Impaired cardiac function and IGF-I response in myocytes from calmodulin-diabetic mice: role of Akt and RhoA

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Duan, Jinhong, Hai-Ying Zhang, Steven D. Adkins, Bonnie H. Ren, Faye L. Norby, Xiaochun Zhang, Joseph N. Benoit, Paul N. Epstein, and Jun Ren. Impaired cardiac function and IGF-I response in myocytes from calmodulin-diabetic mice: role of Akt and RhoA. Am J Physiol Endocrinol Metab 284: E366–E376, 2003; 10.1152/ajpendo.00254.2002.—This study characterized the cardiac contractile function and IGF-I response in a transgenic diabetic mouse model. Mechanical properties were evaluated in cardiac myocytes from OVE26 diabetic and FVB wild-type mice, including peak shortening (PS), time to PS (TPS), time to 90% relengthening (TR90), and maximal velocity of shortening/relengthening (±dL/dt). Instracellular Ca2+ was evaluated as Ca2+-induced Ca2+ release [difference in fura 2 fluorescent intensity (ΔFFI)] and fluorescence decay rate (τ). Sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA2a), phospholamban (PLB), Na+–Ca2+ exchanger (NCX), GLUT4, and the serine-threonine kinase Akt were assessed by Western blot. RhoA and IGF-I/IGF-I receptor mRNA levels were determined by RT-PCR and Northern blot. OVE26 myocytes displayed decreased PS, ±dL/dt, and ΔFFI associated with prolonged TPS, TR90, and τ. SERCA2a, NCX, and Akt activation were reduced, whereas PLB and RhoA were enhanced in OVE26 hearts. GLUT4 was unchanged. IGF-I enhanced PS and ΔFFI in FVB but not OVE26 myocytes. IGF-I mRNA was increased, but IGF-I receptor mRNA was reduced in OVE26 hearts and livers. These results validate diabetic cardiomyopathy in OVE26 mice due to reduced SERCA2a, NCX, IGF-I response, and Akt activation associated with enhanced RhoA level, suggesting a therapeutic potential for Akt and RhoA.

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Cardiomyopathy, is believed to be mainly responsible for the enhanced cardiac death seen in diabetes (19). The most prominent defects of diabetic cardiomyopathy include depressed ventricular contraction, prolonged duration of contraction and relaxation, and reduced compliance (19, 31, 35, 36). The etiology of diabetic cardiomyopathy is complex and may involve metabolic derangements, decreased autonomic function, and abnormalities in various hormones or proteins that regulate intracellular ion homeostasis, particularly Ca2+ (19, 31, 34). Recent investigations have focused on the role of altered insulin sensitivity and glucose metabolism. Our laboratory (38, 39) has shown that elevated glucose may contribute directly to the development of cardiac mechanical dysfunction in diabetes, which can be protected by insulin-sensitizing agents such as thiazolidinediones and metformin. Other evidence suggests that diabetic cardiomyopathy is associated with elevated tyrosine kinase phosphorylation (37, 48). These observations suggest a link(s) between glucose metabolism, insulin signaling, and the development of diabetic cardiomyopathy.

Insulin-like growth factor I (IGF-I) is very similar to insulin in structure and cellular action and can be used as a replacement for insulin to control blood glucose in diabetes, especially under insulin-resistant conditions. IGF-I promotes cardiac growth and myocardial contraction, improves hemodynamics and energy metabolism, and protects the heart against apoptosis induced by ischemia or oxidative stress (33, 41, 47). These cardiac functions of IGF-I are believed to be mediated through phoshatidylinositol (PI) 3-kinase activation, which produces phosphoinositides promoting subsequent activation of the serine/threonine kinase Akt (11, 33). Activated Akt plays a central role in antiapoptosis by modulating the Bcl-2 family of proteins, caspase 9, and Fas ligand (6, 12), further validating the significant role of IGF-I in preserving cardiac function. However, resistance to IGF-I-induced cardiac response has been shown in both chemically induced (43) and diabetic mouse; ventricular myocyte; excitation-contraction coupling; insulin-like growth factor I; sarco(endo)plasmic reticulum Ca2+-ATPase; sodium-calcium exchanger

Ample evidence indicates high cardiovascular morbidity and mortality associated with diabetic populations largely due to diabetes-related macro/microvascular complications, which often lead to structural, metabolic, and functional damage in the heart (20, 39, 41). A specific form of cardiac muscle disease, namely diabetic cardiomyopathy, is believed to be mainly responsible for the enhanced cardiac death seen in diabetes (19). The most prominent defects of diabetic cardiomyopathy include depressed ventricular contraction, prolonged duration of contraction and relaxation, and reduced compliance (19, 31, 35, 36). The etiology of diabetic cardiomyopathy is complex and may involve metabolic derangements, decreased autonomic function, and abnormalities in various hormones or proteins that regulate intracellular ion homeostasis, particularly Ca2+ (19, 31, 44). Recent investigations have focused on the role of altered insulin sensitivity and glucose metabolism. Our laboratory (38, 39) has shown that elevated glucose may contribute directly to the development of cardiac mechanical dysfunction in diabetes, which can be protected by insulin-sensitizing agents such as thiazolidinediones and metformin. Other evidence suggests that diabetic cardiomyopathy is associated with elevated tyrosine kinase phosphorylation (37, 48). These observations suggest a link(s) between glucose metabolism, insulin signaling, and the development of diabetic cardiomyopathy.
genetically predisposed diabetes. Recent evidence also revealed that the GTP-binding protein RhoA, a small ras-related G protein, may be inactivated by insulin and is involved in the regulation of cardiac excitation-contraction coupling, hypertrophy, and apoptosis. Nevertheless, the role of Akt and RhoA in the pathogenesis of diabetic cardiomyopathy and whether there is any correlation with the cardiac IGF-I resistance under diabetes remain unknown.

Most of the studies regarding diabetic cardiomyopathy to date have been focused on the rat models of diabetes. Although rats provide a good model for experimental diabetes, they may not be readily used to evaluate the role of a given gene or its product in the pathogenesis and therapeutics of diabetic complications. On the other hand, although transgenic techniques have been widely used to manipulate diabetes-related genes, characterization of diabetic cardiomyopathy in diabetic mice, especially at the isolated myocyte level, has not been elucidated. The aim of the present investigation was to examine cardiac contractile function in a transgenic diabetic mouse line, the OVE26 mouse with an FVB background, and to determine the levels of crucial cardiac Ca\textsuperscript{2+}-regulating and membrane glucose-transporting proteins [sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA), phospholamban (PLB), Na\textsuperscript{-}Ca\textsuperscript{2+} exchanger (NCX), and glucose transporter 4 (GLUT4)]. Because diabetes is often associated with cardiac IGF-I resistance (34, 43), the cardiac response to IGF-I was also examined in this transgenic line. We developed the OVE26 diabetic transgenic line, and it has been proved to be an excellent model for complications of type 1 diabetes. Overexpression of the Ca\textsuperscript{2+}-binding protein calmodulin in pancreatic β-cells produces diabetes within the 1st wk of life due to pancreatic β-cell damage in these transgenic mice. Although the exact mechanism of β-cell toxicity has not been established, the specificity of damage to the β-cells makes this an extremely useful model of diabetes complications with severe hyperglycemia and hyperlipidemia. They also have a surprisingly long life span, typically 1–2 yr without insulin therapy. In addition, this diabetic model does not have potential extraneous effects on target organs (such as toxin’s direct action on heart), which may produce unwanted artifacts. OVE26 diabetic mice exhibited cardiomyopathy characterized by significantly altered mRNA expression, clear morphological abnormalities, and reduced contractility under ischemic/reperfusion conditions. Our ultimate goal is to establish the OVE26 diabetic line as a tool to evaluate other genes of interest using transgenic techniques.

MATERIALS AND METHODS

Animals. The experiments described here were approved by the Institutional Animal Care and Use Committee of University of North Dakota (Grand Forks, ND). All animal procedures were in accordance with National Institutes of Health animal care standards. Eight- to ten-week-old male OVE26 transgenic diabetic and age-matched wild-type (FVB) mice were housed individually and allowed free access to standard laboratory chow and tap water. Blood glucose levels were measured weekly with a glucose monitor (Accu-ChekII, model 792; Boehringer Mannheim Diagnostics, Indianapolis, IN).

Cell isolation procedures. Hearts were rapidly removed from anesthetized mice and immediately mounted on a temperature-controlled (37°C) perfusion system. After perfusion with modified Tyrode solution (Ca\textsuperscript{2+} free) for 2 min, the heart was digested for 10 min with 0.9 mg/ml collagenase D (Boehringer Mannheim Biochemicals) in modified Tyrode solution. The modified Tyrode solution (pH 7.4) contained the following (in mM): 135 NaCl, 4.0 KCl, 1.0 MgCl\textsubscript{2}, 0.33 NaH\textsubscript{2}PO\textsubscript{4}, 10 glucose, and 10 butanedione monoxime, and the solution was gassed with 5% CO\textsubscript{2}-95% O\textsubscript{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 (isolation medium) was resuspended and allowed to settle for another 20 min at room temperature, during which time extracellular Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} transients, as previously described (17).

Cell shortening/relengthening. Mechanical properties of ventricular myocytes were assessed using a SoftEdge MyoCam system (IonOptix, Milton, MA) (17). In brief, cells were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus, IX-70) and superfused (~1 ml/min at 25°C) with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 glucose, and 10 HEPES, pH 7.4. The cells were field stimulated with suprathreshold voltage, at a frequency of 0.5 Hz and 3 ms duration, by use of a pair of platinum wires placed on opposite sides of the chamber connected to an FHC stimulator (Brunswick, NE). The myocyte being studied was displayed on the computer monitor by means of an IonOptix MyoCam camera. SoftEdge software (IonOptix) was used to capture changes in cell length during shortening and relengthening.

Intracellular fluorescence measurement. 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) as described (17). 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 (bandwidth ±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 the cells were first illuminated 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 Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) were inferred from the ratio of the fluorescence intensity at two wavelengths.

Western analysis of SERCA2a, PLB, NCX, GLUT4, and Akt activation. Membrane proteins from left ventricular myocardium (for SERCA2a, PLB, and NCX measurement) or ventricular myocytes (for basal GLUT4 and Akt measurement) after a 15-min incubation at 37°C with either IGF-I or insulin, both at 10−6 M) were isolated as described (50).
Freshly dissected hearts or ventricular myocytes were homogenized and centrifuged at 1,000 g for 10 min. The supernatants were then centrifuged at 70,000 g for 30 min at 4°C. The 100,000-g pellets were cellular membrane fractions and were used for immunoblotting of SERCA2a, PLB, NCX, GLUT4, and Akt (both total and phosphorylated (p-Akt)). We confirmed that these membrane fractions did not contain any detectable collagens. Membrane proteins (50 μg/lane) were separated on 7% (SERCA2a, NCX, and Akt) or 15% (PLB and GLUT4) SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad) and transferred to polyvinylidene difluoride membranes. The membranes were blocked (4% Block Ace; Dainippon Pharmaceutical, Osaka, Japan) and then incubated with anti-SERCA2a (A7R5) and PLB (2D12 monoclonal antibodies to SERCA2a, NCX, and Akt) or anti-PLB (1:1,000), and anti-GLUT4 (1:4,000) antibodies (4% Block Ace; Dainippon Pharmaceutical, Osaka, Japan) with peroxidase-linked anti-mouse (Amersham) or anti-sheep (Akt) IgG (1:5,000 dilution). After immunoblotting, the membranes were blocked (4% Block Ace; Dainippon Pharmaceutical, Osaka, Japan) and then incubated with anti-SERCA2a (1:1,000 dilution), anti-PLB (1:1,000), and anti-GLUT4 (1:4,000) antibodies (4% Block Ace; Dainippon Pharmaceutical, Osaka, Japan) with peroxidase-linked anti-mouse (Amersham) or anti-sheep (Akt) IgG (1:5,000 dilution). After immunoblotting, the film was scanned, and the intensity of immunoblot bands was detected with a Bio-Rad calibrated densitometer (model GS-800).

Measurement of IGF-I mRNA and IGF-I receptor mRNA. To quantify IGF-I and IGF-I receptor mRNA levels, total RNA was extracted from ventricles by use of the guanidinium thiocyanate method (8). The RNA was visualized using ultraviolet light and ethidium bromide staining to determine RNA integrity and the equal loading of lanes. Differences in loading were normalized using the fluorescence intensity of the 18S band as measured by the gel documentation system (LumiAnalyst, Boehringer Mannheim). The RNA was transferred from the gel to a nylon membrane via capillary action and dried for 2 h at 80°C. Membranes were hybridized with a 376-bp, 32P-labeled antisense riboprobe generated from the IGF-I or IGF-I receptor 3’ cDNA in the pGEM3 vector, kindly provided by Dr. Derek LeRoith at the National Institute of Diabetes and Digestive and Kidney Diseases. Membranes were hybridized overnight at 65°C and then washed at 65°C. Membranes were exposed to film to visualize IGF-I or IGF-I receptor mRNA levels, and band intensities were compared using densitometric measurement to determine relative IGF-I and IGF-I receptor mRNA levels.

Measurement of RhoA mRNA with RT-PCR. Total RNA was isolated from ventricular myocytes by use of TRIzol reagent purchased from Invitrogen (Carlsbad, CA). First-strand cDNA was synthesized using oligo(dT)12-18 primer and each type of total RNA (3 μg) as the template. Reverse transcription was performed according to the instructions of SuperScript First-Strand Synthesis System for RT-PCR from Invitrogen. The thermal cycle profile used in this study was denaturing for 60 s at 92°C, annealing for 60 s at 56°C, extending for 30 s at 72°C, and 26 cycles of amplification. The following primer designs were used as previously reported (30): RhoA forward primer, 5’-ACC AGT TCC CAG AGG TTG ATG T-3’; RhoA reverse primer, 5’-TTT GGT CCT TGC TGA ACC ACA ATG-3’; GAPDH forward primer, GTC TCC TAC AGG ATT GTC AGC AA-3’; GAPDH reverse primer, 5’-AGA TCC ACA AGC GAT ACA TT-3’. The expected sizes of the PCR products for RhoA and GAPDH were 410 and 308 bp, respectively. PCRs for RhoA and GAPDH were performed from the same first-strand cDNA to allow the data to be expressed as a ratio to GAPDH. PCR products were confirmed by sequencing and run on a 2% agarose gel. The gel was stained with ethidium bromide and photographed. By use of UN-SCAN-IT gel quantitation software, the data are expressed as the ratio of RhoA to GAPDH.

Statistical analyses. For each experimental series, data are presented as means ± SE. Statistical significance (P < 0.05) for each variable was estimated by analysis of variance (ANOVA, for data shown in Fig. 4) or f-test, where appropriate (SYSTAT, Evanston, IL).

RESULTS

General features of FVB and OVE26 diabetic animals. The OVE26 diabetic mice exhibited significantly lower body weights and higher plasma glucose levels compared with the age-matched nondiabetic FVB mice. Diabetic mice showed enlarged kidney as well as greater proportional liver and kidney size when normalized to body weight (Table 1).

Cell shortening and relengthening properties of myocytes from FVB and OVE26 mice. The average resting cell length (CL) of ventricular myocytes used in this study was 151 ± 7 μm in FVB and 147 ± 5 μm in OVE26 diabetic groups (n = 124 and 122 cells/group, respectively, P > 0.05). Similar to our earlier observations in chemically induced and genetically predisposed diabetes (35, 36), the peak shortening amplitude (PS) normalized to CL was significantly reduced in myocytes isolated from OVE26 diabetic mice. Myocytes from OVE26 mice also exhibited significantly prolonged time to PS (TPS) and time to 90% relengthening (TR90), associated with significantly reduced maximal velocities of shortening (+dL/dt) and relengthening (−dL/dt; Fig. 1).

Intracellular Ca2+ transient properties in FVB and OVE26 myocytes. We used the membrane-permeant form of fura 2 to evaluate the properties of intracellular Ca2+ transients in myocytes from FVB and OVE26 mice. The time course of the fluorescence signal decay was well described by a single exponential equation, and the time constant (τ) was used as a measure of the rate of decline of free cytoplasmic Ca2+. The fluorescence measurements revealed normal resting fura 2 fluorescent intensity (FFI), decreased Ca2+-induced Ca2+ release (AFFI = peak − resting), and slowed intracellular Ca2+ transient decay rate in myocytes from OVE26 mice compared with those from the FVB group (Fig. 2). These results reveal potential abnormal-

Table 1. General features of FVB and OVE 26 diabetic mice

<table>
<thead>
<tr>
<th>Mouse Group</th>
<th>FVB (n = 53)</th>
<th>OVE26 (n = 55)</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>24.27 ± 0.73</td>
<td>22.06 ± 0.49*</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>1.216 ± 0.037</td>
<td>1.278 ± 0.039*</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>0.397 ± 0.016</td>
<td>0.463 ± 0.018*</td>
</tr>
<tr>
<td>Kidney/body weight, mg/g</td>
<td>16.31 ± 0.36</td>
<td>20.96 ± 0.64*</td>
</tr>
<tr>
<td>Plasma glucose, mg/dl</td>
<td>148.8 ± 7.4</td>
<td>435.4 ± 9.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. *P < 0.05 vs. FVB group.
ities in cytoplasmic Ca\(^{2+}\) handling and clearing mechanisms in OVE26 diabetic mouse hearts. The traces in Fig. 2 were chosen to illustrate that diabetes depressed stimulation-induced increase in intracellular Ca\(^{2+}\) and prolonged \(\tau\). Myocyte shortening was also recorded from fura 2-loaded cells, but the data were used for qualitative comparisons only, to avoid potential effects on contraction from intracellular Ca\(^{2+}\) buffering by fura 2.

Western blotting of SERCA2a, PLB, NCX, GLUT4, and Akt activation. The major mechanical defects representative of diabetic cardiomyopathy are depressed ventricular contractility and prolonged duration of relaxation (20, 34–36). It has been reported that these diabetes-associated defects may be due to alteration of key regulating proteins for intracellular Ca\(^{2+}\) homeostasis (SERCA, NCX, and PLB) as well as membrane glucose transport protein GLUT4 (14, 22, 25). To examine the role of these proteins in the development of diabetic mechanical dysfunctions, the protein levels of SERCA2a, PLB, NCX, and GLUT4 (basal or IGF-I/insulin stimulated) from hearts of FVB and OVE26 mice were measured and are shown in Fig. 3. SERCA2a and NCX protein levels were significantly reduced, whereas PLB protein levels were significantly elevated in OVE26 diabetic mouse hearts compared with the FVB group. The basal GLUT4 protein levels were not different between the two groups and could not be stimulated by a 15-min incubation with IGF-I or insulin (10\(^{-6}\) M). The observations of reduced SERCA2a and NCX associated with enhanced PLB (reduced SERCA-to-PLB ratio) are consistent with the functional data of depressed ventricular contractility, slowed intracellular Ca\(^{2+}\) removal, and prolonged duration of relengthening (TR\(_{90}\)) (25). Our further immunostaining analysis revealed that, although total Akt protein levels were not different between the two groups and could not be stimulated by a 15-min incubation with IGF-I or insulin (10\(^{-6}\) M), the amount of activated (or phosphorylated) Akt, presented as p-Akt-to-total Akt ratio (p-Akt/Akt), was significantly reduced in the
OVE26 diabetic hearts. Meanwhile, IGF-I (10^{-6} M) stimulated Akt phosphorylation (shown as enhanced p-Akt/Akt) in ventricular myocytes from FVB but not OVE26 mouse hearts. A similar concentration of insulin, however, failed to stimulate Akt phosphorylation with a 15-min incubation. Neither IGF-I nor insulin significantly affected the total Akt level in myocytes from the FVB or OVE26 group (Fig. 4). These observations indicated reduced basal and IGF-I-activated Akt in the diabetic hearts.

RT-PCR measurement of RhoA mRNA levels. As mentioned in the previous section, the hallmark of diabetic cardiomyopathy is the depressed ventricular contractility and prolonged duration of relaxation (35, 36). Paradoxically, cardiac-specific overexpression of RhoA has been shown to result in prolonged action potential and diminished ventricular contractility (46). To examine the potential role of RhoA in the development of diabetic mechanical dysfunctions, the RhoA mRNA levels from ventricular myocytes of FVB and OVE26 mice were measured with RT-PCR and are shown in Fig. 5. The RhoA mRNA level (normalized to GAPDH mRNA level) was significantly elevated in the OVE26 diabetic group compared with the FVB group. The housekeeping gene GAPDH mRNA levels were similar in both groups.

Effect of IGF-I on myocyte PS and intracellular Ca^{2+} transients. To evaluate the role of IGF-I on the mechanical and intracellular Ca^{2+} transient properties in myocytes isolated from FVB and OVE26 mouse hearts, myocytes were exposed to IGF-I acutely (5 min), and mechanical as well as intracellular Ca^{2+} properties were studied. Figure 6A shows that IGF-I (10^{-10}-10^{-6} M) caused a concentration-dependent increase in PS, with the threshold being between 10^{-9} and 10^{-8} M. The effect of IGF-I on cell shortening reached maximum at 5 min of exposure and was partially reversible upon washout. Similar to our earlier report on diabetic rats (34, 43), IGF-I failed to exert any response in myocytes isolated from OVE26 diabetic mice. IGF-I did not affect TPS and TR_{90} over the concentration range tested (data not shown). To determine whether the differential response of IGF-I in FVB and OVE26 mouse myocytes was due to changes in [Ca^{2+}]_i, we used the fluorescent dye fura 2 to estimate [Ca^{2+}]_i in the myocytes from both groups. IGF-I caused a concentration-dependent increase in Ca^{2+}-induced Ca^{2+} release (ΔFFI) in myocytes from FVB but not in those from OVE26 mice (Fig. 6B). The effect of IGF-I on ΔFFI achieved steady state at or before 5 min, and cells recovered partially following washout. Neither resting FFI nor τ was affected by IGF-I in the concentration range tested (data not shown).

Expression of IGF-I and IGF-I receptor mRNA in liver and heart. To examine the potential mechanism of action involved in the disparate IGF-I response between the FVB and OVE26 groups, the heart and liver (the major source of IGF-I production) mRNA levels of IGF-I and its receptor were measured. Figure 7 reveals that the IGF-I mRNA levels were significantly elevated in both heart and liver from the OVE26 group compared with the FVB group. Conversely, the IGF-I re-
ceptor mRNA levels were markedly reduced in the heart and liver from OVE26 diabetic mice. The reduced level of cardiac IGF-I receptor mRNA was consistent with the attenuated mechanical response to the hormone observed earlier.

**DISCUSSION**

Our present study using transgenic diabetic mice confirmed results from previous studies in diabetic rat models regarding the existence and phenotype of diabetic cardiomyopathy and its causal relationship to cardiac IGF-I resistance. In addition, we found that the diabetic mechanical dysfunction is correlated with reduced basal and IGF-I-stimulated Akt levels as well as enhanced RhoA mRNA in diabetic hearts. The OVE26 transgenic diabetic model was first described in 1989 (18). These mice develop diabetes within the 1st wk of life due to β-cell damage as a result of overexpression of the Ca²⁺-binding protein calmodulin in pancreatic β-cells. The transgenic line shows a high consistency in the onset and progression of type 1 diabetes with a long life span of up to 2 yr without the need for insulin (26). They can easily be cross-bred with other transgenic mice to evaluate the effect of gene modifications on the development of diabetes and its complications (26).

This diabetic rodent model should offer unique advantages over the chemically induced (such as with streptozotocin, which may possess direct toxic effects on the heart) and genetically predisposed biobreeding rats (which require intensive insulin therapy). Results from the present study reveal that these diabetic mice, like most other models of type 1 diabetes, exhibit typical cardiac contractile dysfunctions associated with altered cardiac IGF-I response. Collectively, these results suggest that OVE26 diabetic mice display overt diabetic cardiomyopathy, possibly due to disrupted Ca²⁺-regulating proteins (SERCA2, PLB, and NCX), reduced Akt levels (both basal and IGF-I stimulated), enhanced RhoA mRNA levels, and cardiac IGF-I resistance shown as blunted IGF-I response and diminished IGF-I receptor levels.

The hallmark of diabetes-induced cardiac dysfunctions is abnormal contraction and relaxation. Characteristics of abnormal function include prolonged duration and reduced rate of contraction and relaxation, as measured in whole hearts (5, 13), isolated papillary muscle (20, 43), and isolated ventricular myocytes under chemically induced and genetically predisposed diabetes (35, 36). Similar to our earlier observations, the present study demonstrated prolonged TPS and
TR90 associated with reduced PS and slowed maximal rate of $dL/dt$ in OVE26 diabetic mice. These mechanical dysfunctions are consistent with our findings of impaired intracellular Ca$^{2+}$ homeostasis shown as reduced intracellular Ca$^{2+}$ clearing and Ca$^{2+}$-induced Ca$^{2+}$ release in diabetic mouse ventricular myocytes. It is worth pointing out that the mechanical as well as intracellular Ca$^{2+}$ defects observed in OVE26 mice are identical to the ones seen in diabetic rat models (35, 36). Several mechanisms have been postulated for these mechanical and intracellular Ca$^{2+}$ defects. It has been reported that the depressed rate of shortening may be associated with diabetes-induced shifts in contractile protein isoforms, such as the shift of myosin isozymes from the fast type (V$_1$) to the slow type (V$_3$) (16, 20). Diabetes may also significantly reduce myofilament Ca$^{2+}$ sensitivity (21, 34). Finally, the prolonged Ca$^{2+}$ transient decay and duration of relaxation may simply be a consequence of impaired SERCA and NCX function (7, 16, 45). This is supported by the reduced SERCA2a and NCX protein levels as well as enhanced PLB levels in OVE26 mouse hearts observed in our study. PLB, expressed mainly in the sarcoplasmic reticulum of cardiac muscle, is closely associated with SERCA2a, whereas in the dephosphorylated form it acts as the main inhibitor of SERCA pump function. Phosphorylation of PLB by cAMP-dependent protein kinase or Ca$^{2+}$-calmodulin-dependent protein kinase II may relieve this inhibition (25). Elevated PLB levels accompanying reduced SERCA2a levels have been reported in diabetic hearts (22). An increase in the PLB-to-SERCA2 ratio (PLB/SERCA2; as in our case) reduces the SERCA Ca$^{2+}$ affinity and activity, leading to prolonged relaxation and reduced contractility. On the other hand, a decreased PLB/SERCA2 improves the cardiac Ca$^{2+}$ cycling and pumping functions (25, 28, 31). Our results clearly show an enhanced PLB/SERCA2 in OVE26 mouse hearts, consistent with the prolonged TR$_{90}$ and reduced PS. The importance of the ratio of PLB to SERCA2 on the regulation of myocardial contractility has been demonstrated in mouse models with variable expression levels of PLB (25). The indifferent GLUT4 levels (either basal or IGF-I/insulin stimulated) between OVE26 and FVB groups suggest that impaired glucose transport is unlikely to be a major factor in the mechanical defects in OVE26 mouse hearts.

Fig. 4. A: representative gels depicting immunostaining using anti-Akt and anti-phosphorylated (p)-Akt antibodies. Western blot shows total nonphosphorylated Akt (B) and the ratio between p-Akt and total Akt (C) under basal condition or after a 15-min incubation (at 37°C) with either IGF-I ($10^{-6}$ M) or insulin ($10^{-6}$ M) in ventricular myocytes from FVB and OVE26 diabetic mouse hearts. Each bar represents the ratio of p-Akt to Akt. Data are means ± SE; n = 5–9 animals. *P < 0.05 vs. basal level; #P < 0.05 vs. FVB group.

Fig. 5. RhoA mRNA expression in ventricular myocytes from FVB and OVE26 diabetic mice. Data are means ± SE; n = 4–5 animals. *P < 0.05 vs. FVB group.
cardiac myocytes. However, caution should be taken when interpreting these results, since we measured only total GLUT4 instead of the translocation of GLUT4, which has been shown to be impaired in diabetic rat hearts (14). Last but not least, activation of Akt and its upstream signal phosphatidylinositol (PI) 3-kinase may dramatically improve cardiac function and protect against apoptosis (9, 11, 24, 34, 47), establishing an important role for Akt in the maintenance of heart morphology and function. Impairment of basal and IGF-I-stimulated Akt activation, as seen in the present study, may be directly responsible for the development of diabetic cardiac mechanical dysfunctions. Although the potential mechanism of action responsible for reduced Akt activation is still unclear, diminished “drive” for Akt activation from certain cardioprotective agents such as IGF-I (as shown in our study), insulin, and gp130-dependent cytokines may play a role. Remarkably, recent data from our group revealed that elevated extracellular glucose (from 5.5 to 25.5 mM) for 12–18 h significantly attenuated IGF-I-stimulated Akt activation in parallel to a diminished cardiac contractile response to IGF-I in isolated rat ventricular myocytes (39a). It is somewhat surprising that insulin failed to stimulate Akt phosphorylation in our study, although the cell type and duration of insulin treatment may be speculated to play a role. Further studies are warranted to elucidate the precise role of Akt activation, especially under diabetic conditions, in cardiac mechanical function regulation, possibly with the assistance of gene transfer techniques using Akt or dominant negative Akt.

Recent evidence suggests that RhoA, a small ras-related G protein, is involved in the regulation of cardiac excitation-contraction coupling, hypertrophy, and apoptosis (23, 46). RhoA alternates between an inactive and an active GTP-bound form. Activated RhoA subsequently stimulates Rho kinase, which regulates cardiac myocytes. However, caution should be taken when interpreting these results, since we measured only total GLUT4 instead of the translocation of GLUT4, which has been shown to be impaired in diabetic rat hearts (14). Last but not least, activation of Akt and its upstream signal phosphatidylinositol (PI) 3-kinase may dramatically improve cardiac function and protect against apoptosis (9, 11, 24, 34, 47), establishing an important role for Akt in the maintenance of heart morphology and function. Impairment of basal and IGF-I-stimulated Akt activation, as seen in the present study, may be directly responsible for the development of diabetic cardiac mechanical dysfunctions. Although the potential mechanism of action responsible for reduced Akt activation is still unclear, diminished “drive” for Akt activation from certain cardioprotective agents such as IGF-I (as shown in our study), insulin, and gp130-dependent cytokines may play a role. Remarkably, recent data from our group revealed that elevated extracellular glucose (from 5.5 to 25.5 mM) for 12–18 h significantly attenuated IGF-I-stimulated Akt activation in parallel to a diminished cardiac contractile response to IGF-I in isolated rat ventricular myocytes (39a). It is somewhat surprising that insulin failed to stimulate Akt phosphorylation in our study, although the cell type and duration of insulin treatment may be speculated to play a role. Further studies are warranted to elucidate the precise role of Akt activation, especially under diabetic conditions, in cardiac mechanical function regulation, possibly with the assistance of gene transfer techniques using Akt or dominant negative Akt.

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contractility and gene expression, probably mediated through the extracellular signal-regulated protein kinase subfamily of the mitogen-activated protein kinase or PI 3-kinase. The effects of RhoA on cellular architecture may be mediated through Rho-dependent serine/threonine kinases (10). Cardiac-specific overexpression of RhoA results in prolonged action potential and diminished ventricular contractility (46). Transfection of activated RhoA stimulates atrial natriuretic factor expression and myofibrillogenesis (10). Angiotensin II is known to activate RhoA (1). In contrast, insulin, an analog of IGF-I, inactivates RhoA in vascular smooth myocytes (2). An imbalance in action of these peptides, i.e., excessive angiotensin II and diminished insulin or IGF-I action, both seen in diabetes (34, 41), may play a role in the onset of cardiac contractile dysfunction. RhoA has recently been shown to be up-regulated in diabetic basilar artery, suggesting a role of RhoA in the pathogenesis of diabetic vascular complications (29). Because RhoA is implicated in the regulation of the actin-myosin cytoskeleton and cardiac contractility (10, 46), further study is warranted in its role in the development of diabetic cardiomyopathy.

IGF-I facilitates glucose metabolism, lowers insulin levels, increases insulin sensitivity, and improves the lipid profile (33, 41), suggesting both a physiological role and a therapeutic potential. The rapid onset, the long duration of effect, and the relatively modest magnitude of its action compared with other endogenous substances suggest that, if IGF-I has an acute cardioregulatory role, it may contribute to the modulation of the inotropic responsiveness of the myocardium over a time frame of minutes to hours. Altered responsiveness to the action of IGF-I has been shown in diabetes, hypertension, and obesity (34, 40, 42, 43), indicating that IGF-I may increase the propensity of certain cardiac disorders (41). The present study supported this notion by revealing reduced IGF-I cardiac responsiveness and IGF-I receptor mRNA levels in OVE26 diabetic mice. IGF-I has been shown to enhance myocardial contractility through binding to its membrane receptor and may act as an endogenous regulator of myocardial mechanics (34, 40, 43). Our earlier study (34) revealed that the effect of IGF-I (≥10⁻⁶ M) on cardiac contractility may be blocked by H-1356, an IGF-I analog that inhibits the autophosphorylation of the IGF-I receptor. Reduction of IGF-I receptor mRNA or abundance is expected to interfere with the physiological signaling of IGF-I, which is initiated with the binding of IGF-I to its membrane receptor, and IGF-II or insulin receptors at high levels of IGF-I (41). In the present study, IGF-I increased myocyte shortening in myocytes from FVB but not in those from OVE26 mice. This disparate pattern was associated with a somewhat similar pattern of response in intracellular Ca²⁺ transients. However, the difference in IGF-I concentration-response curves between Fig. 6A (cell shortening) and Fig. 6B (intracellular Ca²⁺ transient), especially at 10⁻⁶ M IGF-I, may indicate enhanced or reduced myofilament Ca²⁺ sensitivity in response to IGF-I (9) or diabetes (21, 35), respectively. The reduced cardiac IGF-I response in OVE26 diabetic mice may reflect attenuated or abrogated postreceptor signaling pathways activated by the IGF-I/IGF receptor complex such as PI 3-kinase and Akt. As mentioned, activation of PI 3-kinase and Akt is essential for the transduction of metabolic growth and functional effects of IGF-I and insulin, including stimulation of glucose transport (15, 32, 49), protein and glycogen synthesis (27), inhibition of apoptosis (33, 49), and cardiac contractility (9, 24, 34, 49). Although data from the present study indicated that the reduced IGF-I receptor may contribute to the blunted IGF-I response in diabetes, potential alteration in post-IGF-I-receptor mechanisms en route to reduced Akt activation is definitely worthy of further study.

In summary, our findings characterized, for the first time, the impaired cardiac contractile function and cardiac IGF-I response representative of diabetic cardiomyopathy in OVE26 transgenic diabetic mouse hearts. Although our data suggest the potential role of certain cardiac Ca²⁺-regulating proteins such as SERCA, NCX, PLB, and GLUT4 in the mechanical dysfunction, the precise relationship among cardiac IGF-I resistance, reduced cardiac Akt activation, elevated RhoA mRNA, and impaired mechanical function under diabetes may be critical in designing therapeutic targets for diabetes and diabetic heart complications.

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