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Original Contribution

Metallothionein alleviates cardiac dysfunction in streptozotocin-induced diabetes: Role of Ca^{2+} cycling proteins, NADPH oxidase, poly(ADP-Ribose) polymerase and myosin heavy chain isozyme

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Abstract

Diabetic cardiomyopathy contributes to high morbidity and mortality in diabetic populations. It is manifested by compromised ventricular contraction and prolonged relaxation attributable to multiple causative factors including oxidative stress. This study was designed to examine the effect of cardiac overexpression of the heavy metal scavenger metallothionein (MT) on cardiac contractile function, intracellular Ca^{2+} cycling proteins, stress-activated signaling molecules and the myosin heavy chain (MHC) isozyme in diabetes. Adult male wild-type (FVB) and MT transgenic mice were made diabetic by a single injection of streptozotocin (STZ). Contractile properties were evaluated in cardiomyocytes including peak shortening (PS), time-to-PS (TPS), time-to-relengthening (TR_{90}), maximal velocity of shortening/relengthening (±dL/dt) and intracellular Ca^{2+} fluorescence. Diabetes significantly depressed PS, ±dL/dt, prolonged TPS, TR_{90} and intracellular Ca^{2+} clearing, elevated resting intracellular Ca^{2+}, reduced caffeine-induced sarcoplasmic reticulum Ca^{2+} release and dampened stress tolerance at high stimulus frequencies. MT itself exhibited little effect on myocyte mechanics but it significantly alleviated STZ-induced myocyte contractile dysfunctions. Diabetes enhanced expression of the AT_1 receptor, phospholamban, the $p47^{phox}$ NADPH oxidase subunit and poly(ADP-ribose) polymerase (PARP), depressed the level of SERCA2a, Na^+-Ca^{2+} exchanger and triggered a β-MHC isozyme switch. All of these STZ-induced alterations with the exception of depressed SERCA2a and enhanced phospholamban were reconciled by MT. Collectively, these data suggest a beneficial effect of MT in the therapeutics of diabetic cardiomyopathy, possibly through a mechanism related to NADPH oxidase, PARP and MHC isozyme switch.

Keywords: Antioxidant; Diabetes; Myocyte; Contraction; Ca^{2+} cycling protein; PARP; MHC; Free radical

Introduction

Clinical and experimental evidence has demonstrated the existence of a specific type of myopathic alteration, namely diabetic cardiomyopathy, in diabetic populations [1–4]. Diabetic cardiomyopathy occurs independent of any macro- and micro-vascular diseases and is believed to be responsible for the high incidence of heart failure and cardiovascular mortality in diabetes. It is characterized by diminished ventricular function, reduced wall compliance and rate of myocardial relaxation [1–4]. Although several hypotheses have been postulated in an effort to interpret the pathogenesis of diabetic cardiomyopathy including glucose metabolism, oxidative stress and intracellular Ca^{2+} mishandling [1,3,4], the ultimate culprit and effective therapeutic remedies have not yet been elucidated for this devastating myopathic problem in individuals with both type 1 and 2 diabetes mellitus.

The heart possesses a relatively low antioxidant capacity contributed by enzymatic and non-enzymatic free radical scavengers or antioxidants, thus making it a prime target for oxidative insult [4,5]. Several lines of evidence have indicated

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the involvement of oxygen free radicals in the onset and development of diabetic cardiomyopathy in the diabetic state [4–6]. This is further supported by the observation that antioxidant treatment is proven to be beneficial for patients with diabetes [7]. However, most of the antioxidant approaches against diabetes were essentially limited to exogenously administered antioxidants such as α-tocopheral and flavonols [7,8], which may suffer from pitfalls such as route of absorption and cardiac tissue availability/specificity. To circumvent this problem, we took advantage of the transgenic technique to generate a mouse line with overexpression of the heavy metal free radical scavenger metallothionein specifically in the heart using an α-myosin heavy chain (MHC) promoter [6]. An earlier study has revealed antagonizing effects of metallothionein against cardiomyocyte contractile dysfunction and accumulation of oxidative stress in a murine model of genetically-predisposed diabetes [6]. However, the mechanism of action behind metallothionein-offered cardiac protection is still unknown. The present study was designed to examine the influence of metallothionein on cardiomyocyte contractile and intracellular Ca^{2+} homeostasis defects in the chemically-induced streptozotocin (STZ) diabetic model. We also evaluated generation of reactive oxygen species (ROS), oxidative stress, apoptosis and expression of the main cardiac intracellular Ca^{2+} regulating proteins [sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), Na^{+}-Ca^{2+} exchanger and phospholamban] and stress-activated signals including NADPH oxidase and poly(ADP-ribose) polymerase (PARP). Since diabetes is known to trigger the MHC isozyme switch from α-isoform to β-isoform [9], the MHC isozyme distribution was also evaluated in control and diabetic mice with or without metallothionein transgene overexpression.

Materials and methods

Experimental diabetic animals

The experimental procedure was approved by the Institutional Animal Use and Care Committees at the University of North Dakota (Grand Forks, ND, USA) and the University of Wyoming (Laramie, WY, USA). All animal procedures were in accordance with NIH animal care standards. In brief, eight to ten week-old weight-matched male FVB albino and metallothionein cardiac-specific transgenic mice were given a single injection of streptozotocin (STZ, 220 mg/kg., i.p.) dissolved in sterile citrate buffer (0.05 M sodium citrate, pH 4.5). Fur pigmentation (dark grey) was used as a marker for metallothionein transgene expression identification as described [6]. Fasting blood glucose levels were evaluated 7 days later. A supplemental STZ injection at 100 mg/kg (i.p.) was given if the fasting blood glucose was below 12 mM. All diabetic mice (fasting blood glucose level >12 mM) were maintained for a total of 4 weeks (after confirming diabetes) with free access to standard lab chow and tap water. The mortality rate in STZ-injected diabetic animals was ~25% and ~15% in FVB and metallothionein mice, respectively. Control mice received similar volume of sodium citrate buffer. Body weight and fasting blood glucose were measured using a standard lab scale and glucose monitor (Accu-ChekII, model 792, Boehringer Mannheim Diagnostics, Indianapolis, IN, USA), respectively.

Isolation of mouse ventricular myocytes

Hearts were rapidly removed from anesthetized mice and immediately mounted on a temperature-controlled (37°C) Langendorff perfusion system. After perfusion with modified Tyrode solution (Ca^{2+} free) for 2 min, the heart was digested for 10 min with 0.9 mg/ml collagenase D (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) in modified Tyrode solution. The modified Tyrode solution (pH 7.4) contained the following (in mM): NaCl 135, KCl 4.0, $MgCl_2$ 1.0, HEPES 10, NaH_2PO_4 0.33, glucose 10, butanedione monoxime 10, and the solution was gassed with 5% CO_2 −95% O_2. The digested heart was then removed from the cannula and the left ventricle was cut into small pieces in the modified Tyrode solution. These pieces were gently agitated and the pellet of cells was resuspended in modified Tyrode solution and allowed to settle for another 20 min at room temperature during which time extracellular Ca^{2+} was added incrementally back to 1.20 mM. Cell viability was approximately 80% in all four animal groups. Isolated myocytes were used for experiments within 8 hours after isolation. Only rod-shaped myocytes with clear edges were selected for recording of mechanical properties and intracellular Ca^{2+} transients as described [10].

Cell shortening/relengthening measurements

Mechanical properties of ventricular myocytes were assessed using a SoftEdge MyoCam® system (IonOptix Corporation, Milton, MA, USA) [10]. In brief, cells were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus IX-70, Olympus Optical Corporation, Tokyo, Japan) and superfused (~1 ml/min at 25°C) with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, at pH 7.4. The cells were field stimulated with suprathreshold voltage at a frequency of 0.5 Hz, 3 msec duration, using a pair of platinum wires placed on opposite sides of the chamber connected to a FHC stimulator (FHC Incorporation, Bowdoinham, ME, USA). The myocyte being studied was displayed on the computer monitor using an IonOptix MyoCam camera. A SoftEdge software (IonOptix Corp., Milton, MA, USA) was used to capture changes in cell length during shortening and relengthening. Cell shortening and relengthening were assessed using the following indices: peak shortening (PS), time-to-PS (TPS), time- to −90% relengthening (TR_{90}), maximal velocity of shortening (+dL/dt) and maximal velocity of relengthening (−dL/dt). In the case of altering stimulus frequency (0.1, 0.5, 1.0, 3.0 and 5.0 Hz), a steady state contraction of the myocyte was achieved (usually after the first 5–6 beats) prior to recording PS.

Intracellular Ca^{2+} transient measurement and sarcoplasmic reticulum (SR) Ca^{2+} load

A separate cohort of myocytes was loaded with fura-2/AM (0.5 μM) for 10 min and fluorescence measurements were

recorded with a dual-excitation fluorescence photomultiplier system (Ionoptix). Myocytes were placed on an Olympus IX-70 inverted microscope and imaged through a Fluor ×40 oil objective. Cells were exposed to light emitted by a 75W lamp and passed through either a 360 or a 380 nm filter (bandwidths were ±15 nm), while being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480–520 nm by a photomultiplier tube after first illuminating the cells at 360 nm for 0.5 sec then at 380 nm for the duration of the recording protocol (333 Hz sampling rate). The 360 nm excitation scan was repeated at the end of the protocol and qualitative changes in intracellular Ca^{2+} level were inferred from the ratio of the fura-2 fluorescence intensity (FFI) at the two wavelengths (360 nm/380 nm). Fluorescence decay time was also measured as an indication of the intracellular Ca^{2+} clearing rate [10]. The SR load was directly assessed through a 10 second-rapid application of caffeine (10 mM) with a rapid solution switcher to fura-2-loaded myocytes (0.5 μM for 10 min at 30°C). The integration underneath the Ca^{2+} transient curve during the caffeine perfusion was calculated and used as an index of the SR Ca^{2+} load [11].

Glutathione and glutathione disulfide (GSH/GSSG) assay

The ratio of GSH/GSSG was used as an indicator for oxidative stress. Ventricular tissues were homogenized in 4 volumes (w/v) of 1% picric acid. Acid homogenates were centrifuged at 16,000 × g (30 min) and supernatant fractions collected. Supernatant fractions were assayed for total GSH and GSSG by the standard recycling method. The procedure consisted of using one-half of each sample for GSSG determination and the other half for GSH. Samples for GSSG determination were incubated at room temperature with 2 μl of 4-vinyl pyridine (4-VP) per 100 μl sample for 1 hr after vigorous vortexing. Incubation with 4-VP conjugates any GSH present in the sample so that only GSSG is recycled to GSH without interference by GSH. The GSSG (as GSHx2) was then subtracted from the total GSH to determine actual GSH level and GSH/GSSG ratio [12].

Caspase-3 assay

Caspase-3 is an enzyme activated during induction of apoptosis [13]. In brief, 1 ml of PBS was added to flasks containing cardiomyocytes and the monolayer was scraped and collected in a microfuge tube. The cells were centrifuged at 10,000 × *g* at 4°C for 10 min and cell pellets were lysed in 100 μl of ice-cold cell lysis buffer (50 mM HEPES, 0.1% CHAPS, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% NP40). After cells were lysed, 70 μl of reaction buffer was added to cell lysate (30 μl) followed by an additional 20 μl of caspase-3 colorimetric substrate (Ac-DEVD-pNA) and incubated at 37°C for 1 hr, during which time the caspase in the sample was allowed to cleave the chromophore p-NA from the substrate molecule. The samples were then read with a microplate reader at 405 nm. Caspase-3 activity was expressed as picomoles of pNA released per microgram of protein per minute [14].

Generation of intracellular reactive oxygen species (ROS)

Production of cellular ROS was evaluated by analyzing changes in fluorescence intensity resulting from oxidation of the intracellular fluoroprobe 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA). In brief, isolated myocytes from each group were loaded with 1 μmol/L of the non-fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes, Eugene, OR, USA) at 37°C for 30 min. The myocytes were rinsed and the fluorescence intensity was then measured using a fluorescent micro-plate reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm (Molecular Devices, Sunnyvale, CA, USA). Untreated cells showed no fluorescence and were used to determine background fluorescence, which was subtracted from the treated samples. The final fluorescent intensity was normalized to the protein content in each myocyte group [15].

Western blot analysis

The total protein was prepared as described previously [10]. In brief, tissue samples from the heart ventricles were removed and homogenized in a lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS and 1% protease inhibitor cocktail. Samples were then sonicated for 15 sec and centrifuged at 12,000 × g for 20 min at 4°C. The protein concentration of the supernatant was evaluated using Protein Assay Reagent (Bio-Rad, Hercules, CA, USA). Equal amounts (50 μg protein/lane) of the protein and prestained molecular weight markers (Gibco-BRL, Gaithersburg, MD) were separated on 10% or 15% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad); they were then transferred electrophoretically to Nitrocellulose membranes (0.2 μm pore size, Bio-Rad Laboratories, Inc, Hercules, CA, USA). Membranes were incubated for 1 hr in a blocking solution containing 5% milk in TBS-T buffer, and incubated with anti-AT_1 receptor (1:500), anti-AT_2 receptor (1:500), anti-SERCA2a (1:1000), anti-Na^+-Ca^{2+} exchanger (1:1000), anti-phospholamban (1:1000), anti-PARP (1:1000) and anti-β-actin (1:5000) antibodies at 4°C overnight. Anti-AT_1 (monoclonal) and anti-AT_2 (polyclonal) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies to SERCA2a (A7R5) and phospholamban (2D12) were kindly provided by Dr. Larry Jones, Indiana University School of Medicine (Indianapolis, IN, USA). Rabbit polyclonal anti-Na^+-Ca^{2+} exchanger antibody was purchased from Swant (Bellinzona, Switzerland). A monoclonal antibody against the NADPH oxidase subunit $p47^{phox}$ was kindly provided by Dr. Mark T. Quinn from Montana State University (Bozeman, MT, USA). A polyclonal antibody against PARP and a monoclonal antibody against β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). After washing blots to remove excess primary antibody binding, blots were incubated for 1 hr with horseradish peroxidase (HRP)–conjugated

secondary antibody (1:5000). Antibody binding was detected using enhanced chemiluminescence (Amersham Pharmacia), and film was scanned and the intensity of immunoblot bands was detected with a Bio-Rad Calibrated Densitometer (Model: GS-800). For all Western blot analysis experiments, β-actin was used as an internal loading control.

MHC isoform analysis by gel electrophoresis

Samples were prepared for gel electrophoresis according to method described [16]. Each sample (20–30 mg heart tissue) was placed in a microcentrifuge tube and 30 μl of sample buffer was added per mg of tissue. The sample was homogenized for ~10 sec, heated for 2 min at 95°C, chilled on ice for 5 min before being centrifuged. The supernatant was saved and diluted 1:10 with sample buffer. Three μl of the diluted samples were loaded for electrophoresis. The methods for gels and the running conditions were identical to those described by Reiser and Kline [17]. Gel electrophoresis was performed at 8°C in a PROTEAN II unit (Bio-Rad, Hercules, CA, USA). Stacking gels consisted of 4% acrylamide (acrylamide:bis = 50:1) and 5% glycerol (v/v, pH 8.8). Gels were run at a constant voltage of 200 V for 30 hr, fixed for a minimum of 2 hr in 5% glutaraldehyde before being silver-stained and scanned with a Bio-Rad Calibrated Densitometer (Model: GS-800) to determine the amount of MHC-α and MHC-β.

Data analysis

Data are expressed as means ± SE. Statistical comparisons were performed by analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* test. Significance was defined as $p < 0.05$.

Results

Experimental animals

Two weeks of STZ-induced diabetes significantly elevated blood glucose levels and reduced body weight in FVB mice, consistent with the notion of diabetes-induced anabolic effect [18,19]. Interestingly, metallothionein alleviated diabetes-induced body weight loss without affecting the hyperglycemic condition. Diabetes significantly enhanced the liver size (normalized to body weight) without affecting the absolute weight of liver. There is no difference in heart and kidney weight or organ-to-body weight ratio between control and diabetic FVB mice. Metallothionein elicited little effect on the absolute organ weights and organ sizes (Table 1).

Mechanical and intracellular Ca^{2+} properties of myocytes from control and diabetic mice

Diabetes is known to increase the propensity of cardiac hypertrophy [19]. Our results exhibited significantly longer resting cell length in STZ-induced FVB diabetic mice compared to FVB control mice, which was abolished by cardiac overexpression of metallothionein. PS and ±dL/dt were significantly depressed in myocytes from the FVB diabetic group compared to those from control mice. Myocytes from STZ-induced FVB diabetic mice displayed significantly prolonged TPS and TR_{90} compared to those from control mice. Interestingly, the antioxidant metallothionein effectively protected cardiomyocytes from STZ-induced mechanical dysfunctions (Fig. 1). Our further experiments using intracellular fura-2 fluorescence revealed significantly elevated resting fura-2 fluorescent intensity (FFI), indication of overloaded resting intracellular Ca^{2+}, and reduced intracellular Ca^{2+} clearing rate in myocytes from STZ-induced FVB diabetic myocytes, consistent with prolongation in TR_{90} in these diabetic myocytes. Similar to its effect on cell shortening, STZ treatment significantly depressed caffeine-induced SR Ca^{2+} release without affecting electrically-stimulated rise of FFI (ΔFFI) in the FVB group. Consistent with its effect on cell shortening, metallothionein abolished STZ-induced intracellular Ca^{2+} homeostasis abnormalities (Fig. 2). Lastly, metallothionein transgene itself exhibited little effect on cardiomyocyte mechanical and intracellular Ca^{2+} properties with the exception of a decreased resting intracellular Ca^{2+} levels (Fig. 2).

Effect of metallothionein on diabetes-induced alteration in stimulus frequency response

Murine hearts contract at high frequencies whereas our mechanical evaluation was conducted at 0.5 Hz. To evaluate

Table 1
General features of adult control and diabetic FVB and metallothionein (MT) mice

Mice group	Body Wt (g)	Heart Wt (mg)	Heart Wt/ Body Wt (mg/g)	Liver Wt (g)	Liver Wt/ Body Wt (mg/g)	Kidney Wt (g)	Kidney/ Body Wt (mg/g)	Blood Glucose (mM)
FVB-Control	26.8 ± 0.8	187 ± 6	6.78 ± 0.35	1.22 ± 0.10	43.3 ± 3.1	0.53 ± 0.02	18.8 ± 0.6	6.5 ± 0.4
FVB-Diabetic	23.2 ± 1.0*	176 ± 12	7.58 ± 0.55	1.21 ± 0.06	51.3 ± 1.8*	0.49 ± 0.02	20.8 ± 0.7	20.9 ± 1.3*
MT-Control	26.4 ± 1.1	169 ± 12	6.19 ± 0.38	1.35 ± 0.09	48.7 ± 2.1	0.49 ± 0.02	18.0 ± 0.7	7.2 ± 0.4
MT-Diabetic	26.1 ± 1.0	197 ± 16	7.01 ± 0.57	1.50 ± 0.09	53.5 ± 3.3*	0.56 ± 0.02	20.2 ± 0.5	20.5 ± 1.4*

Mean ± SEM, $n = 21–26$ mice per group.
* $p < 0.05$ vs. FVB control group.

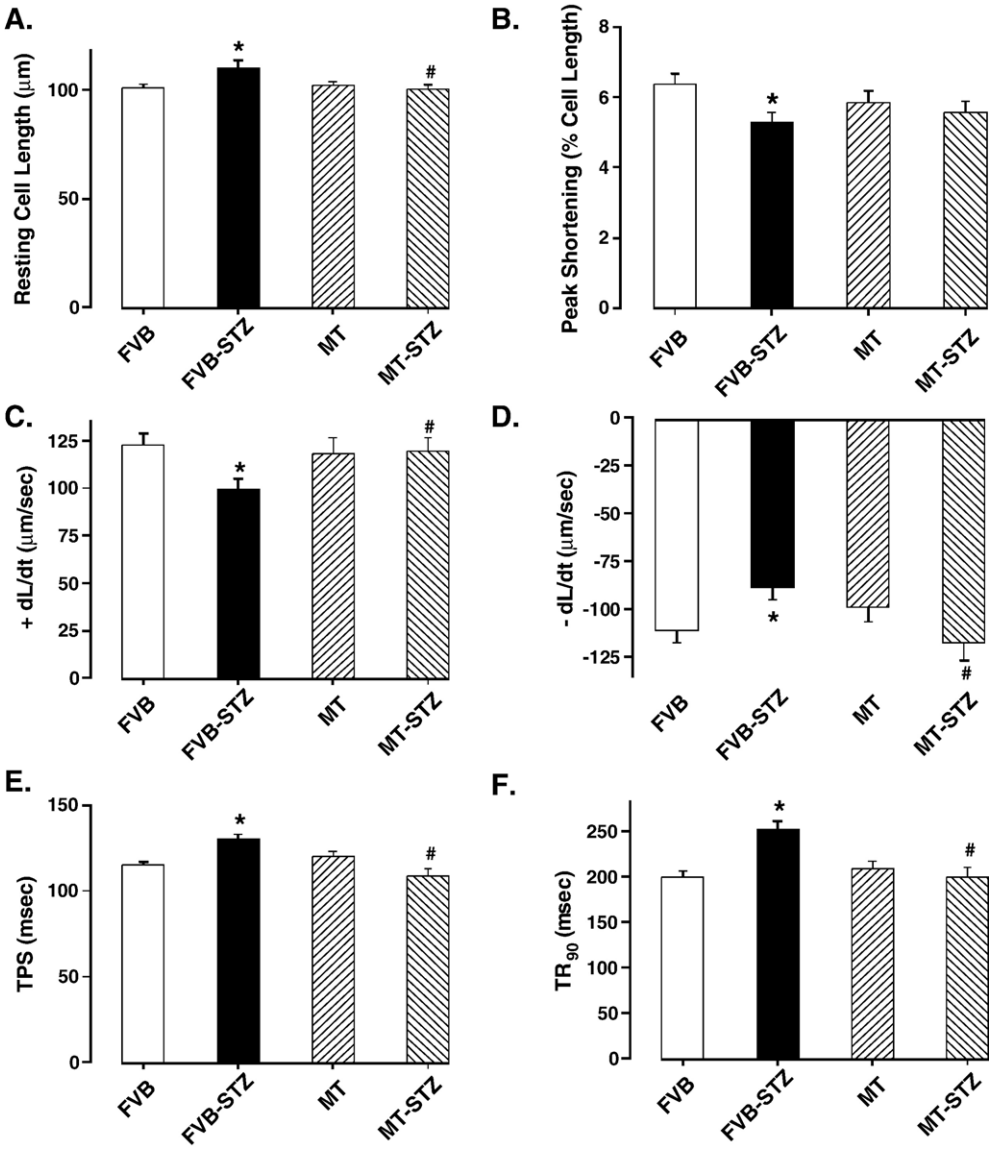

Fig. 1. Contractile properties of cardiomyocytes from control and streptozotocin (STZ)-treated FVB and metallothionein (MT) mice. A: Resting cell length; B: Peak shortening (PS, normalized to resting cell length); C: Maximal velocity of shortening (+dL/dt); D: Maximal velocity of relengthening (−dL/dt); E: Time-to-PS (TPS); and F: Time- to −90% relengthening (TR_{90}). Mean ± SEM, n = 153 cells/group, *$p < 0.05$ vs. FVB control group, #$p < 0.05$ vs. FVB-STZ group.

the impact of diabetes and metallothionein on cardiac function at higher pacing rates, we increased the field stimulus frequency to 5.0 Hz (300 beats/min) and recorded the steady-state PS. Cells were initially stimulated to contract at 0.5 Hz for 5 min to ensure steady-state before commencing the frequency response study. All the recordings were normalized to PS at 0.1 Hz of the same myocyte. Fig. 3 shows a much steeper decline of PS in FVB-STZ myocytes with increasing stimulus frequency (1.0–5.0 Hz) compared with myocytes from the FVB control mice. Consistent with its effect on cell shortening and intracellular Ca^{2+} handling, metallothionein abolished the STZ-induced steeper decline in PS at higher stimulus frequencies. These data indicated that metallothionein may antagonize STZ-induced intracellular Ca^{2+} cycling abnormalities or diminished stress tolerance capacity.

Effect of metallothionein on diabetes-induced ROS generation, oxidative stress and apoptosis

Diabetic cardiomyopathy has been demonstrated to be associated with enhanced ROS generation, oxidative stress and apoptotic cell death [4,15]. To verify the antioxidant effect of metallothionein in diabetic hearts, ROS generation, oxidative stress status and apoptosis in myocardium or cardiomyocytes from control and diabetic mice were examined by DCF fluorescence, GSH/GSSG assay and caspase-3 activity, respectively. Results shown in Fig. 4 display that STZ treatment enhanced ROS generation, reduced the GSH/GSSG ratio (with a higher proportional rise in GSSG) and enhanced caspase-3 activity in the FVB group, indicating substantial oxidative stress and apoptosis. As expected, the antioxidant metallothionein effectively alleviated STZ-induced ROS generation, oxidative stress and apoptosis.

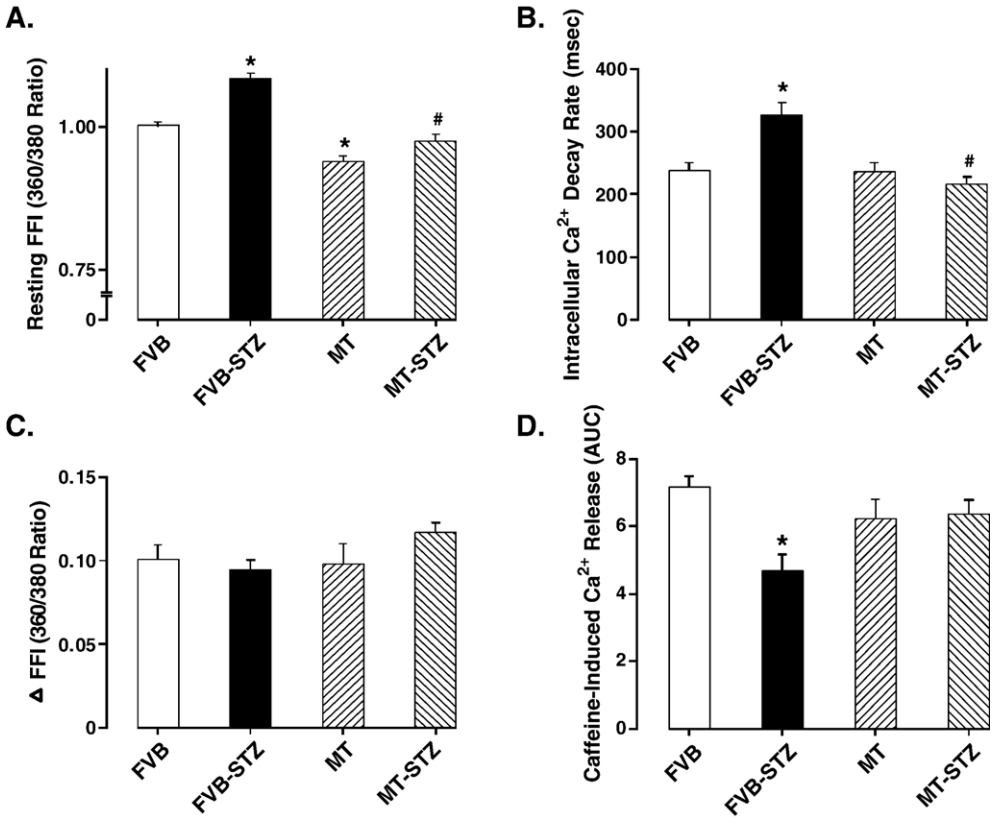

Fig. 2. Intracellular Ca^{2+} transient properties in cardiomyocytes from control and streptozotocin (STZ)-treated FVB and metallothionein (MT) mice. A: Resting intracellular Ca^{2+} fura-2 fluorescence intensity (FFI); B: Intracellular Ca^{2+} transient decay rate. C: Electrically-stimulated increase in FFI (ΔFFI); and D: Caffeine-induced sarcoplasmic reticulum Ca^{2+} release presented as area underneath curve (AUC). Mean ± SEM, $n = 91$ cells/group (panel A through C) and $n = 18–19$ cells/group for panel D, $*p < 0.05$ vs. FVB group, $\#p < 0.05$ vs. FVB-STZ group.

Protein expression in AT_1, AT_2 receptors, SERCA2a, Na^+-Ca^{2+} exchanger, phospholamban, $p47^{phox}$, PARP and MHC isozyme distribution in control and diabetic hearts

Diabetic cardiomyopathy has been demonstrated to be closely linked to up-regulation of the angiotensin II receptor, interrupted function of the key intracellular Ca^{2+} regulatory/cycling proteins including SERCA2a, Na^+-Ca^{2+} exchanger and phospholamban, and elevated expression of the stress signaling molecules NADPH oxidase $p47^{phox}$ subunit and PARP [4,10,15,20]. To causally relate these protein markers to change of cardiomyocyte contractile function in diabetes with or without metallothionein transgene, we examined the expression of AT_1 receptor, AT_2 receptors, SERCA2a, Na^+-Ca^{2+} exchanger, phospholamban, $p47^{phox}$ and PARP in myocardium from control or STZ-treated FVB and metallothionein transgenic mice. Our results indicated that STZ treatment significantly up-regulated AT_1 but not AT_2 receptor expression. Expression of SERCA2a and Na^+-Ca^{2+} exchanger was significantly reduced whereas that of phospholamban was enhanced in myocardium of STZ-treated FVB mice. Interestingly, the antioxidant metallothionein ablated STZ-induced up-regulation of AT_1 receptor and down-regulation of Na^+-Ca^{2+} exchanger without affecting expression of SERCA2a, phospholamban and AT_2 receptor. The SERCA2a-to-phospholamban ratio was significantly lower in the STZ-induced diabetic group. Metallothionein transgene failed to reconcile the diabetes-induced reduction in SERCA2a-to-phospholamban ratio (FVB: 1.97 ± 0.16; FVB-STZ: 1.05 ± 0.21; Metallothionein: 1.70 ± 0.28; Metallothionein-STZ: 1.07 ± 0.27, $n = 8–11$ per group, $p < 0.05$ between control and diabetic groups). Metallothionein transgene itself did not elicit any overt effect on the levels of AT receptors or intracellular Ca^{2+} cycling proteins (Fig. 5). In addition,

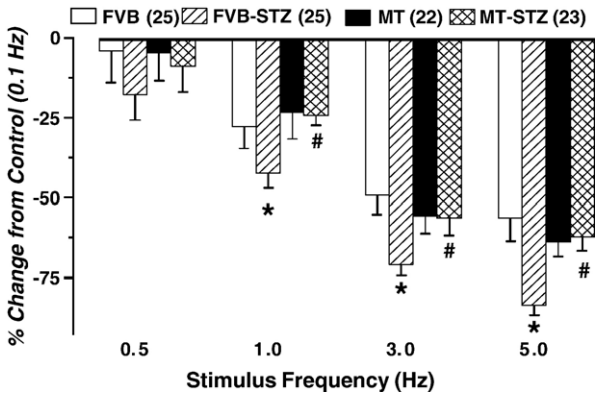

Fig. 3. Peak shortening (PS) amplitude of cardiomyocytes from control and streptozotocin (STZ)-treated FVB and metallothionein (MT) mice at different stimulus frequencies (0.1–5.0 Hz). PS was shown as % change from PS at 0.1 Hz of the same cell. Mean ± SEM, numbers in parenthesis indicate cell number per group, $*p < 0.05$ vs. FVB group, $\#p < 0.05$ vs. FVB-STZ group.

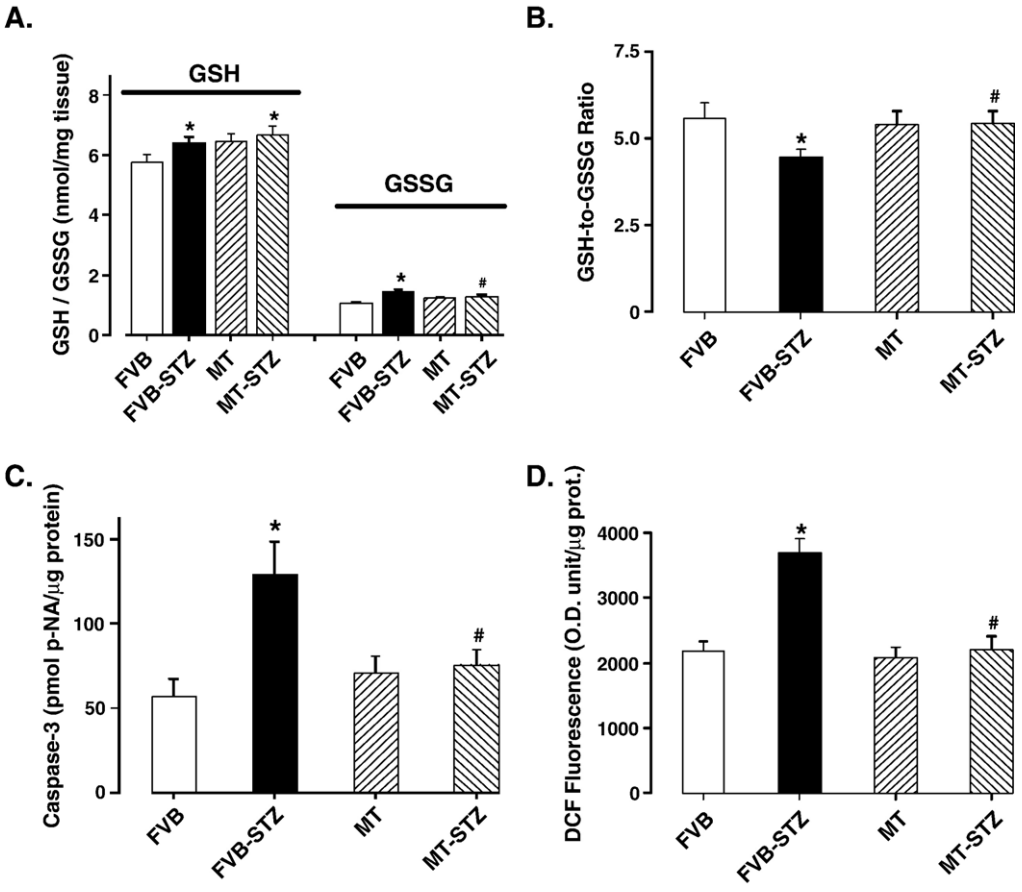

Fig. 4. Effect of metallothionein (MT) on streptozotocin (STZ)-induced oxidative stress (evaluated by GSH/GSSG ratio) and apoptosis (evaluated by Caspase-3 activity) in mouse hearts as well as accumulation of reactive oxygen species (measured by DCF fluorescence) in isolated cardiomyocytes. A: Levels of GSH and GSSG; B: GSH-to-GSSG ratio; C: Caspase-3 activity; and D: DCF fluorescence staining for ROS. Mean ± SEM, $n = 9$–14 hearts per group for panel A–C and $n = 6$ isolations per group for panel D, $*p < 0.05$ vs. FVB group, $\#p < 0.05$ vs. FVB-STZ group.

STZ treatment significantly enhanced expression of the oxidative stress signaling molecule $p47^{phox}$ subunit of NADPH oxidase and the DNA nick sensor PARP, both of which were ablated by metallothionein (Fig. 6). Our study also revealed that STZ-induced diabetes triggers an α- to β-MHC isozyme switch, consistent with a previous report [9]. Interestingly, metallothionein restored the low levels of β-MHC expression in STZ-treated diabetic myocardium and itself reduced β-MHC isozyme distribution under the non-diabetic condition (Fig. 6).

Discussion

The major findings of our present study are that the heavy metal antioxidant scavenger metallothionein rescues STZ-induced diabetic cardiomyocyte contractile and intracellular Ca^{2+} handling dysfunctions. Diabetes-induced cardiac contractile and intracellular Ca^{2+} defects were associated with enhanced ROS generation, oxidative stress and apoptosis, up-regulation of AT_1 receptor, phospholamban, $p47^{phox}$ NADPH oxidase subunit and PARP, as well as down-regulation of SERCA2a (both SERCA2a expression and SERCA2a-to-phospholamban ratio) and Na^+-Ca^{2+} exchanger. Interestingly, the STZ diabetes-induced oxidative stress, apoptosis and alterations in oxidative stress-related signaling molecules (AT_1 receptor, $p47^{phox}$ NADPH oxidase subunit and PARP) were effectively alleviated by metallothionein. However, diabetes-induced changes in intracellular Ca^{2+} cycling protein SERCA2a, its locker phospholamban and SERCA2a-to-phospholamban ratio were unaffected by metallothionein, contrary to that of Na^+-Ca^{2+} exchanger. Since metallothionein did not affect the hyperglycemic condition in diabetes, our data suggest that STZ-induced diabetes may elicit cardiac contractile dysfunction and intracellular Ca^{2+} mishandling likely through enhanced oxidative stress and cell injury.

Data from our study revealed that ventricular myocytes from STZ-induced diabetic mice exhibited depressed peak shortening, reduced maximal velocity of shortening/relengthening, prolonged duration of contraction and relaxation associated with elevated resting intracellular Ca^{2+}, delayed intracellular Ca^{2+} clearing and compromised SR Ca^{2+} release. These data are somewhat consistent with our previous findings using both chemically-induced and genetically-predisposed diabetic models [6,10,19,21,22]. The fact that our early observation showed normal resting cell length, PS and ±dL/dt associated with prolonged TPS and TR_{90} in STZ-treated diabetic cardiomyocytes [19,22] may be related to older age (10 week-old versus 8 week-old used in current study) and lower STZ administration

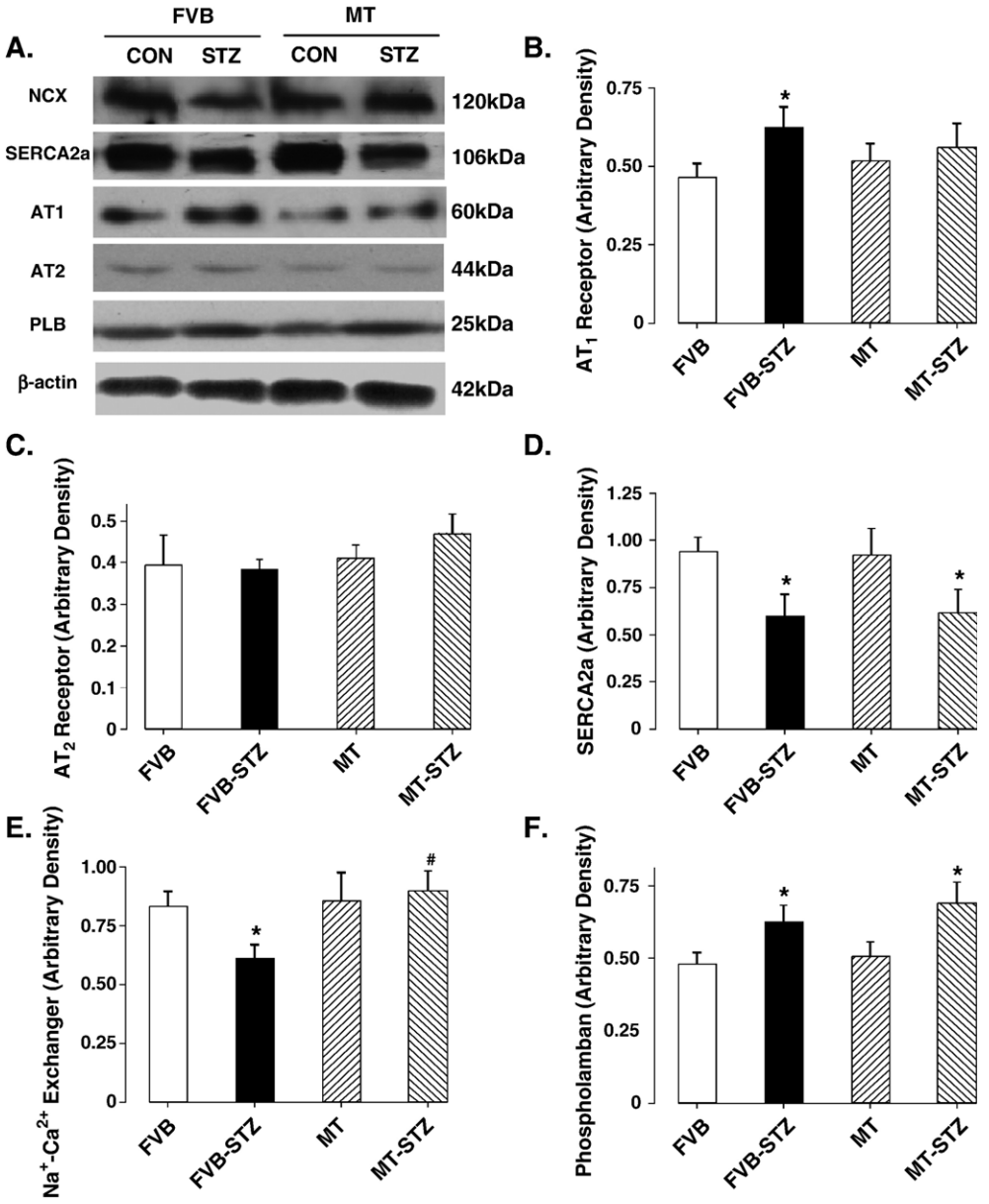

Fig. 5. Western blot analysis exhibiting expression of AT_1 receptor, AT_2 receptor, SERCA2a, Na^+-Ca^{2+} exchanger (NCX) and phospholamban (PLB) in ventricles from control and diabetic FVB and metallothionein (MT) mice. A: Represent gel blots depicting expression of above mentioned proteins using specific antibodies. B: AT_1 receptor expression; C: AT_2 receptor expression; D: SERCA2a expression; E: Na^+-Ca^{2+} exchanger expression and F: phospholamban expression. Mean ± SEM, n = 7–11 samples per group, *$p < 0.05$ vs. FVB group, #$p < 0.05$ vs. FVB-STZ group.

(200 mg/kg versus 220 mg/kg used in the current study). Cardiomyocytes from STZ-induced FVB diabetic mice had slightly but significantly longer resting cell length although the heart weight or size was unchanged. This apparent disparity indicates that these diabetic hearts were possibly going through a cardiac remodeling state prior to ventricular hypertrophy. The mechanical defects observed in our study are quite similar to those reported in ventricular myocytes from full blown diabetes (both chemically-induced and genetically-predisposed), all of which are characteristic of diabetic cardiomyopathy [2,6,10,19,21]. The impaired intracellular Ca^{2+} handling in the FVB-STZ diabetic group shown as reduced intracellular Ca^{2+} clearance rate and caffeine-induced SR Ca^{2+} release appears to be responsible for prolonged relaxation duration (TR_{90}) and reduced peak shortening (PS) in these cells. The diminished SR Ca^{2+} release in conjunction with normal electrically-stimulated rise in intracellular Ca^{2+} (ΔFFI) in FVB diabetic myocytes suggests that certain voltage-independent machineries may be at fault under the diabetic state. Another plausible explanation for STZ-induced cardiomyocyte dysfunction in our study is the MHC isozyme switch from the fast α-isoform to the slow β-isoform. Altered MHC isozyme distribution is present in myocardial remodeling, cardiac hypertrophy and cardiomyopathy [23,24], indicating that cardiac contractile function may be directly regulated by the relative amounts of MHC-α and MHC-β isozyme [17]. A decrease in the MHC-α

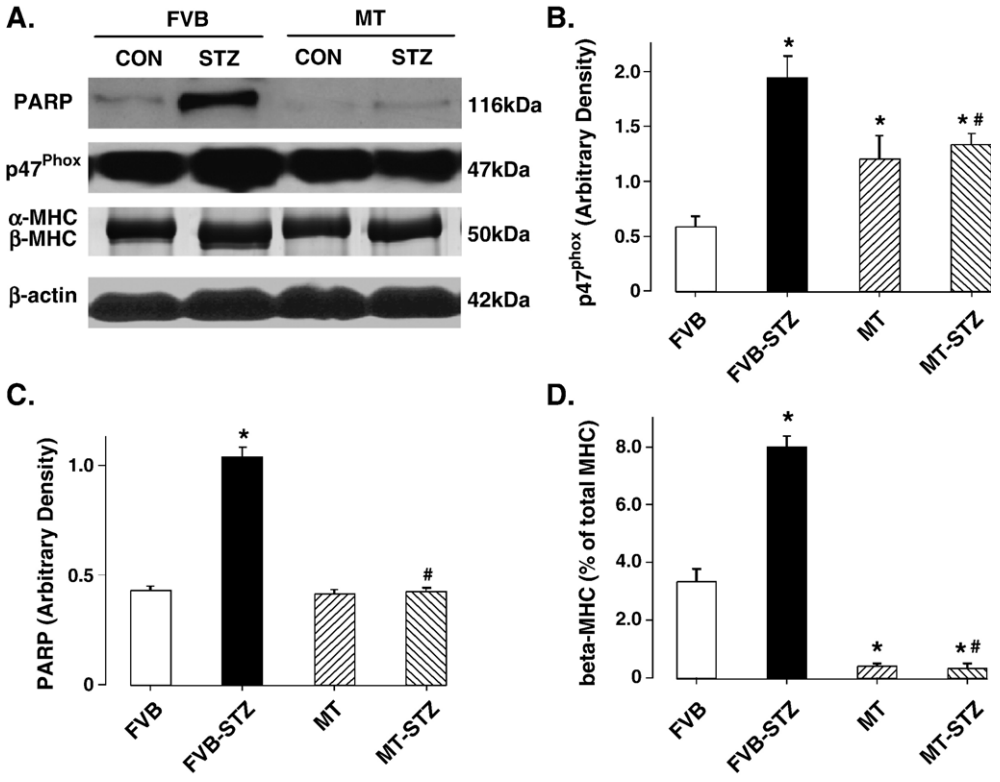

Fig. 6. Western blot analysis exhibiting expression of the p47^{phox} subunit of NADPH oxidase, PARP and myosin heavy chain (MHC) isozyme (α-MHC, β-MHC) distribution in ventricles from control and diabetic FVB and metallothionein (MT) mice. A: Represent gel blots depicting expression of above mentioned proteins using specific antibodies. B: p47^{phox} expression; C: PARP expression and D: Levels of β-MHC as a percentage of total MHC (α-MHC + β-MHC). Mean ± SEM, n = 3–6 samples per group, *$p < 0.05$ vs. FVB group, #$p < 0.05$ vs. FVB-STZ group.

to MHC-β ratio has been demonstrated in diabetic, hypertrophic and senescent hearts and plays a key role in cardiac dysfunction under diabetes, cardiac hypertrophy and senescence [17,23,24]. To the best of our knowledge, this is the first report to correlate cardiac contractile dysfunction with cardiac MHC isozyme switch in diabetic hearts with endogenous antioxidant treatment.

Our observation revealed that the mechanical and intracellular Ca^{2+} defects triggered by STZ may be greatly alleviated by metallothionein, in a manner similar to its effect on cardiac contractile dysfunctions in genetically-predisposed diabetes [6] and diet-induced insulin resistance [25]. Although our experimental results indicated that an alteration of the crucial cardiac Ca^{2+} cycling protein Na^{+}-Ca^{2+} exchanger may play a role in metallothionein-offered cardiac protection against STZ-induced diabetes. This is consistent with the earlier observation that diabetes induces compromised Na^{+}-Ca^{2+} exchanger function [26]. Our result of diminished SERCA2a levels associated with elevated phospholamban expression (manifested as a reduced SERCA2a-to-phospholamban ratio) is consistent with earlier reports [10,27,28], which indicates a role of SERCA2a and phospholamban in cardiomyocyte contractile and intracellular Ca^{2+} dysregulation in STZ-induced diabetic condition. SERCA2a and phospholamban are two key intracellular Ca^{2+} cycling proteins in charge of sarcoplasmic reticulum (SR) Ca^{2+} re-uptake and cardiac diastolic function [29]. However, the lack of effect from metallothionein on SERCA2a and phospholamban in STZ-treated mouse hearts in the presence of improved cardiac contractile function seems to rule out these two Ca^{2+} cycling proteins as contenders for a metallothionein-induced protective mechanism in STZ-induced diabetes. It is possible that enhanced Na^{+}-Ca^{2+} exchanger expression in the STZ-treated metallothionein mice may play a compensatory role for cytosolic Ca^{2+} extrusion, albeit SERCA2a levels and the SERCA2a-to-phospholamban ratio remained decreased in diabetic metallothionein mice. It should be mentioned that possible beneficial effects of metallothionein on SERCA activity cannot be ruled out at this point. Our additional results suggest that ROS accumulation, oxidative stress and apoptosis accompany STZ-induced mechanical dysfunction and contribute to STZ-induced diabetic cardiac contractile dysfunction. Although enhanced ROS generation in diabetic cardiomyocytes is likely attributed to STZ-induced chronic hyperglycemia in our study, the direct role of STZ in ROS formation and heart dysfunction cannot be discounted as shown previously [30]. Metallothionein reconciled STZ-induced cardiac dysfunction in parallel with its effect on ROS generation, oxidative stress and apoptosis. Oxidative stress has been demonstrated to be closely associated with impaired cardiac contractile function in diabetes, hypertension and obesity [4–6,25,31]. Possible sources of increased ROS in diseased myocardium include NADPH oxidase (such as p47^{phox}), cyclooxygenase, xanthine oxidase and the mitochondrial electron transport chain and activated neutrophils [4,5]. In addition to ROS, antagonism

against reactive nitrogen species (RNS) and nitrosative damage in diabetes has also been demonstrated to contribute to the beneficial effect of metallothionein in the development of diabetic cardiomyopathy [32].

Data from our present study suggest that up-regulation of AT_1 (not AT_2) receptor, $p47^{phox}$ subunit of NADPH oxidase and PARP may contribute to cardiac oxidative stress and contractile dysfunctions. Our earlier study revealed that hyperglycemia may directly lead to cardiomyocyte contractile dysfunction through a AT_1 receptor-NADPH oxidase-dependent mechanism [15]. Treatment with a AT_1 receptor antagonist not only alleviates high glucose-induced elevation in the $p47^{phox}$ subunit of NADPH oxidase but also protects cardiomyocytes from glucose toxicity [15]. Perhaps the most interesting observation in our current study was that metallothionein not only abolished STZ-induced elevation in AT_1 receptor and $p47^{phox}$ NADPH oxidase, but also nullified STZ-induced up-regulation of PARP. It has been suggested that free radical and oxidant production in cardiac myocytes during ischemia/reperfusion, aging and cardiomyopathy leads to DNA strand-breakage, which in turn activates the nuclear enzyme PARP [33]. Both RNS and NADPH oxidase-mediated ROS have been demonstrated to turn on PARP [34]. Activation of the nuclear enzyme PARP triggers an energy consuming, inefficient cellular metabolic cycle resulting in relocation of the ADP-ribosyl moiety of NAD^+ to protein acceptors, a process detrimental to cardiomyocyte survival and function [33]. Our observation that metallothionein prevented STZ-induced up-regulation also supports the notion that PARP is a critical effector downstream of oxidative and nitrosative stress. Abnormal activation of PARP has been shown to contribute to the pathogenesis of a number of cardiovascular dysfunctions including myocardial infarction, heart failure, diabetes, atherosclerosis, hypertension, shock and aging [33,34]. Pharmacological inhibition of superoxide formation, neutralization of peroxynitrite and inhibition of PARP may offer significant benefit to cardiovascular injury [33,34]. Although our study sheds some light on the interaction of oxidative stress, diabetes and diabetes-associated cardiac defects, the pathogenesis of cardiac contractile dysfunction under diabetes, especially the clinical value of the nuclear enzyme PARP as a therapeutic target for diabetic heart diseases, still deserves further in-depth investigation.

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