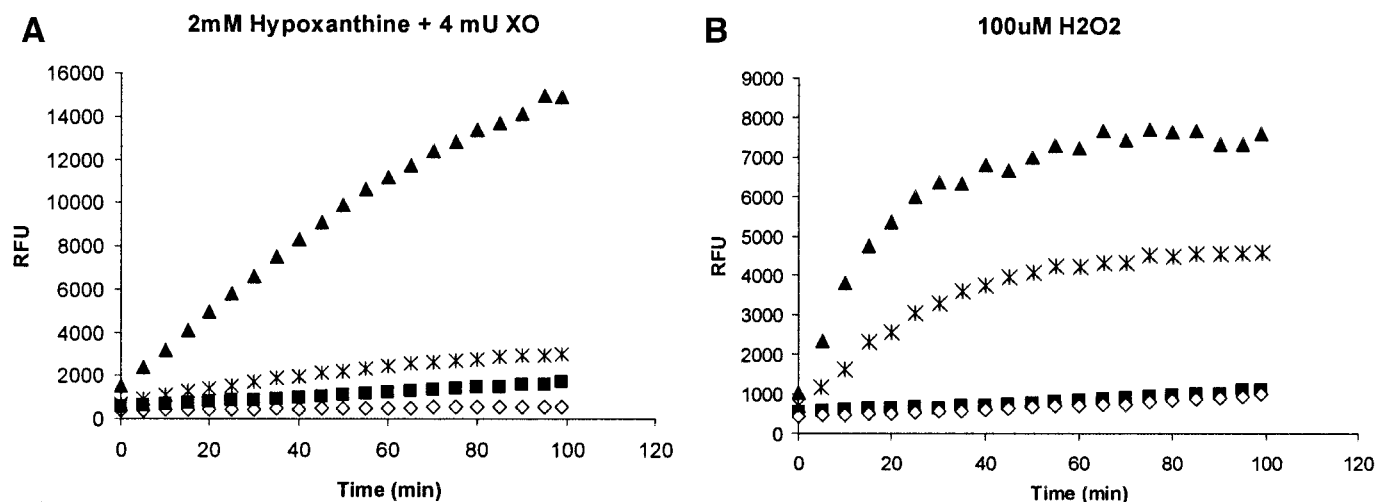


FIG. 3. MnSOD overexpression reduces superoxide- and hydrogen peroxide-induced ROS in isolated cardiomyocytes. FVB control and MnSOD transgenic cardiomyocytes were treated with the indicated concentrations of hypoxanthine and xanthine oxidase (A) or hydrogen peroxide (B). Hank's buffer was used to measure basal values. ROS production in the myocytes was measured by DCFDA fluorescence as described in RESEARCH DESIGN AND METHODS. ■, FVB basal; ▲, FVB treated; ◇, MySOD basal; ✕, MySOD treated. Data are typical of three or four independent experiments.

relengthening. Myocyte contractility was assessed by percentage of peak shortening and maximal velocities of shortening and relengthening (\pm dL/dt). **Data analysis.** Data are expressed as the means \pm SE. Statistical comparisons were performed by Student's *t* test for comparison between two groups and one- or two-way ANOVA for comparison among several groups using Sigma-Stat software. Significance was defined as $P < 0.05$.

RESULTS

MnSOD transgenic line development. The MySOD transgene for cardiac-specific expression of human MnSOD was coinjector into FVB embryos along with a tyrosinase transgene. Expression of tyrosinase provides a coat color marker for transgenesis on an albino background (20). Two founder lines, MySOD3 and MySOD4, were kept for further studies because of their stable inheritance of the transgene, high SOD activity (Fig. 1), and cointegration of the tyrosinase coat color marker. The MySOD transgene increased SOD activity by 20- and 10-fold in MySOD3 and MySOD4 hearts, respectively, as shown in Fig. 1. Cardiomyocytes of MySOD3 mice displayed normal morphology, contractility, and mitochondrial function (Figs. 4–6 and 8). Unless otherwise stated, subsequent results were obtained from MySOD3 mice.

Endogenous MnSOD is located in mitochondria. To confirm the subcellular location of transgenic MnSOD, Western blots were carried out using proteins extracted from mitochondrial and cytoplasmic fractions of FVB and MnSOD hearts. Blots were probed with anti-human MnSOD antibody, which gave a band of 28 kDa. The result confirmed the targeting of transgenic MnSOD overexpression to mitochondria (Fig. 2). No human MnSOD band was detected in control mice, presumably because the MnSOD antibody was anti-human and the endogenous MnSOD was too weak for detection on this exposure.

MnSOD reduces ROS in isolated cardiomyocytes. To determine whether overexpression of MnSOD enhanced ROS scavenging, isolated cardiomyocytes from control and MySOD transgenic mice were exposed to different concentrations of superoxide generated by hypoxanthine and xanthine oxidase or to hydrogen peroxide. The change in ROS level in the myocytes was monitored by the increase of the signal intensity for the fluorescent probe DCFDA. As shown in Fig. 3, MnSOD effectively scavenged

superoxide and significantly reduced intracellular ROS level during exposure to hydrogen peroxide. The reduction in hydrogen peroxide-induced fluorescence may have been due to the induction of catalase determined in transgenic hearts (Fig. 7). Very similar results were obtained at other doses of hypoxanthine and xanthine oxidase or hydrogen peroxide (not shown).

MnSOD protects morphology in diabetic hearts. After characterization of the MySOD transgenic lines, we crossed it with our type 1 diabetes model OVE26. Measurement of blood glucose levels were similar in OVE26 mice and in double transgenic OVE26-MySOD mice: non-fasted hyperglycemia almost always exceeded >600 mg/dl (data not shown). Excess production of superoxide by mitochondria has been implicated as a key step in diabetes complications (8). We have previously demonstrated that OVE26 diabetic hearts exhibit many characteristics of cardiomyopathy (16), which makes this a suitable model to test whether increased mitochondrial SOD activity protects from diabetic cardiomyopathy. Female MySOD3 mice were bred to male OVE26 mice. We first examined the effect of MnSOD overexpression on cardiac ultrastructure (Fig. 4). FVB control and MySOD morphology were indistinguishable. As we previously reported (16), OVE26 hearts contained many focal areas of damage with swollen mitochondria, mottled matrix, and broken mitochondrial double membrane. In double transgenic OVE26-MySOD mice, such severely damaged areas were never detected.

Improved mitochondrial function and reduced mitochondrial mass in OVE26-MySOD hearts. We further tested whether the better morphology of OVE26-MySOD mitochondria coincided with improved function. Consistent with our prior studies (14), RCR and state 3 respiration were significantly impaired in OVE26 mitochondria (Fig. 5). MnSOD overexpression significantly improved RCR and state 3 respiration in OVE26-MySOD mitochondria, although it did not completely return respiration to normal. OVE26 hearts have been shown to have greater mitochondrial mass, as indicated by significantly increased protein and DNA content, as well as greater area and number (14). This was confirmed in our current study (Fig. 6). We proposed that this was a compensatory response to impaired function of diabetic mitochondria (14). If our hypothesis is correct, then the

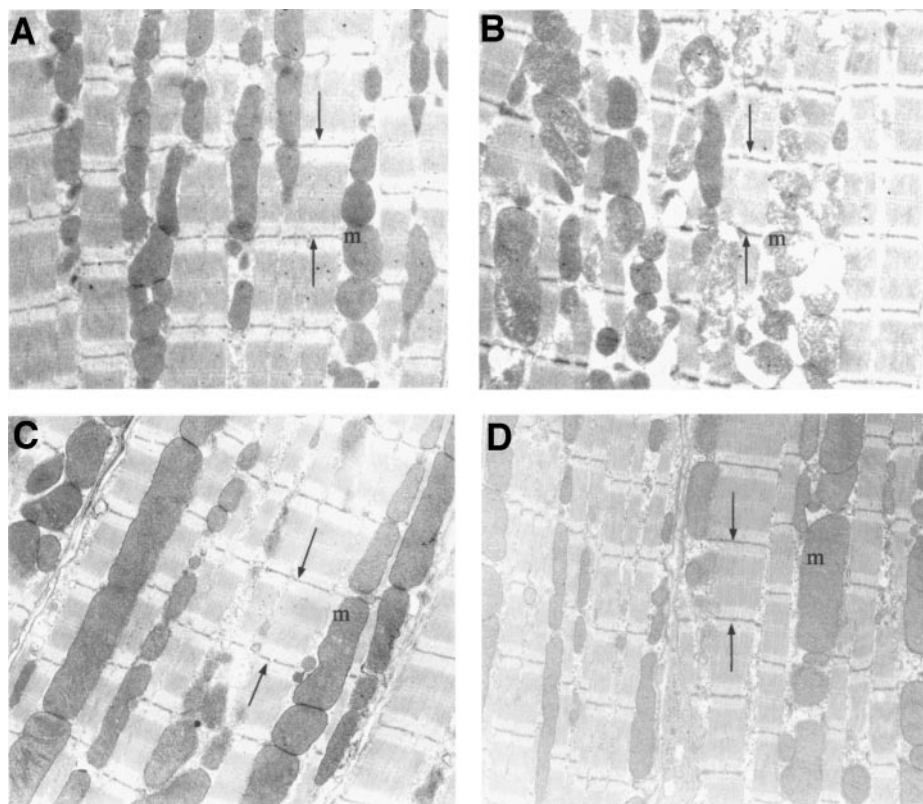


FIG. 4. MnSOD prevented damaged morphology in diabetic hearts. Electron micrographs ($\times 3,800$) from left ventricle of FVB (A), OVE26 (B), OVE26-MySOD (C), and MySOD (D) mice at 5 months of age. M, mitochondria; arrowhead, z lines.

improved function of OVE26-MySOD mitochondria should inhibit the diabetes-induced increase in mitochondrial mass. As shown in Fig. 6, MnSOD overexpression completely prevented the diabetes-induced increase in mitochondrial area, number, protein, and DNA content.

MySOD increased cardiac catalase activity and mitochondrial GSH levels. Catalase activity was measured in cardiac extracts from 4- to 5-month-old FVB, OVE26, OVE26-MySOD, and MySOD transgenic mice. As shown in Fig. 7A catalase activity was increased by both diabetes from the OVE26 transgene and by the presence of the MySOD transgene. The effects of the two transgenes were approximately additive in double transgenic OVE26-MySOD mice.

We previously reported decreased mitochondrial GSH levels in 10-month-old OVE26 heart (14). In this study, we measured mitochondrial GSH levels in 4-month-old control and transgenic mice. At this age, the drop in mitochondrial GSH in OVE26 hearts was only 10% (Fig. 7B) and did not reach statistical significance. However, a two-way ANOVA using the presence of the MySOD transgene and diabetes as factors indicated that presence of the MySOD gene increased mitochondrial GSH content ($P = 0.03$).

Improved contractility in OVE26-MySOD cardiomyocytes. In addition to the morphological damage, diabetes also significantly impairs contractility of isolated cardiomyocytes, as indicated by reduced percentage of peak shortening, rate of contraction, and rate of relaxation (23). As shown in Fig. 8, MnSOD overexpression in diabetic hearts completely normalized all of these contractile parameters. These results were obtained from the MySOD3 line. Diabetic cardiomyocytes from the MySOD4 line (data not shown) also showed a very similar degree of protection.

DISCUSSION

We have previously reported cardiac mitochondrial damage and biogenesis in our chronic model of type 1 diabetes (14) and proposed that oxidative stress, in particular, mitochondria-derived ROS were involved in diabetic cardiomyopathy. In the present study, we overexpressed the mitochondrial antioxidant protein MnSOD specifically in heart to determine whether reducing mitochondrial superoxide would protect from diabetes-induced injury. MnSOD overexpression was able to completely reverse diabetes-damaged cardiac morphology and impaired contractility. It also provided partial protection from the effects of diabetes on mitochondria, including reduced mitochondrial RCR and abnormal mitochondrial mass.

MnSOD is the primary mitochondrial antioxidant enzyme and is essential for maintaining normal cell development and function. Overexpression of MnSOD has been shown to be beneficial in various animal models and cultured cells (24,25). In our MnSOD transgenic mice, overexpression was directed to the cardiomyocyte by the α -MHC promoter. This promoter has been widely used to achieve high-level cardiac-specific expression of transgenes (16,26). The MySOD transgene elevated SOD enzymatic activity 10- to 20-fold, and the increased MnSOD protein expression was shown to be confined to mitochondria. MnSOD overexpression also elevated myocyte catalase activity and mitochondrial GSH levels. These changes may act together with MnSOD against oxidative insults. Overexpression of MnSOD did not change morphology, contractility, and mitochondrial function in nondiabetic transgenic hearts. This indicates that normal mitochondrial superoxide production does not mediate essential cardiac cell signaling processes.

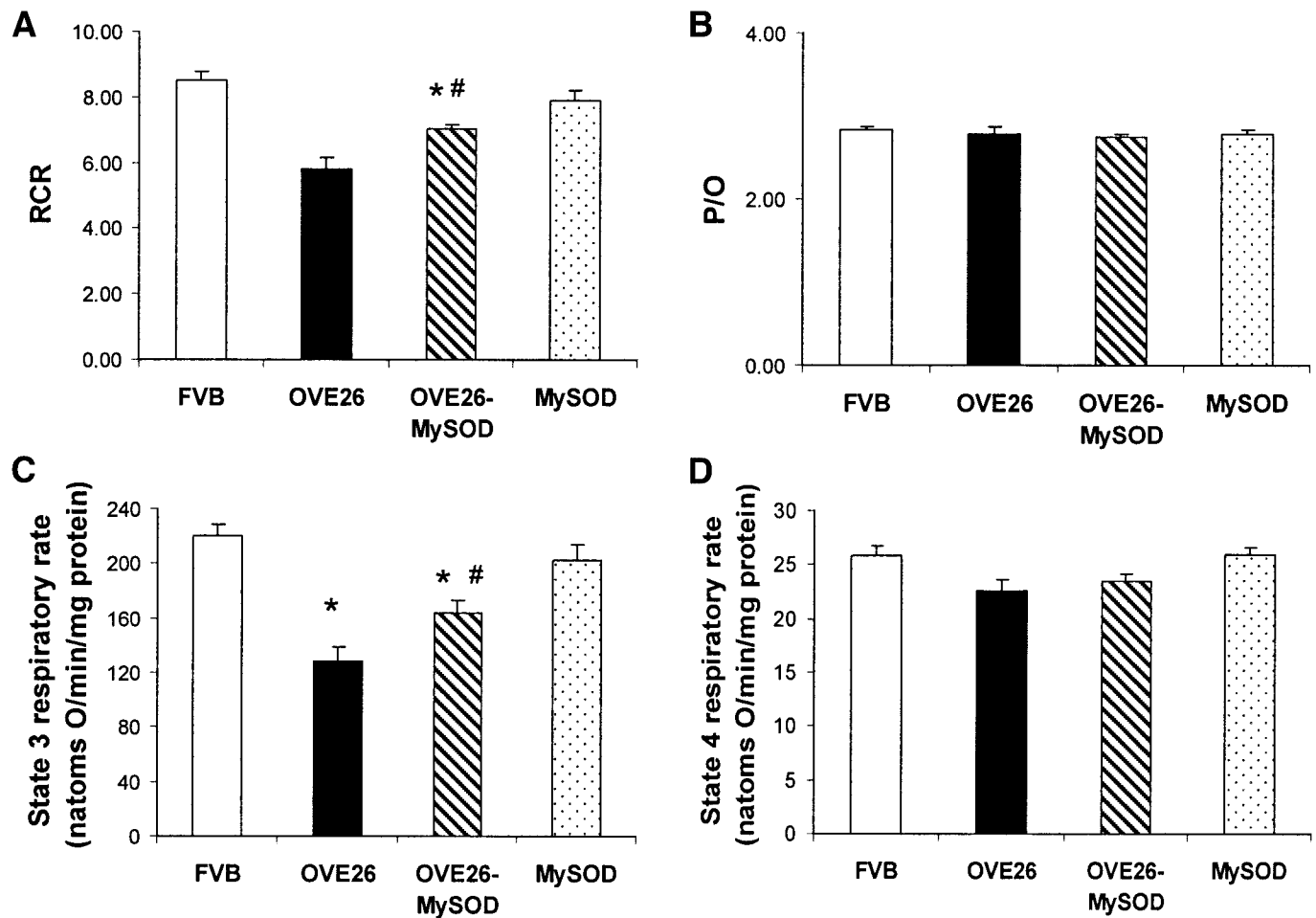


FIG. 5. MnSOD partially restored impaired mitochondrial function in OVE26 heart. RCR (A) and state 3 respiration (C) are reduced in OVE26 mitochondria and partially restored by overexpression of MnSOD in OVE26-MySOD mitochondria. P/O (B) and state 4 respiration (D) were not significantly different in any group. Assays were performed in cardiac mitochondria from 3-month-old mice as described in RESEARCH DESIGN AND METHODS. Results were from at least 12 assays performed with mitochondria isolated from four animals per group. Vertical bars indicate the SE. * $P < 0.05$ vs. FVB by one-way ANOVA. # $P < 0.05$ vs. OVE26 by one-way ANOVA.

MnSOD transgenic cardiomyocytes generated a weaker ROS signal when treated with exogenous oxidants. The major function of MnSOD is to scavenge superoxide radicals. Therefore it was not unexpected that MnSOD transgenic cardiomyocytes, challenged with superoxide produced by hypoxanthine and xanthine oxidase, produced markedly reduced ROS levels. This reduction was almost 10-fold compared with nontransgenic myocytes. The MySOD transgene produced a lesser reduction in the hydrogen peroxide-induced ROS signal. This reduction was ~40%. Hydrogen peroxide is not a substrate of MnSOD, so we expect that the reduction was due to the ~twofold induction of catalase activity in MySOD myocytes. The induction of catalase may be secondary to the increased hydrogen peroxide produced by elevated MnSOD activity in transgenic mitochondria, because hydrogen peroxide is a known inducer of catalase activity (27). We also observed that diabetes produced a significant induction of catalase activity, and this is consistent with our previous finding that catalase mRNA is elevated in OVE26 hearts (23).

Under most conditions, mitochondria are the major site of ROS production. Many laboratories have generated data suggesting that diabetic damage is a consequence of elevated production of ROS by the mitochondrial respira-

tory chain during hyperglycemia (28,29). Brownlee and colleagues (8) have shown that normalizing mitochondrial ROS levels by overexpressing MnSOD in endothelial cells prevented high glucose-induced activation of at least three different potential pathological pathways. Data from our own laboratory have also shown that mitochondrial ROS are important for diabetic heart complications (14,23). Cardiac structural damage is most extreme in mitochondria, and we observed many areas of diabetic heart in which mitochondria were severely damaged, whereas the adjacent myofibrils in the same myocytes were normal (14). We also found that mitochondrial GSH was more sensitive to diabetes than whole-heart GSH (16). In addition, the increased levels of ROS generated in diabetic cardiomyocytes could be prevented when we incubated myocytes with rotenone or thenoyltrifluoroacetone, inhibitors of mitochondrial electron transport chain complexes I and II (23). All of the above findings support the hypothesis that mitochondrial ROS mediate diabetes-induced cardiac defects. If this is correct, then we should be able to protect the diabetic heart by augmenting mitochondrial antioxidant defense systems.

We tested this by crossing MnSOD transgenic mice with diabetic OVE26 mice. MnSOD was effective in preventing many aspects of diabetic cardiomyopathy in OVE26-

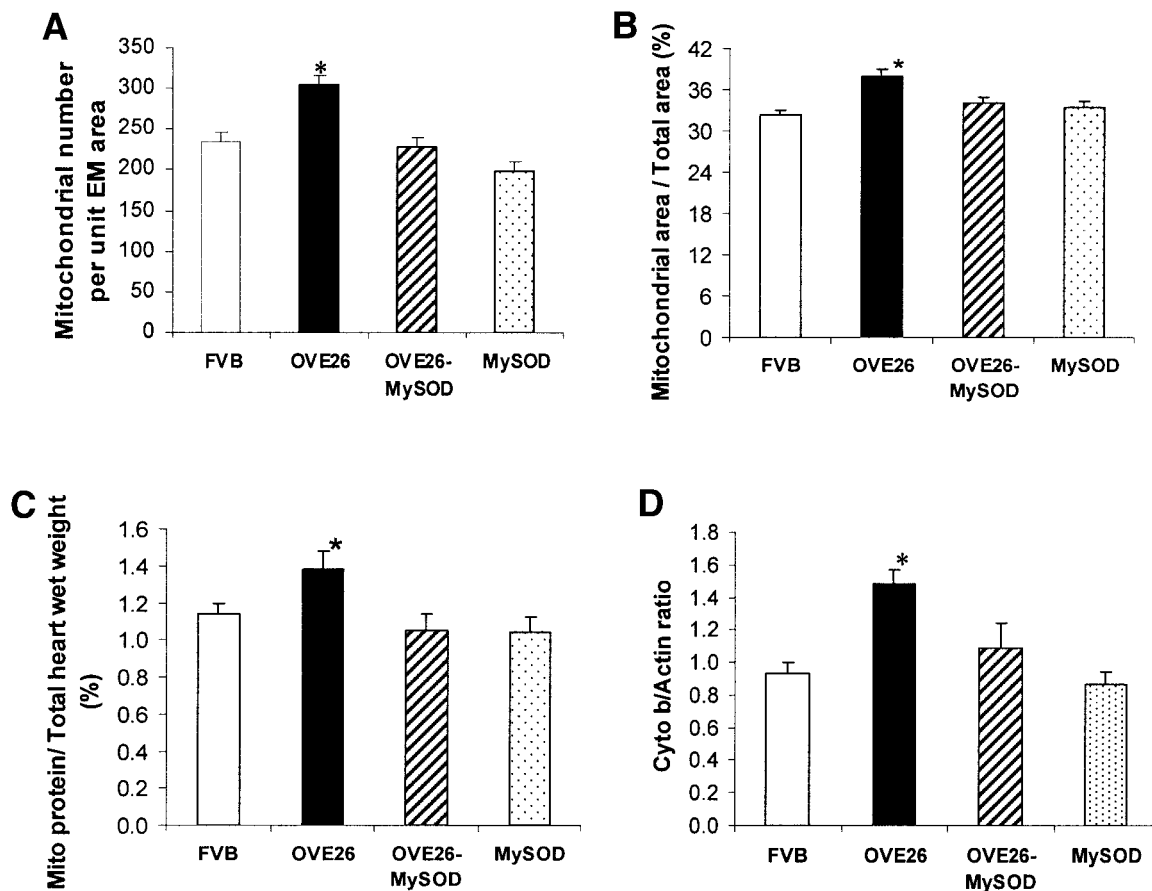


FIG. 6. MnSOD normalized increased mitochondrial mass in diabetic heart. Mitochondrial area (A), number (B), protein (C), and DNA content (D) in 5-month-old FVB, OVE26, OVE26-MySOD, and MySOD hearts were measured as described in RESEARCH DESIGN AND METHODS. Values are means from at least five animals in each group. Vertical bars indicate the SE. * $P < 0.05$ vs. all other groups by one-way ANOVA.

MySOD mice. The MySOD transgene eliminated all identifiable areas of severe mitochondrial damage found in diabetic myocytes. MnSOD overexpression prevented injury to mitochondrial matrix and membrane and reduced mitochondrial swelling. In addition, the expanded mitochondrial area, number, protein, and DNA content were all

completely normalized by MnSOD overexpression. This indicated that hyperglycemia alone did not induce increased mitochondrial mass, because the OVE26-MySOD transgenic mice had the same levels of glucose as OVE26 mice. Therefore excess superoxide must be important in these mitochondrial abnormalities. We have observed that

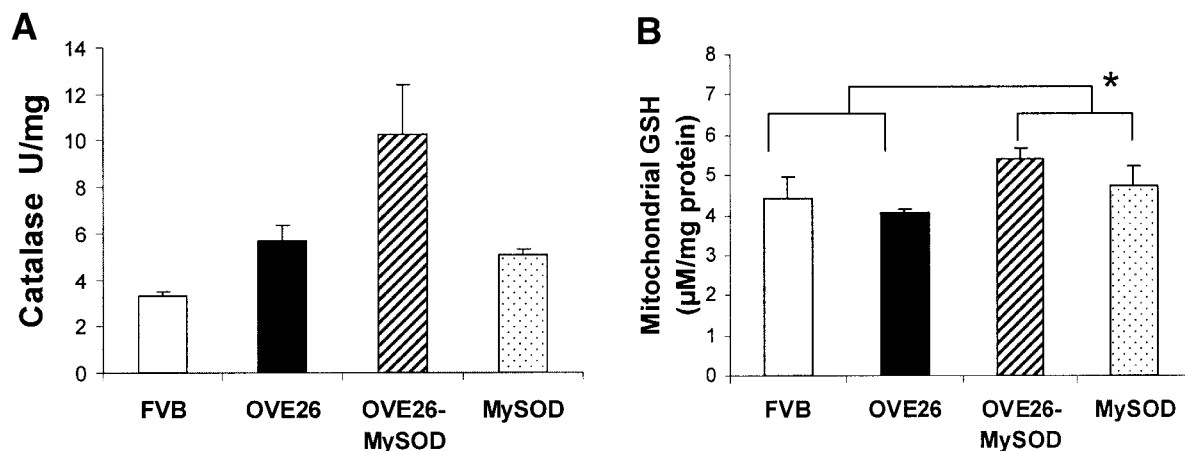


FIG. 7. The MySOD transgene increased cardiac catalase and cardiac mitochondrial GSH level. A: Catalase levels were measured using the catalase kit from Cayman Chemicals. Two-way ANOVA performed on the log of catalase activity (to equalize variance between groups) showed that either the presence of the OVE26 gene or the presence of the MySOD gene significantly increased catalase activity ($P < 0.01$). In addition, all pairwise comparisons between groups were significant, $P < 0.01$; catalase activity in MySOD mice was greater than in FVB, OVE26 was greater than MySOD, and OVE26-MySOD was greater than OVE26. B: Mitochondrial GSH levels were measured with the GSH kit from Oxis Research. * $P = 0.03$ for the comparison between the two groups carrying the MySOD gene vs. the two groups that did not have the gene by two-way ANOVA. Values are means from four animals between 4 and 5 months of age in each group.

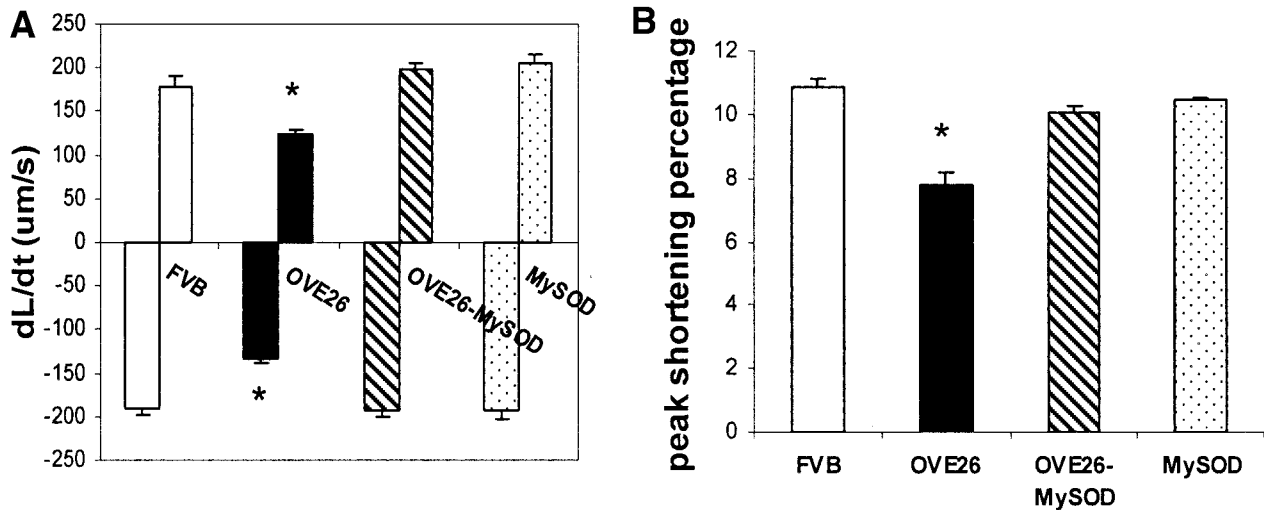


FIG. 8. MnSOD protected diabetic cardiomyocytes from reduced contractility. A: Rates of contraction (+dL/dt) and relaxation (-dL/dt). **B:** Percentage of peak shortening of isolated cardiomyocytes from 5-month-old FVB, OVE26, OVE26-MySOD, and MySOD mice. Values are means from ≥ 80 myocytes derived from five mice per group. Vertical bars indicate SE. *OVE26 is different from all other three groups ($P < 0.05$) by one-way ANOVA.

mitochondrial morphological damage is much worse in our oldest OVE26 heart specimens (from mice >1 year old; X.S., E.C. Carlson, P.N.E., unpublished results), which suggests that initial damage may accelerate the process of superoxide generation and/or destruction in mitochondria.

Consistent with our previous report (14), diabetic mitochondria had significantly reduced RCR due to impaired state 3 respiration. Overexpression of MnSOD in diabetic mice restored about one-half of the deficit in state 3 respiration and RCR. This shows that excess superoxide contributes to the impaired mitochondrial function. The fact that respiration was not completely normalized by MnSOD implies that superoxide overproduction may not be the only source for mitochondrial dysfunction in the diabetic state. Excess free fatty acid metabolism has been implicated in diabetes-induced mitochondrial abnormalities (30), and OVE26 mice have elevated serum triglycerides (16). Because MnSOD overexpression is not likely to affect abnormal free fatty acid metabolism, this could explain the remaining impairment in respiration. However, the significant beneficial effect of MnSOD on diabetic respiration provides the most direct demonstration that impaired mitochondrial function in diabetic hearts is due, at least in part, to superoxide and that it can be improved by an antioxidant enzyme. Reduced state 3 respiration (14) and excess superoxide are both caused by electron transport chain dysfunction, and protection of the electron transport chain may explain most benefits of MnSOD overexpression.

The MySOD transgene also restored normal rates of contraction, relaxation, and peak shortening in isolated diabetic cardiomyocytes. This was not necessarily expected, because MnSOD is localized within diabetic mitochondria, physically separated from the contractile sarcomere. We have previously shown that two cytoplasmic antioxidants, catalase (23) and metallothionein (22), both of which are excluded from mitochondria (31,32), also protect sarcomere function in diabetic myocytes. Interestingly, catalase and metallothionein also protected mitochondrial structure in diabetic hearts. Thus cytoplasmic antioxidants could protect the mitochondria, and mitochondrial antioxidants could protect cytoplasmic

structures. The basis for this interorganelle antioxidant protection is not known, but it implies that diffusible ROS, such as hydrogen peroxide, are important in diabetic cardiomyopathy. It is also possible that the twofold induction of catalase that we observed contributed to the protection of cytoplasmic structures in MySOD transgenic hearts.

These studies are a part of our continued efforts (14,16,22,23) to understand the basis of diabetic cardiomyopathy in type 1 diabetes. All of our results support an essential pathological role of ROS in diabetes-induced cardiotoxicity that can be prevented with adequate, continuous antioxidant protection of mitochondria.

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