Metallothionein Prevents Diabetes-Induced Deficits in Cardiomyocytes by Inhibiting Reactive Oxygen Species Production

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Diabetes is a major risk factor for the development of congestive heart failure (1). Diabetic cardiomyopathy, a diabetes-related myopathic state and a major cause of disability and morbidity for patients with diabetes, has been documented to lead to congestive heart failure even in the absence of any other risk factors such as hypertension or coronary artery disease, suggesting that diabetic cardiomyopathy is a specific cardiomyopathy associated with diabetes and independent of macrovascular complications. Results from chemically induced insulinopenic (2) as well as in genetically predisposed insulin-resistant (3) rodent models (4) and human histological (5) and clinical observations (6) provide support for this concept of a specific diabetic cardiomyopathy, although its mechanism remains poorly understood. The hallmarks of diabetic cardiomyopathy, in experimental or clinical settings, include prolongation of contraction and relaxation, compromised relaxation velocity, and impaired contractility (3,4).

Reactive oxygen species (ROS), such as the superoxide radical, the hydroxyl radical, and H$_2$O$_2$, are continuously produced in most cells under physiological conditions, and their levels are regulated by a number of enzymes and physiological antioxidants such as superoxide dismutase, glutathione peroxidase, and catalase as well as by other nonenzymatic antioxidants. However, when the production of ROS becomes excessive, oxidative stress will develop and impose a harmful effect on the functional integrity of biological tissue. There is convincing experimental and clinical evidence that the generation of ROS is increased in both types of diabetes and that the onset of diabetes and its complications, including diabetic cardiomyopathy, are closely associated with oxidative stress (7,8).

Metallothionein (MT) is a cysteine-rich protein that can bind heavy metal ions such as copper and zinc (9). It also has a strong effect in scavenging free radicals because of its high thiol content (10). The concept is supported by the fact that MT can scavenge hydroxyl radicals in vitro (11) and provide protection against radiation damage (12). Also, some chemicals that stimulate the production of ROS can increase MT levels (13). However, it was observed that MT in the heart of adult rodents was the lowest of any organs when compared with the liver, kidney, small intestine, testes, and brain (14), indicating that the heart tissue may be susceptible to oxidative stress as a result of low antioxidant defense (15). To address whether increased ROS is involved in the pathogenesis of diabetic cardiomyopathy and the potential protection of MT against harmful effects of ROS in the development of diabetic cardiomyopathy, we used a transgenic line that overexpresses MT specifically in the heart from the α-myosin heavy chain promoter. MT transgenic mice were crossbred with OVE26 diabetic mice, and the offspring were used for study. In a previous report, we demon-
stated that MT benefits diabetic heart morphology and whole-heart ischemic contractility (16). In the current study, we show that MT protection of mechanical performance and calcium metabolism is exerted at the level of the individual ventricular myocyte. We also found that prolonged diabetes increases cardiomyocyte ROS production in response to angiotensin II and high glucose and that MT overexpression prevents this damaging response of the heart to diabetes.

RESEARCH DESIGN AND METHODS
Animals. OVE26 diabetic mice have been previously described (17). MT mice that overexpress MT 16-fold in the heart were also previously described (18). Transgenic mice of both lines were identified by the presence of phenotypic markers. OVE26-positive mice were recognized by the presence of small eyes as a result of the co-integration of the GR19 gene, which is expressed in the eye. MT-positive mice were recognized by the presence of coat color as a result of co-integration of the tyrosinase gene that corrects the mutant tyrosinase gene of FVB mice (19). Age-matched OVE26, OVE26MT, and FVB mice of both sexes were obtained at 20 weeks of age. All transgenic and nontransgenic animals were maintained on the inbred FVB background. Mice were maintained on a 12-h light/dark cycle and received food (Purina Laboratory Rodent Diet 5001) and water ad libitum. Serum triglycerides were measured with a Sigma triglyceride (UV) kit. The U.S. Department of Agriculture certified institutional animal care committee approved all animal procedures.

Isolation of ventricular myocytes. Single ventricular myocytes were isolated by enzymatic dissociation with collagenase II (Worthington, NJ) from adult mice by modification of described procedures (4). Briefly, animals received an injection of heparin (1,000 units/kg, i.p.) and were anesthetized with ketamine (150 mg/kg, i.p.) and xylazine (22.5 mg/kg, i.p.). Their hearts were rapidly removed and perfused at 37°C with oxygenated (5% CO2/95% O2) Krebs-Henseleit (KH) calcium-free buffer (in mmol: 135 NaCl, 4.0 KCl, 1.0 MgCl2, 0.33 NaH2PO4, 10 HEPES and glucose, and 10 Butanedione [pH 7.4]). Hearts were then perfused with the same Ca2+-free KH buffer with collagenase II (0.9 mg/ml) for 15–20 min until the heart became flaccid. After perfusion, the left ventricle was removed, minced, and resuspended with same Ca2+-free KH at room temperature. Extracellular Ca2+ was added incrementally back to 1.2 mmol/l. Isolated myocytes were maintained at room temperature in a serum-free medium consisting of medium 199 (GIBCO) with Hanks’ salts containing 25 mmol HEPES for additional mechanical and fluoroscent studies. Mechanical properties remained relatively stable in myocytes for 8–10 h in the serum-free medium. Cell yield was ~50–70%. There was no notable difference of yield between normal FVB and transgenic mice. Myocytes with rod-shaped sarcomemal blebs or spontaneous contractions were not used. Only rod-shaped myocytes with clear edges were used for recording of mechanical properties, intracellular Ca2+ transients, and ROS fluorescent measurement.

Cell shortening/relenetheng. Mechanical properties of ventricular myocytes were assessed using a video-based edge-detection system (IonOptix, Milton, MA) (4). Cells were placed in a Teflon glass coverslip dish (Harvard Apparatus, Holliston, MA) mounted on the stage of an inverted microscope (Olympus, IX-70). The cells were field-stimulated at a frequency of 1.0 Hz, 4-ms duration, using a pair of platinum wires placed on opposite sides of the dish chamber connected to the MyoPacer Field Stimulator (IonOptix). The polarity of the stimulatory electrodes was reversed frequently to avoid possible build-up of electrolyte by-products. The myocytes being studied were displayed on the computer monitor using an IonOptix MyoCam camera, which rapidly scans the image area every 8.3 ms such that the amplitude and velocity of shortening/relenetheng was recorded with good fidelity. The soft-edge software (IonOptix) was used to capture changes in cell length during shortening and relenetheng. Cell shortening and relenetheng were assessed using the following indexes: peak shortening (PS), time to 90% PS (TPS90), time to 90% relenetheng (TR90), and maximal velocities of shortening/relenetheng ($\Delta$$L$/dt).

Intracellular fluorescence measurement of Ca2+. A separate cohort of myocytes was loaded with fura-2/AM (1.0 $\mu$mol/l) for 30 min, and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (IonOptix). Myocytes were placed in a dish chamber on the stage of an Olympus IX-70 inverted microscope and imaged through a Fluor 100× oil objective. Cells were exposed to light emitted by a 75-W lamp and passed through either a 360- or a 380-nm filter (bandwidths were ±15 nm), while being stimulated to contract at 1.0 Hz. Fluorescence emissions were detected between 480 and 520 nm by a photomultiplier tube after first peak emission (510 nm). Changes in intracellular Ca2+ were expressed as fluorescence intensity (ratio of emitted light at 510 nm to 485 nm) and determined using the software (IonOptix).
overexpression occurred despite the fact that MT does not
versus OVE26,
Data analysis.
images were captured from live cells. For results in Fig. 6, myocytes were
washed twice and images were captured by Olympus IX70 inverted micro-
intracellular oxidation by ROS such as hydrogen peroxide and hydroxyl
radical (20). Intracellular oxidant stress was monitored by measuring changes
in fluorescence resulting from intracellular probe oxidation. Isolated myo-
cytes were loaded with 1 μmol/l CM-H2DCFDA (Molecular Probes, Eugene,
OR) for 30 min. After myocytes were washed, fluorescence intensity from
individual cells was measured using an excitation wavelength of 485 nm and
an emission wavelength of 530 nm. Cells were sampled at random in each
preparation using an Olympus IX70 inverted microscope equipped with a
digital cooled charged-coupled device camera and ImagePro image analysis
software (Media Cybernetics, Silver Spring, MD). Fluorescence was calibrated
with InSpeck microspheres (Molecular Probes). Calibration curves were
generated, and cell brightness was measured. The InSpeck microspheres were
used as internal standards. The intensities of cells and beads were calibrated
by subtraction of the background, and the final result for comparison was
assessed by ratio of myocyte fluorescent intensities divided by intensities of
internal fluorescent beads. The cardiomyocytes from each group were incu-
bated at room temperature separately in M199 medium with normal glucose
concentration as control (5.5 mmol/l), high glucose (25 mmol/l), normal
glucose plus angiotensin II (50 nmol/l), and normal glucose plus hydrogen
peroxide as a positive control (H2O2, 40 μmol/l). Some incubations included 1
μmol/l diphenyleneiodonium (DPI) or 0.1 μmol/l losartan. The different
culture conditions were begun 30 min before CM-H2DCFDA was added to the
medium. After 30 min of incubation of CM-H2DCFDA, the myocytes were
washed twice and images were captured by Olympus IX70 inverted micro-
scope equipped with a charged-coupled device camera. For results in Fig. 5,
images were captured from live cells. For results in Fig. 6, myocytes were
fixed in 4% parafomaldehyde after incubation and stored at 4°C for up to 16 h
before imaging. There were 100 cells in each group.
Data analysis. Data are expressed as means ± SE. Statistical comparisons
were performed by ANOVA and Student-Newman-Keuls post hoc tests among
different groups. Significance was defined as P < 0.05.

RESULTS
Cell shortening and relengthening in ventricular
myocytes from FVB, OVE26, and OVE26MT mice. Similar rod-shaped cardiomyocytes with good striation
and edges were obtained from FVB, OVE26, and OVE26MT mice. The averaged resting myocyte length was not signifi-
cantly different among the two transgenic and the wild-
type mouse groups (OVE26 113.13 ± 2.83 μm, OVE26MT
118.44 ± 2.72 μm, and FVB 121.09 ± 3.04 μm). As shown
in Figs. 1 and 2, cardiomyocyte contractile function from
OVE26 mice was markedly depressed. The cardiomyo-
cytes from OVE26 mice showed significantly reduced peak
shortening (OVE26 versus FVB, P < 0.01; Fig. 3B) and
±dL/dtmax (OVE26 versus FVB, P < 0.01; Fig. 2A and B) as
compared with those from FVB mice. Myocytes from OVE26
mice also exhibited significantly prolonged TPS90 (OVE26
versus FVB, P < 0.05; Fig. 1C) and TBS90 (OVE26
versus FVB, P < 0.01; Fig. 1D). Overexpressed MT in the
heart significantly reversed the impaired myocyte contractile
performance (PS, Fig. 1B; ±dL/dtmax, Fig. 2A and B) and
prolongation of myocyte contraction and relengthening
(TPS90 and TBS90, Fig. 1C and D) by diabetes (OVE26MT
versus OVE26, P < 0.01). The benefits provided by MT
overexpression occurred despite the fact that MT does not
reduce diabetic blood glucose, as we previously reported
(16), or elevated serum triglyceride levels (OVE26 349.9 ±
62.9, OVE26MT 467.7 ± 29.5, and FVB 107.6 ± 7.7, P <
0.001 for FVB versus both other groups by ANOVA and
Bonferroni test).
Intracellular Ca2+ transient in ventricular myocytes
from FVB, OVE26, and OVE26MT mice. Myocytes from
the three groups exhibited similar peak and baseline FFI
(data not shown) and no differences of ∆FFI (Fig. 3B).
As shown in Fig. 3C, recovery of the Ca2+ transient occurred
more rapidly in control FVB cardiomyocytes than in
OVE26 myocytes (τ, FVB 178.78 ± 8.77 ms vs. OVE26
261.97 ± 14.23 ms; P < 0.01). However, overexpressed MT
significantly facilitated the calcium-clearing rate in dia-
abetic cardiomyocytes (OVE26MT 190.34 ± 10.26 ms vs.
OVE26 261.97 ± 14.23 ms; P < 0.01).
Intracellular ROS levels in ventricular myocytes from
FVB, OVE26, and OVE26MT mice. Figures 4 and 5 show
ROS production measured with CM-H2DCFDA. Normal
extracellular glucose produced similar ROS intensities
among the cardiomyocytes from all three groups. After
exposure of myocytes to 40 μmol/l H2O2, the fluorescent
intensities were significantly increased in the cardiomyo-
cytes from the three groups compared with normal glu-
cose incubation, but there were no significant differences
among the three groups. Treatment of myocytes with high
glucose (25 mmol/l) or angiotensin II (50 nmol/l) for 60
min did not affect the ROS level in control cardiomyocytes
but caused a significantly increased ROS in the OVE26
cardiomyocytes compared with those in the normal con-
dition of glucose (OVE26 HG versus OVE26 NG, P <
0.001). However, the effects of high glucose or angiotensin
II on ROS production were completely abolished by over-
expressed MT (OVE26MT versus OVE26, P < 0.001).
To ascertain the source of increased ROS in diabetic cells, we incubated OVE26 and FVB myocytes with 10 μmol/l DPI and 0.1 μmol/l losartan. DPI is an inhibitor of flavoprotein-containing enzymes, including NADPH oxidase. Losartan is a specific angiotensin II type I receptor antagonist. As shown in Fig. 6, both DPI and losartan eliminated the increased fluorescence induced in OVE26 myocytes by either angiotensin II or high glucose. These inhibitors had no significant effect on fluorescence in FVB myocytes.

DISCUSSION

This is the first study to evaluate the efficacy of permanent antioxidant protection of individual cardiomyocytes from diabetes. These studies were carried out in myocytes from the OVE26 mouse. Although this model has not been widely used to study diabetes complications, it provides several advantages: all OVE26 mice have a very consistent onset and severity of diabetes, their duration of diabetes is long, they can live with hyperglycemia for 2 years without insulin therapy, and specific damage to the β-cell occurs without use of toxic drugs. Diabetes is produced in heterozygous OVE26 animals, allowing these mice to be cross-bred easily to other strains of mice (16).

OVE26 diabetic cardiomyocytes had reduced contractility and prolonged calcium transients. The amplitude of cell shortening was decreased, the duration of contraction was increased, and the rates of contraction and relaxation were reduced. This demonstrates that the impaired contractility of the intact diabetic heart is due to defects in the individual myocyte, rather than just increased cardiac fibrosis. These results are in agreement with data obtained in other models of diabetes (21,22) and confirm the validity of the OVE26 model.
Calcium transients in diabetic cardiomyocytes were also abnormal. As seen in other diabetic models (22), decay of intracellular Ca\(^{2+}\) transients in OVE26 myocytes was significantly slowed. The slow Ca\(^{2+}\) clearing is consistent with and may be the cause of the prolonged duration of myocyte relengthening. Slow relengthening is a hallmark of diabetic cardiomyopathy. In contrast to decay rate, resting and peak Ca\(^{2+}\) levels were similar between the control and diabetic cardiomyocytes. Reports regarding intracellular Ca\(^{2+}\) level in diabetic cardiomyocytes are somewhat controversial. Early studies reported an increase in resting Ca\(^{2+}\) (23), whereas Noda et al. (24) reported a reduced basal Ca\(^{2+}\) in diabetic cardiomyocytes. Several reports demonstrated no differences in basal Ca\(^{2+}\) or peak Ca\(^{2+}\) levels between diabetic and control cardiomyocytes (25–27), which is consistent with our results. Therefore, there may not be significant alteration of Ca\(^{2+}\) levels that correspond to the depressed contraction in diabetic cardiomyocytes. Attenuated calcium sensitivity of the contractile apparatus or changes in cardiac contractile proteins may be more important than absolute Ca\(^{2+}\) levels to the reduced shortening of diabetic cardiomyocytes (28).

Many studies report that diabetic hearts exhibit characteristics of oxidative stress. OVE26 hearts have increased levels of oxidized glutathione (16), and hearts of BB (29) and streptozotocin (30) diabetic rats show induction of antioxidant enzymes. This ROS stress of diabetes can produce oxidative damage including lipid peroxidation (31,32) and nitrotyrosine formation (20). To determine whether oxidative stress was a cause or consequence of diabetic cardiomyopathy, we placed the cardiac transgene for the antioxidant protein MT on the OVE26 background (16). MT overexpression was remarkably effective in ameliorating functional deficits in OVE26 diabetic cardiomyocytes, despite that MT overexpression in no way reduced the exposure of cardiomyocytes to the severe hyperglycemic environment in these mice. MT overexpression completely reversed diabetes-induced diminished PS amplitude, prolonged duration and reduced maximal velocities of contraction and relaxation, and the slower intracellular Ca\(^{2+}\) transient decay. This indicates that ROS is essential to the pathogenesis of diabetic cardiomyopathy. Our preliminary data showing that two other antioxidant transgenes (unpublished results) protect from OVE26 diabetic cardiomyopathy confirm that it was the antioxidant actions of MT that protected the diabetic heart.

Increased oxidative damage suggests elevated ROS production in diabetic cardiomyocytes. Consistent with this, OVE26 myocytes produced more ROS in the presence of angiotensin II than myocytes from control mice or mice protected by the MT transgene. Bendall et al. (33) recently showed that angiotensin II stimulates cardiac NADPH oxidase, an enzyme that produces superoxide at the expense of the electron donor NADPH. Losartan blocked the response to angiotensin II in OVE26 myocytes. Consistent with a possible role for NADPH oxidase DPI, an inhibitor of flavoprotein enzymes such as NADPH oxidase blocked excess ROS production produced by angiotensin II.

High glucose also stimulated ROS production in OVE26 myocytes. Several sources have been proposed for enhanced ROS formation in hyperglycemia. These include mitochondrial electron transport, NADPH oxidase, and autooxidation of glucose. Mitochondria are the major source of ROS in most cells as a result of superoxide-generating electron-transport intermediates such as ubisemiquinone. Increased substrate for electron transport increases the mitochondrial membrane potential, which results in increased ROS production (34). The Brownlee laboratory has shown that hyperglycemia increases electron transport–generated superoxide in endothelial cells, which activates several pathways thought to lead to complications of diabetes (35). Glucose autooxidation and protein glycation both are processes that are accelerated by high glucose and produce superoxide radical or other ROS (36,37). NADPH oxidase has been shown to be stimulated by hyperglycemia in several cell types (38,39), and its mRNA in endothelial cells is induced sevenfold by high glucose (39). High glucose can turn on NADPH oxidase by de novo synthesis of diacylglycerol (40), which activates PKC, which in turn can activate NAD(P)H oxidase by phosphorylation of p47(phox) (41). The response of OVE26 cardiomyocytes to high glucose was similar to their response to angiotensin II. Glucose, like angiotensin II, increased ROS levels only in diabetic cardiomyocytes, and in both cases the response was prevented by DPI, MT overexpression, and losartan. This similarity suggests that high glucose acts via angiotensin II or that high glucose requires the simultaneous activation of the angiotensin II system. That losartan and DPI blocked the high glucose response suggests involvement of NADPH oxidase. However, the DPI results cannot be
considered a definitive indication for the involvement of NADPH oxidase, because DPI inhibits other flavoprotein-containing enzymes that may play a role in ROS production. This includes inhibition of complex II of the mitochondrial electron transport chain.

In summary, our data demonstrate that 5 months of diabetes resulted in OVE26 cardiomyocytes with reduced mechanical performance and slow intracellular Ca\(^{2+}\) clearing. Permanent antioxidant treatment by MT overexpression prevents these defects. OVE26 myocytes exhibited a significantly greater ROS response to high glucose or angiotensin II, and this was also prevented by MT overexpression. Acute treatment with DPI or losartan blocked the enhanced diabetic ROS production, suggesting that high glucose requires angiotensin II activity and that ROS are produced by a flavoprotein-containing enzyme. Chronic ROS elevation in diabetic cardiomyocytes damages contractility, impairs calcium handling, and may predispose the myocyte to produce greater levels of ROS.

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