Overexpression of Metallothionein Reduces Diabetic Cardiomyopathy

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Many diabetic patients suffer from cardiomyopathy, even in the absence of vascular disease. This diabetic cardiomyopathy predisposes patients to heart failure and mortality from myocardial infarction. Evidence from animal models suggests that reactive oxygen species play an important role in the development of diabetic cardiomyopathy. Our laboratory previously developed a transgenic mouse model with targeted overexpression of the antioxidant protein metallothionein (MT) in the heart. In this study we used MT-transgenic mice to test whether an antioxidant protein can reduce cardiomyopathy in the OVE26 transgenic model of diabetes. OVE26 diabetic mice exhibited cardiomyopathy characterized by significantly altered mRNA expression, clear morphological abnormalities, and reduced contractility under ischemic conditions. Diabetic hearts appeared to be under oxidative stress because they had significantly elevated oxidized glutathione (GSSG). Diabetic mice with elevated cardiac MT (called OVE26MT mice) were obtained by crossing OVE26 transgenic mice with MT transgenic mice. Hyperglycemia in OVE26MT mice was indistinguishable from hyperglycemia in OVE26 mice. Despite this, the MT transgene significantly reduced cardiomyopathy in diabetic mice: OVE26MT hearts showed more normal levels of mRNA and GSSG. Typically, OVE26MT hearts were found to be morphologically normal, and elevated MT improved the impaired ischemic contractility seen in diabetic hearts. These results demonstrate that cardiomyocyte-specific expression of an antioxidant protein reduces damage to the diabetic heart. Diabetes 51:174–181, 2002

A leading cause of death for diabetic patients is cardiac failure. Also, diabetic patients have an especially poor prognosis after myocardial infarction. Based on postmortem findings of heart failure in patients free of coronary artery disease or other known cardiac risk factors, Rubler et al. (1) first suggested the existence of a specific diabetic cardiomyopathy in 1972. Similar findings have been reported in other clinical studies (2,3). In addition, the increased risk of diabetic heart failure reported in the Framingham Heart Study (4) could not be explained by such diabetes-associated risk factors as obesity, hypertension, hypercholesterolemia, and coronary artery disease. Preclinical cardiac studies of diabetic patients (rev. in 5) have also reported left ventricular abnormalities independent of other risk factors. Together, these studies support the existence of a specific diabetic cardiomyopathy with origins in diabetic cardiac muscle.

Reactive oxygen species (ROS) have been proposed as a cause of many complications of diabetes (6,7). Hyperglycemia can elevate levels of ROS by increasing mitochondrial superoxide anion production (8,9) or by the process of glucose auto-oxidation (10). The heart is a susceptible target because it contains low levels of free radical scavengers (11). Available evidence implicating ROS in diabetic cardiomyopathy include increased cardiac lipid peroxidation (12,13) and elevated expression of genes for the antioxidants catalase (12,14,15) and heme oxygenase (16). Also, functional or morphological damage to diabetic hearts can be reduced with oral or systemic antioxidant administration (12,17). However, much of the current evidence lacks specificity. For example, the gene expression data are open to alternative interpretations because oxidative stress is not the only type of stress that can increase the activity of antioxidant genes. Also, the antioxidants that have been used to reduce cardiomyopathy lower circulating lipids (18,19), which may explain their beneficial actions in diabetic cardiomyopathy. Our laboratory has developed a transgenic model that expresses the potent antioxidant protein metallothionein (MT) specifically in cardiomyocytes. This model is ideal to test the significance of chronic ROS exposure because the transgene is overexpressed permanently and specifically in the heart.

RESEARCH DESIGN AND METHODS

Animals. We have previously described the development of OVE26 diabetic mice (20). MT mice that overexpress MT 10-fold in the heart were also previously described (21). Transgenic mice of both lines were identified by the presence of phenotypic markers. OVE26-positive mice were recognized by the presence of small eyes caused by the cointegration of the GR19 gene (20), which is expressed in the eye. MT-positive mice were recognized by the presence of coat color due to cointegration of the tyrosinase gene that corrects the mutant tyrosinase gene of FVB mice (22). 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. A U.S. Department of Agriculture–certified institutional animal care committee approved all animal procedures.

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ANF, atrial natriuretic factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; GSSG, oxidized GSH; M2VP, 1-methyl-2-vinylpyridinium trifluoromethane sulfonate; MT, metallothionein; ROS, reactive oxygen species.
Glucose, insulin, triglyceride, and ketone assays. Glucose assays of fasted mice were performed with a Fast-Take (Lifescan) glucometer. Unfasted blood glucose values in OVE26 mice commonly exceeded the maximum of the glucometer and were assayed with a Sigma HK2O kit. Serum insulin levels were determined by double-antibody radioimmunoassay (16) using rat insulin (Eli Lilly) as standard. Serum triglycerides were determined by an automated method (Cobas Fara; Roche Diagnostic Systems). Plasma ketones were determined with Accet Reagent Tablets (Bayer).

Reduced and oxidized glutathione. A Bioxytech GSH/GSSG-412 kit from Oxis Health Products was used to measure reduced and oxidized glutathione (GSSG). Hearts were homogenized in six volumes of 0.5% metaphosphoric acid with or without 33 mmol/l 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate (M2VP), a scavenger of reduced glutathione (GSH). The homogenate was centrifuged at 1,000 °C for 5 min. For GSH estimation, 5 μl supernatant was mixed with 355 μl assay buffer containing 100 mmol/l NaPO4 and 5 mmol/l EDTA, pH 7.5. For GSSG estimation, 10 μl homogenate with M2VP was mixed with GSSG buffer containing 100 mmol/l NaPO4 and 5 mmol/l EDTA, pH 10.05. The samples were mixed with 1.262 mmol/l 5,5′-dithiobis-(2-nitrobenzoic acid) and 15 units/ml GSH reductase. The mixture was incubated at room temperature for 5 min, and the absorbance was recorded at 412 nm for 3 min after the addition of 9.8 μmol NADPH.

RNA analysis. Northern blots were prepared as previously described (23) using 10 μg heart RNA from transgenic or control mice at 150 days of age. The membrane was prehybridized in 10 ml of Church buffer (1% bovine serum albumin, 7% SDS, 100 μg/ml salmon sperm DNA, and 0.5 mol/l NaH2PO4, pH 7.0) for 2 h. Then, the probe was added, and the blot was hybridized for 24 h. All procedures were carried out at 50 °C. Oligonucleotide probes for mouse mRNAs for skeletal actin (TGGAGCAAAACAGAATGGCTGGCTTTAATGCT TCAAGTTTTCTCATTCCTTTTCCACAGGG), atrial natriuretic factor (ANF; AATGTGACCAAGCTGCGTGACACACCACAAGGGCTTAGGATCTTTTGAGA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (GGCACTGTAGACCATGTAGTTGAGGTCAATGAAG) were labeled with 32P-ATP and polyadenylate kinase. The strength of the hybridization signal was determined on a Molecular Dynamics model 440 SI phosphorimager. Signals for skeletal actin and ANF were normalized to the signals for GAPDH.

Measurement of contractility. Cardiac contractility was measured in Langendorff mode by a method we previously described (24). In brief, the heart was retrogradely perfused with Krebs-Henseleit (KH) buffer (120 mmol/l NaCl, 20 mmol/l NaHCO3, 4.6 mmol/l KCl, 1.2 mmol/l KH2PO4, 1.5 mmol/l MgCl2, 2.5 mmol/l CaCl2, and 11 mmol/l glucose). Hearts were paced throughout the procedure at 5 Hz (6 V, 3 ms), which exceeded the spontaneous rates observed in our Langendorff preparations. Contractile force and heart rate are measured by means of an Grass FT03 force transducer hooked to the apex of the heart and connected to a ETH 400 bridge amplifier that fed into a Powerlab/400 amplifier. Once mounted, cardiac contractility was allowed to stabilize, which usually required 10–20 min. Baseline contractility was determined during the subsequent 30 min of recording. Next, the heart was subjected to a 60-min period of low-flow ischemia. This was achieved by switching a three-way valve to another perfusion line running from a buffer reservoir bubbled with 95% N2 and 5% CO2 to ensure hypoxia and a buffer pH of 7.4. The ischemic flow rate was 0.15 ml/min, which was one-tenth of the preischemia flow rate. For the last hour, the heart was again perfused with 95% O2 and 5% CO2–equilibrated KH buffer at 1.5 ml/min. Perfusion pressure, heart rate, contractile force, and the differential of force were continuously monitored. Data were analyzed using AD Instruments Chart for Windows version 3.3.5. Analysis of contractility during the ischemia and reperfusion periods was performed on the average of measurements made at 2-min intervals during the first 10 min of the period and at 10-min intervals for the remainder of the 60-min period. Basal contractility was determined from the average of measurements made at 10-min intervals throughout the 30-min basal period.

Electron microscopy. Fixation was carried out by vascular perfusion through the left ventricle. Mice were anesthetized with an intraperitoneal injection of pentobarbital (100 mg/kg). The right atrium was incised to allow exsanguination and fluid drainage. The perfusates included washouts (at 4°C) of 35 ml half-strength Karnovsky’s (25) fixative with 1% procaine, and 75 ml full-strength Karnovsky’s final fix. After perfusion, hearts were removed and immersed in chilled Karnovsky’s fixative and then immediately cut transversely to produce ventricular “rings” (1- to 2-mm wide). The rings were minced to form small (1-mm) tissue blocks. Left ventricular tissue blocks were fixed at 4°C for 2–4 h. This was followed by rinsing in 0.2 mol/l sodium cacodylate buffer (pH 7.4), postfixation in 1% OsO4 (90 min), rinsing in distilled water, and en bloc staining in 0.5% aqueous uranyl acetate (90 min), all at 4°C. Dehydration proceeded through graded ethanol and propylene oxide. Tissue blocks were embedded in Epon/Araldite (26) and cured 48 h at 60°C. Epoxy blocks were trimmed, and 1-μm thick sections were made and stained with toluidine blue (1% in 1% sodium borate) for determination of tissue position and orientation. Thin sections were cut on an RMC MTXI ultramicrorotome equipped with a Diatome diamond knife. Sections were collected on 300 mesh naked copper grids and stained with lead citrate (27) and uranyl acetate (4% in absolute ethanol). These were observed in a Hitachi 7500 transmission electron microscope at initial magnifications of 4,000–15,000 diameters.

To quantify the morphological features of cardiac tissues in each of the animal groups, transmission electron micrographs of randomly selected areas of left ventricular thin sections were printed at identical magnifications. Two observers who were unaware of their origin rated these prints. On a scale of 1–4, observers rated the morphology relative to a figure exhibiting near-perfect cardiac morphology. Figures were rated for overall morphology (which included the presence of edematous areas, myelin figures, and overall organization), mitochondrial morphology (which included mitochondrial packing, density, and shape), and myofilament morphology (which examined linearity of the Z lines, linearity of myofilaments, regularity of myofilaments, and continuity of myofilaments).

Statistical analysis. Statistical comparisons of GSH, RNA expression, and cardiac contractility were performed by one-way analysis of variance and Bonferroni’s post hoc tests, unless otherwise indicated in the text. The significance of differences for morphology ratings were determined by the Kruskal-Wallis test followed by Dunn’s multiple comparison test. Comparisons between two groups were performed with Student’s t test.

RESULTS

Diabetes in OVE26 and OVE26MT transgenic mice. The transgenic line OVE26 was used to evaluate diabetic cardiomyopathy. As previously described (20), these mice exhibit very high blood glucose concentrations and reduced serum insulin values (Figs. 1A and B). Figure 1C also shows that OVE26 mice possess elevated serum triglycerides. Assays of seven diabetic plasma samples for the presence of ketones were negative (not shown). MT overexpression was produced in diabetic mice by crossing OVE26 mice with an MT transgenic line that we previously described (21) and that has a 10-fold increase in cardiac MT. Fasting blood glucose levels were measured to rule

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<th>FIG. 1. Diabetes in OVE26 mice. Blood glucose (A), serum insulin (B), and serum triglycerides (C), measured as described in RESEARCH DESIGN AND METHODS, are shown. Values are from ad libitum–fed mice and show the mean ± SE for four to eight control or diabetic mice. In all panels, OVE26 values are different from control (P &lt; 0.05 by Student’s t test).</th>
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out any effect of the MT transgene on diabetes. As shown in Fig. 2, OVE26 and OVE26MT mice had elevated and indistinguishable levels of hyperglycemia. Random assays of unfasted blood samples also demonstrated no effect of the MT transgene on glycemia (not shown).

**Reduced GSH and GSSG in heart tissue.** Conversion of GSH to GSSG is important for several endogenous antioxidant reactions, and the antioxidant properties of GSH and MT are both dependent on sulfhydryl groups. Therefore, we measured reduced GSH and GSSG to assess oxidative stress in diabetic hearts and to determine whether MT protects the heart from oxidative stress. GSH levels in OVE26 and OVE26MT hearts were similar to levels in control FVB hearts (Fig. 3). However, a significantly increased level of GSSG demonstrated oxidative stress in diabetic hearts. Importantly, the elevation in GSSG was completely eliminated in OVE26MT mice.

**RNA levels.** A common finding in cardiomyopathy is upregulation of mRNAs for skeletal actin and ANF. These transcripts were increased two- to threefold in the hearts of OVE26 mice (Fig. 4), confirming a diabetes-induced cardiomyopathy. MT overexpression significantly reversed the increase in skeletal actin mRNA in hearts of OVE26MT mice. MT also reduced ANF expression, but this reduction was partially caused by a high degree of variability in ANF expression, which has been observed by others (28).

**Morphology.** Morphological analyses of left ventricular cardiac tissues from three groups of mice ~150 days old were carried out by transmission electron microscopy (Fig. 5). Cardiomyocytes from control FVB mice (Fig. 5A) showed ultrastructural features considered typical for this cell type. In contrast, heart tissue from diabetic OVE26 animals (Fig. 5H) showed clearly the detrimental effects of the disease. Disorganized collections of mitochondria randomly interspersed between disrupted myofibrils in an edematous sarcoplasm were common. In addition, numerous myelin figures suggested membrane disruption and cellular damage in these tissues. Overexpression of MT had a striking protective effect against the myocardial injury seen in diabetic animals (Fig. 5C). In cardiomyocytes of OVE26MT animals, mitochondria and myofibrillar fine structure were essentially indistinguishable from FVB controls. Moreover, myelin figures and other evidence of myocardial injury were not seen. As described in RESEARCH DESIGN AND METHODS, semiquantitative ratings were made of random electron micrographs by observers blind to the origin of the picture. These figures were judged for overall morphology, mitochondrial structure, and myofibrillar substructure. Figure 5D shows that in this unbiased evaluation of morphology, the OVE26 diabetic group was significantly compromised relative to either of the other groups. This analysis also confirmed that MT was highly protective to the diabetic heart, because there were no significant differences between ultrastructural features of cardiomyocytes in FVB control and OVE26MT hearts.

**Contractility.** To assess the impact of diabetes and potential protection by MT overexpression, cardiac contractility was measured in Langendorff-perfused hearts (Fig. 6). During the basal period of perfusion, there was a trend toward reduced contractility in OVE26 and OVE26MT hearts, but this trend did not reach significance in either case. Under ischemic conditions, diabetes clearly reduced contractility in OVE26 hearts, and this deficit was strikingly reversed in OVE26MT hearts by MT overexpression. During the reperfusion phase, there was also a decrement in diabetic contractility compared with FVB hearts. This trend was not significant by the Bonferroni post hoc test. However, when this trend was tested with Dunnett’s one-way post hoc test, all three parameters of contractility were significantly (P < 0.05) diminished in
OVE26 hearts compared with FVB hearts. OVE26MT contractility was not significantly different from either of the two other groups during reperfusion, as assessed by either the Bonferroni test or the Dunnett test. Thus, it is not possible to determine from the present data whether MT has an effect on contractility during reperfusion.

DISCUSSION
Our results demonstrate obvious cardiomyopathy in OVE26 diabetic mice. Disruption of normal cardiac function was apparent as aberrant gene expression, disrupted cellular morphology, and decreased ischemic and reperfusion contractility. MT overexpression markedly reduced the degree of cardiac damage for each of these parameters, except for reperfusion contractility.

The OVE26 mouse has not been used extensively for the study of diabetes complications, despite the fact that this transgenic diabetic model was first described 12 years ago (20). Early onset, insulinopenic diabetes results from a transgene that overexpresses calmodulin only in pancreatic β-cells (20). The pathology of these animals has at least one feature similar to type 1 diabetes: hyperglycemia is a direct result of damage to the β-cell. However, unlike type 1 diabetes, this damage is not immune-mediated, and a low but significant level of insulin secretion is retained. These mice provide several advantages for the study of chronic cell and tissue damage resulting from diabetes: all OVE26 mice have a very consistent onset and severity of diabetes. Their duration of diabetes is long; they can live with diabetes for 2 years without insulin therapy. In these mice, diabetes is caused by a known genetic alteration whose direct actions are specific to the β-cell. Also, potential artifacts secondary to the use of diabetogenic chemicals such as streptozotocin and alloxan are avoided. However, despite these advantages for the study of complications, it is essential to recognize a major difference between OVE26 diabetes and human diabetes: the complications in humans develop in a much less severe diabetic environment because of insulin administration to these patients. Therefore, the findings identified in OVE26 mice should be replicated in milder models of diabetes, such as db/db and streptozotocin-induced diabetic mice.

Diabetic cardiomyopathy in the OVE26 mice is similar in most respects to the cardiomyopathy described in other diabetic models. Morphological damage comparable to what we observed in OVE26 mice has been reported in other models (12,30), and diabetes-induced changes in cardiac contractile gene expression have also been reported (31). In most studies of rodent diabetes, basal contractility was significantly reduced. In OVE26 mice, we only observed a trend toward reduced basal contractility.
However, this trend may reflect a real underlying defect in basal contractility, because we recently observed impaired basal contractility in isolated OVE26 cardiomyocytes (32). In the current study, diabetic contractility was more impaired during ischemia and reperfusion than in control animals. Increased sensitivity of cardiac contractility to ischemia has been described in other rodent models of diabetes (33). Our results show that the OVE26 mouse develops a cardiomyopathy that is suitable for testing other transgenes by simple breeding strategies. We used this strategy in the current study to demonstrate the benefit of MT overexpression.

MT was effective in reversing many aspects of diabetic cardiomyopathy. However, MT had little or no effect on the diabetic deficit in contractility seen during basal or reperfusion phases. The failure of MT to protect contractility under oxidative perfusion conditions raises the possibility that this component of failure may be caused by factors other than ROS. Indeed, many mechanisms other than ROS have been implicated in diabetic complications. For example, pyruvate dehydrogenase activity is markedly reduced in diabetic hearts (34). This may pose a serious disadvantage to the diabetic heart under our perfusion conditions, which used glucose as the sole energy source. Pyruvate dehydrogenase is essential for maximal ATP production from glucose under oxidative conditions. Because MT overexpression would not restore pyruvate dehydrogenase activity, this could contribute to the lack of benefit of MT on contractility during oxidative conditions.

Because the contractility data obtained in this study was

FIG. 5. A and C: MT overexpression protects cardiac morphology from chronic diabetes. Representative transmission electron micrographs of cardiac tissues from left ventricles of FVB (A), OVE26 (B), and OVE26MT (C) mice are shown. FVB and OVE26MT–cross animals (A and C) show normal myocardial fine structure, with myofilaments comprised of regular and continuous sarcomeres. The latter are demarcated by Z lines (arrowheads), which are in register with adjacent myofilaments. Rows of moderately electron dense mitochondria (M) intervene between myofilaments. OVE26 diabetic myocardium (B) shows randomly distributed mitochondria (M) between poorly organized myofilaments (arrowheads indicate Z lines) in an electron-lucent sarcoplasm. Numerous myelin figures (My) suggest myocardial cytopathology. All figures have a magnification of 8,500 ×. D: Quantitation of cardiac morphology. Two blind observers rated ultrastructural features of myocardial tissues in 16–25 randomly selected electron micrographs from each of three OVE26 diabetic mice, three OVE26MT mice, and three nontransgenic FVB control mice. The parameters used for the overall ratings and ratings for mitochondria (Mito) and myofilaments (Myofil) are described in RESEARCH DESIGN AND METHODS. *OVE26 differs from OVE26MT and FVB (P < 0.001). Vertical bars indicate the SE.
derived from Langendorff perfused hearts, extrapolation of these results to cardiac function in the whole diabetic animal should be made with caution. Unlike in the intact animal, Langendorff hearts perform little work. In addition, Langendorff as well as perfused working hearts beat at about half the rate of the normal mouse heart (35,36), are perfused with incomplete media, and demonstrate markedly decreased rates of pressure development (36). Sophisticated systems for analysis of cardiac function in vivo have been developed for the mouse (35,37). These systems provide a more realistic evaluation of normal and pathological function that should be applied to murine models of diabetic cardiomyopathy.

Disagreement exists over the question of whether diabetic cardiomyopathy is a direct effect of diabetes on the heart. Some propose that diabetic heart failure is secondary to vascular dysfunction (38,39). Decreased delivery of fuel and oxygen by the coronary circulation could result in cumulative damage to the heart. Our results show that direct manipulation of the cardiomyocyte can alter the course of diabetic cardiomyopathy. Because the e-myosin heavy chain promoter is active only in cardiomyocytes, and expression data confirm this in these transgenic mice (21,40), MT could not have exerted its protective actions in the vasculature by altering other components of diabetes. The fact that protection was obtained using a protein expressed exclusively in the cardiomyocyte confirms that diabetic cardiomyopathy is a pathology intrinsic to the myocyte.

Our results strengthen the hypotheses that diabetes increases oxidative stress in the heart, which contributes to diabetic cardiomyopathy. Prior studies using cardiac tissue or cardiomyocytes from animal models of diabetes demonstrated upregulation of antioxidant enzymes (14), increased production of hydrogen peroxide (41), and increased lipid peroxidation (12,13). Recently, oxidative stress in diabetic human hearts has been shown (42) by the finding of elevated levels of the marker of oxidative damage, nitrotyrosine, in biopsies from diabetic patients. In the current study, we demonstrated that GSSG is elevated in diabetic hearts. GSSG is produced by oxidation of GSH during several antioxidant reactions, including the detoxification of hydrogen peroxide by GSH peroxidase. To our knowledge it has not previously been shown that GSSG is increased in diabetic hearts. We saw no corresponding change in GSH concentration. This stability of GSH levels in diabetic hearts was not surprising because a 40% increase in GSSG would be expected to have little impact on GSH, which was present in 20-fold excess. Other authors have also examined GSH in diabetic hearts (43,44) and found it to be unaltered.

Overexpression of MT was effective in both preventing the diabetes-induced change in GSSG levels and reducing several components of diabetic cardiomyopathy. MT has
been shown to defend against many agents known to act through ROS, including radiation, GSH depletors, and xanthine oxidase (rev. in 45,46). The antioxidant action of MT is 50–100 times greater than GSH on a molar basis (47,48). In the current transgenic model, Kang and colleagues (21,49) have also shown that MT protects the heart from ROS damage caused by doxorubicin and hydrogen peroxide. Because ROS damage is prominent in experimental (12) and clinical (42) samples of diabetic hearts, it is likely that the antioxidant actions of MT are the source of the protection demonstrated for morphology, gene expression, and ischemic contractility. These results support continued efforts to find an antioxidant regimen effective for the prevention of cardiac complications of diabetes.

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