Sex difference in cardiomyocyte function in normal and metallothionein transgenic mice: the effect of diabetes mellitus
Asli F. Ceylan-Isik, Karissa H. LaCour and Jun Ren

You might find this additional information useful...

This article cites 43 articles, 20 of which you can access free at:
http://jap.physiology.org/cgi/content/full/100/5/1638#BIBL

Updated information and services including high-resolution figures, can be found at:
http://jap.physiology.org/cgi/content/full/100/5/1638

Additional material and information about Journal of Applied Physiology can be found at:
http://www.the-aps.org/publications/jappl

This information is current as of April 26, 2006.
Sex difference in cardiomyocyte function in normal and metallothionein transgenic mice: the effect of diabetes mellitus

Asli F. Ceylan-Isik, Karissa H. LaCour, and Jun Ren
Division of Pharmaceutical Sciences and Center for Cardiovascular Research and Alternative Medicine, University of Wyoming, Laramie, Wyoming
Submitted 4 October 2005; accepted in final form 4 January 2006

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

DIABETIC CARDIOMYOPATHY, a unique deterioration of myocardial function found in the diabetic population, is a prominent form of diabetic complications independent of macroand microvascular complications of diabetes mellitus (9, 23). It is characterized by impaired ventricular contraction, relaxation, and wall compliance (9, 23, 24, 41). Both clinical and experimental evidence has revealed impaired intracellular Ca^2+ homeostasis, reduced contractility, prolonged duration of contraction and relaxation associated with enhanced free radical accumulation, and oxidative damage in the heart (7, 9, 11, 12, 22, 24, 31, 32, 41). Interestingly, a “female advantage” exists in premenopausal women with regard to the propensity and severity of diabetic heart diseases (23, 35, 36). Sex differences in myocardial and cardiomyocyte contractile function have long been established, mainly manifested as differences in myocardial contractility, duration, and velocity of contraction and relaxation as well as stress tolerance to ischemia-reperfusion injury (1, 5, 23, 42). Although differences in ovarian sex hormones, especially the potent antioxidant capacity associated with estrogen, have been speculated to play a major role in this “sex bias” of myocardial contractile function (1, 23, 26), the precise mechanism of action behind sex differences in intrinsic (i.e., basal physiological) cardiac function and diabetic cardiomyopathy still remains unclear.

One major mechanism responsible for the pathogenesis of diabetic cardiomyopathy is enhanced oxidative stress and oxidative damage in diabetic hearts (3, 11, 40). Oxidative stress, usually a result of imbalance between reactive oxygen species generation and antioxidant defense, has been thought to contribute to a wide variety of cardiovascular diseases including cardiac hypertrophy and heart failure (34, 37, 38). Increased production of reactive oxygen species including superoxide, hydrogen peroxide, and hydroxyl radical has been demonstrated in both patients and animals with cardiac hypertrophy and heart failure (34, 37, 38). Several mechanisms have been speculated to be the major sources of enhanced reactive oxygen species and oxidative stress including xanthine and NADPH oxidase, cyclooxygenase, and mitochondrial electron transport (34, 38). In addition, reactive nitrogen species and nitrosative stress due to excessive nitric oxide production also play a significant role in the alteration of cardiac morphology and function in heart failure, diabetes, atherosclerosis, hypertension, and aging (38). Not surprisingly, antioxidant therapy including exogenous administration and endogenous enzyme expression or induction has shown promise in the treatment of heart diseases in heart failure, hypertension, aging, and diabetes (11, 19, 20, 34, 38). To better understand the role of antioxidant defense in diabetic heart dysfunction and the potential of a sex difference, the present study was designed to examine the impact of cardiac overexpression of the heavy metal scavenger metallothionein (in an effort to “enhance” the intrinsic antioxidant capacity of the heart) on intrinsic and diabetic cardiomyocyte contractile function in both sexes. We also evaluated the status of oxidative stress, protein damage, activation of the essential cardiac survival factor Akt, and stress-related transcription factor c-Jun. The serine/threonine protein kinase Akt, also named protein kinase B, plays an essential role in the regulation of cardiac hypertrophy, preservation of cardiac contractile function, angiogenesis, and apop-
tosis (7, 15, 21). Short-term activation of Akt1 by physiological stimuli, such as insulin, insulin-like growth factor 1 (IGF-1), and exercise, promotes physiological cardiac hypertrophy via mammalian target of rapamycin whereas long-term Akt1 activation triggers a maladaptive cardiac hypertrophic response and angiogenesis en route to heart failure (18, 21, 33). Both sex and diabetes have been shown to alter myocardial Akt phosphorylation status (4, 7). On the other hand, transcription factor c-Jun has been shown to participate in cardiac oxidative stress and hypertrophic response (17, 39), although its role in sex- and diabetes-associated alteration of cardiac contractile function remains largely unknown.

MATERIALS AND METHODS

Experimental diabetic animals. The experimental procedures were approved by the Institutional Animal Use and Care Committee at the University of Wyoming (Laramie, WY). All animal procedures were in accordance with National Institutes of Health animal care standards. In brief, 8- to 10-wk-old weight-matched FVB albino and metallothionein cardiac-specific transgenic mice of both sexes were given a single dose injection of streptozotocin (STZ, 220 mg/kg ip) dissolved in a sterile citrate buffer (0.05 M sodium citrate, pH 4.5). Metallothionein overexpression was ~10-fold of its level in the wild type. The pigmentation of fur (dark gray) was used as a marker for metallothionein transgene expression identification as described (41).

Fasting blood glucose level was evaluated 7 days later. A supplemental STZ injection of 100 mg/kg (ip) was given if the fasting blood glucose was below 12 mM. Control mice received a similar volume of sodium citrate buffer. All diabetic (fasting blood glucose level >12 mM) and control mice were maintained for a total of 2 wk with free access to standard lab chow and tap water. Body weight and fasting blood glucose were measured by using a standard lab scale and glucose monitor (Accu-ChekII, model 792, Boehringer Mannheim Diagnostics, Indianapolis, IN), 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 a modified Tyrode’s solution (Ca2+ free) for 2 min, the heart was digested for 10 min with 0.9 mg/ml collagenase D (Boehringer Mannheim Biochemicals, Indianapolis, IN) modified Tyrode’s solution. The modified Tyrode’s solution (pH 7.4) contained the following (in mM): 135 NaCl, 4.0 KCl, 1.0 MgCl2, 10 HEPES, 0.33 Na2HPO4, 10 glucose, 10 butanedione monoxime, and the solution was gassed with 5% CO2-95% O2. The digested heart was then removed from the cannula and the left ventricle was cut into small pieces in the modified Tyrode’s solution. These pieces were gently agitated and the pellet of cells was resuspended in modified Tyrode’s solution and allowed to settle for another 20 min at room temperature, during which time extracellular Ca2+ was added incrementally back to 1.20 mM. Isolated myocytes were used for experiments within 8 h after isolation. Only rod-shaped myocytes with clear edges were selected for recording of mechanical properties and intracellular Ca2+ transients as described (7).

Cell shortening-relengthening measurements. Mechanical properties of ventricular myocytes were assessed using a SoftEdge MyoCam system (IonOptix, Milton, MA) (7). In brief, cells were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus IX-70, Olympus Optical, Tokyo, Japan) and superfused (~1 ml/min at 25°C) with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 CaCl2, 1 MgCl2, 10 glucose, 10 HEPES, at pH 7.4. The cells were field stimulated with a suprathreshold voltage at a frequency of 0.5 Hz, 3 ms duration, using a pair of platinum wires placed on opposite sides of the chamber connected to a FHC stimulator (FHC Incorporation, Bowdoinham, ME). The myocyte being studied was displayed on the computer monitor using an IonOptix MyoCam camera. A SoftEdge software (IonOptix, Milton, MA) was used to capture changes in cell length during shortening and relengthening. Cell shortening and relengthening were assessed using the following indexes: peak shortening (PS), time to PS (TPS) and time to 90% relengthening (TR90), and maximal velocities of shortening (+dUT/dt) and relengthening (-dUT/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 each myocyte was achieved (usually after the first 5–6 beats) before recording of PS.

Intracellular Ca2+ transient measurement and sarcoplasmic reticulum (SR) Ca2+ load. A separate cohort of murine 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 75-W 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 and 520 nm by a photomultiplier tube after first illuminating the cells at 360 nm for 0.5 s and 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 Ca2+ concentration were inferred from the ratio of the fura-2 fluorescence intensity (FFI) at two wavelengths. Fluorescence decay time was also used as an indication of the intracellular Ca2+ clearing rate (7). The SR load was directly assessed through a 10-s 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 Ca2+ transient curve during the caffeine perfusion was calculated and used as an index of the SR Ca2+ load (27).

GSH and GSSG assay. Ventricular tissues were homogenized in four volumes (wt/vol) of 1% picric acid. Acid homogenates were centrifuged at 16,000 g for 30 min and supernatant fractions were collected. Supernatant fractions were assayed for total reduced (GSH) and oxidized (GSSG) glutathione 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-vinylpyridine (4-VP) per 100 μl sample for 1 h 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-to-GSSG ratio (GSH/GSSG), which is used as an indicator for oxidative stress (28).

Protein carbonyl assay. The carbonyl content of protein was determined as described (28). Briefly, proteins were extracted and mimized to prevent proteolytic degradation. Nucleic acids were eliminated by treating the samples with 1% streptomycin sulfate for 15 min, followed by a 10-min centrifugation (11,000 g). Protein was precipitated by adding an equal volume of 20% trichloroacetic acid (TCA) (protein (0.5 mg) and centrifuged for 1 min. The TCA solution was removed and the sample resuspended in a 10 mM 2,4-dinitrophenylhydrazine solution. Samples were incubated at room temperature for 15–30 min. After a 500 μl addition of 20% TCA, samples were centrifuged for 3 min. The supernatant was discarded, and the pellet washed in ethanol-ethyl acetate and allowed to incubate at room temperature for 10 min. The samples were centrifuged again for 3 min and the ethanol-ethyl acetate steps repeated two more times. The precipitate was resuspended in a 6 M guanidine solution and centrifuged for 3 min, and the insoluble debris was removed. The maximum absorbance (360–390 nm) of the supernatant was read against appropriate blanks (water, 2 M HCl), and the carbonyl content was calculated by using the molar absorption coefficient of 22,000 M⁻¹·cm⁻¹.
Western blot analysis of Akt, phosphorylated Akt (p-Akt), and c-Jun. The total protein was prepared as described previously (7). In brief, heart ventricular samples 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 s and centrifuged at 12,000 g for 20 min at 4°C. The protein concentration of the supernatant was evaluated by use of Protein Assay Reagent (Bio-Rad, Hercules, CA). Equal amounts (50 μg protein/lane) of the protein from the whole cell extraction or mitochondria and prestained molecular weight markers (GIBCO-BRL, Gaithersburg, MD) were separated on 10% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad); they then were transferred electrophoretically to nitrocellulose membranes (0.2 μm pore size, Bio-Rad Laboratories, Hercules, CA). Membranes were incubated for 1 h in a blocking solution containing 5% milk in TBS, and then membranes were washed briefly in TBS and incubated overnight at 4°C with anti-Akt (1:1,000), anti-p-Akt (1:1,000), anti-phospho-c-Jun (Ser63) II, and anti-phospho (Ser473), anti-phospho-c-Jun (Ser63) II, and anti-β-actin (1:5,000) antibodies. Anti-Akt, anti-p-Akt (Ser473), anti-phospho-c-Jun (Ser63) II, and anti-β-actin antibodies were obtained from Cell Signaling (Beverly, MA). After blots were washed to remove excess primary antibody binding, they were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:5,000). Antibody binding was detected by enhanced chemiluminescence (Amersham Pharmacia), and the films were 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.

Data analysis. Data are expressed as means ± SE. Statistical comparisons were performed by ANOVA followed by the Newman-Keuls post hoc test. Significance was defined as P < 0.05.

RESULTS

Experimental animals. There was no significant difference in body weight of control and diabetic FVB or metallothionein mice of both sexes. Heart weight and heart-to-body weight ratio were similar between male and female FVB mice. Metallothionein transgene significantly increased the heart weight and heart-to-body weight ratios, although the increase was more pronounced in males. STZ treatment enhanced heart weight and heart-to-body weight ratio in FVB mice of both sexes and female metallothionein mice. Diabetes triggered hepatic and renal hypertrophy in FVB and metallothionein mice of both sexes. Females displayed reduced liver and kidney weights or ratios in metallothionein mice whereas only reduced kidney weight or size was seen in FVB counterparts. As expected, STZ treatment significantly and equally increased blood glucose levels in FVB and metallothionein mice of both sexes (Table 1).

Mechanical and intracellular Ca2+ properties of myocytes from male and female control and diabetic mice. The resting cell length was similar in cardiomyocytes from control and diabetic FVB and metallothionein mice of both sexes (Fig. 1A). PS and maximal velocities of shortening and lengthening (± dL/dt) were both significantly lower, whereas TPS and T90 were significantly longer in female FVB mice compared with their male counterparts. Interestingly, these sex differences in cardiomyocyte mechanical function were absent in metallothionein mice (Fig. 1, B–F). Metallothionein transgene itself significantly prolonged T90 in male mice (Fig. 1F) whereas it significantly enhanced ± dL/dt in female mice (Fig. 1, C and D). STZ treatment depressed PS and ± dL/dt and prolonged T90 in myocytes from male FVB group. However, only diminished PS was observed in the female diabetic FVB group (Fig. 1, B–F). Metallothionein nullified diabetes-induced cardiac myocyte mechanical dysfunction equally in male and female mice (Fig. 1, B–F). Our further study using intracellular fura-2 fluorescence technique revealed reduced electrically stimulated ΔFF (Fig. 2B) and slowed intracellular Ca2+ clearing rate (Fig. 2C) associated with similar resting FFI (Fig. 2A) and caffeine-induced SR Ca2+ release (Fig. 2D) in myocytes from female FVB mice compared with their male counterparts. Similar to the findings of cell shortening, the sex difference in ΔFFI and intracellular Ca2+ decay rate was no longer present in metallothionein mice. Metallothionein itself significantly enhanced resting FFI and reduced SR Ca2+ release in male mice whereas it significantly enhanced ΔFFI and reduced SR Ca2+ release in female mice without affecting intracellular Ca2+ decay rate in either sex. Such action of metallothionein resulted in a sex difference in the resting FFI but not any other indexes evaluated (Fig. 2, A–D). STZ treatment depressed SR Ca2+ release and prolonged intracellular Ca2+ decay in myocytes from FVB mice of both sexes (Fig. 2, C–D). In addition, STZ triggered a significantly elevated ΔFFI in the female FVB group without affecting ΔFFI in the male FVB group (Fig. 2B). Consistent with its action on mechanical indexes, metallothionein transgene prevented diabetes-induced alteration in ΔFFI, intracellular Ca2+ decay rate, and SR Ca2+ release equally in male and female mouse myocytes (Fig. 2, B–D).

Effect of changes in stimulation frequency on PS in myocytes from male and female control and diabetic mice. To investigate possible derangement of cardiac excitation-contraction coupl

Table 1. General features of male and female, control and diabetic FVB and metallothionein mice

<table>
<thead>
<tr>
<th>Mice Group</th>
<th>Body Wt, g</th>
<th>Heart Wt, mg</th>
<th>Heart/Body Wt, mg/g</th>
<th>Liver Wt, g</th>
<th>Liver Wt/Body Wt, mg/g</th>
<th>Kidney Wt, g</th>
<th>Kidney Wt/Body Wt, mg/g</th>
<th>Blood Glucose, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male FVB-control</td>
<td>25.7±0.8</td>
<td>113±7</td>
<td>4.38±0.23</td>
<td>1.41±0.04</td>
<td>15.7±0.5</td>
<td>5.18±0.58</td>
<td>0.58±0.58</td>
<td>16* 7.20</td>
</tr>
<tr>
<td>Female FVB-control</td>
<td>53.8±0.8</td>
<td>114±7</td>
<td>4.51±0.21</td>
<td>1.32±0.08</td>
<td>12.8±0.5</td>
<td>5.66±0.41</td>
<td>0.20</td>
<td>16* 9.23</td>
</tr>
<tr>
<td>Male FVB-diabetic</td>
<td>26.2±0.7</td>
<td>188±16*</td>
<td>7.20±0.62</td>
<td>1.65±0.08</td>
<td>18.2±0.6</td>
<td>17.8±1.7*</td>
<td>0.59*</td>
<td>10* 5.20</td>
</tr>
<tr>
<td>Female FVB-diabetic</td>
<td>24.4±0.8</td>
<td>225±16*</td>
<td>9.23±0.59</td>
<td>1.39±0.04</td>
<td>15.2±0.5</td>
<td>10.2±2.1*</td>
<td>0.5*</td>
<td>16* 5.66</td>
</tr>
<tr>
<td>Male MT-control</td>
<td>28.7±1.0</td>
<td>206±26‡</td>
<td>7.20±0.89</td>
<td>1.54±0.07</td>
<td>15.6±0.6</td>
<td>5.24±2.0</td>
<td>0.20</td>
<td>16* 5.24</td>
</tr>
<tr>
<td>Female MT-control</td>
<td>26.2±0.6</td>
<td>144±12‡</td>
<td>5.58±0.53</td>
<td>1.26±0.07</td>
<td>12.2±0.3</td>
<td>5.26±0.10</td>
<td>0.59</td>
<td>16* 5.20</td>
</tr>
<tr>
<td>Male MT-diabetic</td>
<td>27.4±1.0</td>
<td>240±30</td>
<td>8.75±1.01</td>
<td>1.79±0.10</td>
<td>20.7±0.8</td>
<td>20.4±0.5*</td>
<td>0.5*</td>
<td>16* 5.20</td>
</tr>
<tr>
<td>Female MT-diabetic</td>
<td>26.2±1.2</td>
<td>235±10*</td>
<td>9.24±0.71</td>
<td>1.53±0.05</td>
<td>17.1±0.9</td>
<td>15.9±1.5*</td>
<td>0.46</td>
<td>16* 5.20</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10–15 mice per group. MT, metallothionein; Wt, weight. *P < 0.05 vs. respective nondiabetic group; ‡P < 0.05 vs. respective male counterpart; †P < 0.05 vs. respective FVB counterpart.
pling in control and diabetic mouse myocytes at higher frequencies, we incrementally enhanced the stimulating frequency to 5.0 Hz (300 beat/min) and recorded the steady-state peak shortening. Cells were initially stimulated to contract at 0.5 Hz for 5 min to ensure the steady state before commencement of the frequency study. All recordings were normalized to the PS obtained at 0.1 Hz for the same myocyte. Figure 3A shows comparable negative staircases in PS with increasing stimulating frequency in the four FVB myocytes from control and diabetic mice of both sexes. The threshold of significant reduction in PS compared with PS obtained at 0.1 Hz was between 0.1 and 0.5 Hz (between 0.5 and 1.0 Hz for the male diabetic group). Although myocytes from control and diabetic metallothionein groups of both sexes displayed negative staircases in PS with rising stimulus frequency from 0.1 to 5.0 Hz, the threshold of attaining significant depression in PS in myocytes from metallothionein mice was between 0.5 to 1.0 Hz, exhibiting a rightward shift compared with that of FVB mouse myocytes (Fig. 3B).

Oxidative stress status and protein carbonyl formation in myocardium from male and female control and diabetic mice. The GSH levels and GSH/GSSG ratio (reduced ratio depicts enhanced oxidative stress) were significantly higher in female FVB myocardium compared with their male counterparts (Fig. 4, A and C). There was no sex difference in GSSG (Fig. 4B) and protein carbonyl formation (Fig. 4D). Metallothionein transgene significantly increased GSH levels and the GSH/GSSG ratio in male myocardium with little effect on female myocardium, thus nullifying the sex difference in GSH and the GSH/GSSG ratio. Metallothionein transgene did not affect GSSG levels although it significantly diminished protein carbonyl levels in male myocardium. STZ treatment facilitated GSSG production, protein carbonyl formation, and decreased GSH/GSSG ratio equally in both sexes, which may be alleviated by the metallothionein transgene, with the exception that the diabetes-induced decrease in GSH/GSSG ratio was partially improved by metallothionein (Fig. 4, A–D). The GSH/GSSG ratio was significantly different between the FVB diabetic and metallothionein diabetic groups of both sexes (Fig. 4C).

Protein expression in Akt, p-Akt, and c-Jun in male and female control and diabetic mice. Both diabetes and sex may be associated with change of cardiac survival and stress signaling molecules Akt and c-Jun (4, 7, 10). We examined the effect of metallothionein on cardiac expression of Akt, p-Akt, and c-Jun in male and female control and diabetic myocar-
Our results indicated that the p-Akt level and p-Akt-to-total Akt (p-Akt/Akt) ratio was significantly higher in female FVB myocardium compared with male FVB myocardium (Fig. 5, B and C). To the contrary, expression of phosphorylated c-Jun was significantly lower in female FVB myocardium (Fig. 5D). There was no sex difference in total Akt expression (Fig. 5A). Interestingly, expression of total Akt and p-Akt and upregulated p-Akt/Akt ratio were enhanced in male metallothionein myocardium. In contrast, total Akt, p-Akt levels, and p-Akt/Akt ratio were depressed in female metallothionein myocardium (Fig. 5, A–C). Such opposite effects of metallothionein on Akt and p-Akt expression between males and females created a sex difference in total Akt and p-Akt as well as the p-Akt/Akt ratio between male and female metallothionein mice. In addition, metallothionein transgene diminished c-Jun expression in male myocardium without affecting expression in the female myocardium or the sex difference in c-Jun expression (Fig. 5D). Diabetes significantly reduced the p-Akt/Akt ratio in male but not female FVB myocardium without significantly affecting expression of Akt or p-Akt independently. Although the p-Akt level and p-Akt/Akt ratio were still depressed by diabetes in male metallothionein myocardium, they were significantly higher than those found in male FVB diabetic myocardium (Fig. 5, B and C). STZ treatment did not significantly affect Akt, p-Akt, or the p-Akt/Akt ratio in either FVB or metallothionein female mice (Fig. 5, A–C). Our further study revealed that STZ treatment significantly enhanced myocardial expression of c-Jun in both sexes the effect of which was blunted by metallothionein overexpression (Fig. 5D).

DISCUSSION

To the best of our knowledge, this is the first study to examine the effect of antioxidant on sex and streptozotocin-diabetic myocardium. Our results indicated that the p-Akt level and p-Akt-to-total Akt (p-Akt/Akt) ratio was significantly higher in female FVB myocardium compared with male FVB myocardium (Fig. 5, B and C). To the contrary, expression of phosphorylated c-Jun was significantly lower in female FVB myocardium (Fig. 5D). There was no sex difference in total Akt expression (Fig. 5A). Interestingly, expression of total Akt and p-Akt and upregulated p-Akt/Akt ratio were enhanced in male metallothionein myocardium. In contrast, total Akt, p-Akt levels, and p-Akt/Akt ratio were depressed in female metallothionein myocardium (Fig. 5, A–C). Such opposite effects of metallothionein on Akt and p-Akt expression between males and females created a sex difference in total Akt and p-Akt as well as the p-Akt/Akt ratio between male and female metallothionein mice. In addition, metallothionein transgene diminished c-Jun expression in male myocardium without affecting expression in the female myocardium or the sex difference in c-Jun expression (Fig. 5D). Diabetes significantly reduced the p-Akt/Akt ratio in male but not female FVB myocardium without significantly affecting expression of Akt or p-Akt independently. Although the p-Akt level and p-Akt/Akt ratio were still depressed by diabetes in male metallothionein myocardium, they were significantly higher than those found in male FVB diabetic myocardium (Fig. 5, B and C). STZ treatment did not significantly affect Akt, p-Akt, or the p-Akt/Akt ratio in either FVB or metallothionein female mice (Fig. 5, A–C). Our further study revealed that STZ treatment significantly enhanced myocardial expression of c-Jun in both sexes the effect of which was blunted by metallothionein overexpression (Fig. 5D).

Fig. 2. Intracellular Ca\(^{2+}\) transient properties in ventricular myocytes from control and STZ-induced diabetic FVB and metallothionein mice of both sexes. A: resting intracellular Ca\(^{2+}\) fura-2 fluorescence intensity (FFI). B: electrically stimulated increase in FFI (ΔFFI). C: intracellular Ca\(^{2+}\) transient decay rate. D: sarcoplasmic reticulum (SR) Ca\(^{2+}\) release shown as area underneath the curve. Values are means ± SE; n = 52 cells from 5–6 mice per group. *P < 0.05 vs. respective nondiabetic group, #P < 0.05 vs. respective male counterpart, **P < 0.05 vs. respective FVB counterpart.

Fig. 3. PS amplitude of cardiomyocytes from control and STZ-induced diabetic FVB (A) and metallothionein (B) mice of both sexes at different stimulus frequencies (0.1–5.0 Hz). PS was shown as percent change from PS at 0.1 Hz of the same cell. Values are means ± SE; numbers in parenthesis depict sample size per group. *P < 0.05 vs. respective nondiabetic group, **P < 0.05 vs. respective FVB counterpart.
associated alterations in cardiomyocyte contractile function. The major findings of our work include that metallothionein transgene enhanced cardiac glutathione levels and nullified the sex differences in cardiac glutathione antioxidant capacity, intrinsic cardiomyocyte contractile function, as well as diabetes-induced mechanical defect and impaired intracellular Ca^{2+} homeostasis. The sex difference in intrinsic cardiomyocyte contractile and intracellular Ca^{2+} properties was accompanied with elevated Akt activation and reduced c-Jun phosphorylation, respectively, in female myocardium compared with their male counterparts. The heavy metal scavenger metallothionein reversed the sex difference in Akt phosphorylation without...
affecting that of the transcription factor c-Jun. On the other hand, STZ-induced diabetes reduced the p-Akt/Akt ratio in male but not female myocardium, whereas STZ treatment upregulated c-Jun phosphorylation in myocardium of both sexes. These diabetes-induced alterations in phosphorylation of Akt and c-Jun were nullified by metallothionein in myocardium of both sexes, to a somewhat similar extent. Because metallothionein transgene itself did not affect blood glucose levels or metabolism, our data suggest that both sex and diabetes-associated alterations in cardiac contractile function and phosphorylation of Akt and c-Jun may be casually associated with a weaker antioxidant capacity and/or a higher oxidative stress status in myocardium from the males or under diabetes.

It has long been known that a sex-associated difference exists in myocardial contractile function including duration and maximal velocity of contraction and relaxation (2, 5, 23). This is consistent with our present observation of reduced peak shortening amplitude, lessened maximal velocity of shortening and relengthening, and prolonged duration of shortening and relengthening in female murine cardiomyocytes compared with their male counterparts. These myocardial divergences suggest the presence of an intrinsic cardiomyocyte contractile difference independent of neurohormonal regulation between sexes, which may possibly contribute to the “female advantage” in cardiac morbidity and mortality. Interestingly, the female gender-elicted influence on cardiomyocyte contractile function is somewhere reminiscent of that triggered by STZ-induced diabetes, which may explain the “lessened STZ insult” to female cardiomyocytes. Several mechanisms may contribute to the sex difference under both the control and diabetic conditions. Diabetes is associated with a cardiac myosin heavy chain isozyme switch from the fast type V1 (α-α) to the slow or diseased type V3 (β-β) (6). Coincidentally, male hearts display significantly higher levels of the β-isoform of myosin heavy chain compared with their female counterparts (43), thus making male hearts work at low gear. This “low gear” state may predispose the male hearts more susceptible to diabetic insults. We did not observe any reduction in electrically stimulated changes of intracellular Ca$^{2+}$ transients (ΔF/F) in diabetic cardiomyocytes of either sex (ΔF/F was enhanced in female diabetic myocytes). Such data, in conjunction with reduced peak myocyte shortening in male diabetic myocytes, indicates a likely reduction in myofilament Ca$^{2+}$ sensitivity in diabetes as reported previously (14). The scenario of impaired myofilament Ca$^{2+}$ sensitivity is also supported by normal frequency response of peak cell shortening in diabetic cardiomyocytes found in our study. The equally dampened SR Ca$^{2+}$ release along with unaltered ΔF/F in diabetic groups of both sexes indicates involvement of certain voltage-independent mechanism(s) in SR Ca$^{2+}$ release under the diabetic condition. Another potential mechanism that may contribute to the sex difference in intrinsic cardiomyocyte contractile function is the relatively higher glutathione antioxidant capacity in female hearts. This is supported by the observation that elevated antioxidant capacity with cardiac metallothionein overexpression not only increased glutathione capacity in males, therefore nullifying the sex difference in glutathione capacity, but also cancelled the sex difference in cardiomyocyte contractile function. Finally, our study depicted elevated cardiac protein carbonyl formation after STZ treatment in both sexes although there was no sex difference in basal cardiac protein carbonyl levels. These data favored a significant role of cardiac protein damage in the onset of diabetic heart dysfunction but not the sex difference in cardiomyocyte contractile function.

Data from our study revealed that cardiomyocytes from STZ-treated diabetic male mice exhibited depressed peak shortening, reduced maximal velocity of shortening and relengthening, and prolonged duration of relaxation associated with normal duration of contraction. These alterations are characteristic of diabetic cardiomyopathy and are consistent with our earlier findings using both genetically predisposed and chemically induced diabetic models (7, 22, 24, 29, 41, 42). The impaired intracellular Ca$^{2+}$ handling shown as reduced intracellular Ca$^{2+}$ clearance rate and reduced SR Ca$^{2+}$ release in diabetic FVB mouse myocytes are most likely responsible for prolonged relaxation (TR$_{90}$) and reduced peak shortening in these cells. Several machineries responsible for cytosolic Ca$^{2+}$ extrusion [such as sarco(end)plasmic reticulum Ca$^{2+}$-ATPase and Na$^+$/Ca$^{2+}$ exchanger] are known to be defective in diabetics and may play an essential role in the mechanical defects in diabetes (7, 23, 29–31, 40). Both mechanical and intracellular Ca$^{2+}$ defects triggered by diabetes were alleviated by metallothionein in both sexes, in a manner similar to its effect found in the genetically predisposed diabetes (41) and diet-induced prediabetic insulin resistance (8). In addition to its beneficial effects on myocyte shortening and intracellular Ca$^{2+}$ transients, metallothionein transgene elicited a rightward shift in the threshold of frequency-related depression in peak shortening (from <0.5 Hz in FVB group to >0.5 Hz in metallothionein group) regardless of the diabetic state (Fig. 3B). This finding indicated that intracellular Ca$^{2+}$ resequestration may not be affected by sex or diabetes; however, antioxidant metallothionein may enhance the intracellular Ca$^{2+}$ recycling ability. Metallothionein protection of cardiomyocyte function in genetic Type 1 diabetes and prediabetic insulin resistance was shown to be related to its antioxidant action (8, 16, 41). It appears that reduction in cardiac oxidative stress and protein damage was responsible for metallothionein-elicted protection against STZ-induced diabetic cardiomyocyte contractile dysfunction, supported by our observation that metallothionein alleviated diabetes-induced reduction in GSH/GSSG ratio and an increase in protein carbonyl formation. Although oxidative stress has been postulated to play a central role in the pathogenesis of diabetic cardiomyopathy including disarray in glucose metabolism and function of sarco(end)plasmic reticulum Ca$^{2+}$-ATPase and Na$^+$/Ca$^{2+}$ exchanger (3, 40), the role of oxidative stress or redox status in the sex difference of myocardial function and reduced susceptibility in female hearts to diabetic insult remains virtually unknown. Finally, it is worth mentioning that cardiac hypertrophy was observed in the 8- to 10-wk-old metallothionein transgenic mice in our study. Because cardiac hypertrophy was not seen in the same transgenic mice at 16 wk of age or older (8, 41), it is possible that cardiac hypertrophy observed in our 8- to 10-wk-old metallothionein mice may reflect a transient physiological hypertrophic response due to Akt activation (Fig. 5C). Short-term Akt activation has been shown to trigger physiological cardiac hypertrophy (21). The hypertrophied metallothionein hearts may contribute to the subtle but significant difference in intrinsic cardiomyocyte function (±dL/dt in females and TR$_{90}$ in males).
Akt is essential for cardiac morphology, contractile function, and cell survival (15, 21, 33). Short-term activation of Akt promotes physiological cardiac hypertrophy whereas long-term Akt activation triggers maladaptive cardiac hypertrophic response and angiogenesis en route to heart failure (18, 21, 33). Dampered Akt activation has been shown in diabetes (13, 25), suggesting a crucial role of Akt activation in the onset of diabetic heart dysfunction. More interestingly, sex differences in Akt activation exist, with female myocardium exhibiting a higher level of Akt phosphorylation (Ser473) (4). This is consistent with the fact that estrogen and phytoestrogen may both stimulate Akt phosphorylation at serine 473 in cardiac myocytes (4). Therefore, the hyperactivation of Akt in female myocardium may “prime” the chronic Akt activation-induced maladaptive cardiac morphological and functional response. In our present study, Akt hyperphosphorylation in female hearts may directly antagonize diabetes-induced downregulation of Akt phosphorylation. Our data also revealed that phosphorylation of the transcription factor c-Jun may be involved in altered cardiomyocyte contractile dysfunction in diabetes but is less likely responsible for the sex difference in cardiomyocyte function. Phosphorylation of c-Jun was reduced by metallothionein in male but not female myocardium. Although a sex difference in myocardial c-Jun phosphorylation has not been established at this point, the ovarian hormone estrogen was reported to directly participate in the regulation of transcription factor expression including c-Jun (10). Nevertheless, whether hyperactivated Akt or hypophosphorylated c-Jun in female hearts plays any direct role in the sex difference in both intrinsic and diabetic myocardial contractile function is unknown and deserves further study.

Our present study indicated that the antioxidant metallothionein may help to nullify the sex difference in myocardial glutathione capacity and cardiomyocyte contractile properties under both normal and diabetic conditions. Our data further revealed that phosphorylation of Akt and c-Jun may be involved in sex and diabetes-associated discrepancies in cardiomyocyte contractile function. Although the sex- and diabetes-associated differences in Akt and c-Jun phosphorylation may be interrupted in metallothionein transgenic mice, it is still pertinent to delineate the precise cardiac stress and survival signaling mechanisms in diabetes to understand the role of antioxidant defense and oxidative stress in sex and diabetes-associated alteration in cardiac dynamics.

ACKNOWLEDGMENTS

The authors acknowledge Cindy X. Fang, Bonnie H. Ren, Jesse Zhang, and Dong An for assistance in Western blot analysis and glutathione assay.

GRANTS

This work was supported in part by the American Heart Association Pacific Mountain Affiliate (no. 0355521Z) to J. Ren.

REFERENCES


