Altered SR protein expression associated with contractile dysfunction in diabetic rat hearts

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Zhong, Yan, Saadia Ahmed, Ingrid L. Grupp, and Mohammed A. Matlib. Altered SR protein expression associated with contractile dysfunction in diabetic rat hearts. Am J Physiol Heart Circ Physiol 281: H1137–H1147, 2001.—The goal of this study was to examine whether alteration of sarcoplasmic reticulum (SR) protein levels is associated with early-onset diastolic and late-onset systolic dysfunction in streptozotocin (STZ)-induced diabetic rat hearts. Four-week diabetic rat hearts exhibited slow relaxation, whereas 6-wk diabetic rat hearts exhibited slow and depressed contraction. Total phospholamban level was increased, and phosphorylated level was decreased in 4- and 6-wk diabetic rat hearts. Sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA2) protein level was unchanged in 4-wk but decreased in 6-wk diabetic rat hearts. Only the apparent affinity of SR Ca2+ uptake for Ca2+ was decreased in 4-wk diabetic rat hearts, but the apparent affinity and the maximum rate was decreased in 6-wk diabetic rat hearts. Insulin treatment of the diabetic rats normalized SR protein expression and function. It was concluded that an increase in nonphosphorylated phospholamban and a decrease in the apparent affinity of SR Ca2+ pump for Ca2+ are associated with early-onset diastolic dysfunction and decreases in SERCA2 protein level and apparent affinity and maximum velocity of SR Ca2+ pump are associated with late-onset systolic dysfunction in diabetic rats.

phospholamban; Ca2+-ATPase; ryanodine receptor; calsequestrin; diabetic cardiomyopathy; sarcoplasmic reticulum

HEART FAILURE is the leading cause of death in diabetic patients (29). Cardiomyopathy has been shown to be an important contributing factor of heart failure in diabetic patients independent of atherosclerosis, hypertension, and other complications (26). Cardiomyopathy in diabetic patients is characterized by early diastolic dysfunction, followed by late systolic dysfunction (5). However, the mechanisms underlying the sequential development of cardiac contractile dysfunction and a rational treatment of the disease remain unknown. Cardiac contractile dysfunction has been observed in streptozotocin (STZ)-induced diabetic rats after 6–8 wk of diabetes (5, 7, 24, 31), but the underlying mechanism remains unclear. Furthermore, no study has been conducted in any animal model of diabetes to determine whether the relaxation process is altered at an early stage of diabetes before the contraction process is affected. To examine potential mechanisms underlying the development of cardiomyopathy in diabetes, it is necessary to identify an animal model of diabetes that mimics the sequential development of cardiomyopathy in diabetic patients. Because STZ-induced diabetic rats have been found to develop cardiac contractile dysfunction after a range of duration of diabetes (5, 7, 24, 31), it is logical to examine this animal model to determine whether a slow relaxation develops before slow or depressed contraction. If a sequential development of contractile dysfunction in this animal model of diabetes is established and the underlying mechanisms are understood, the information can be valuable to understand the mechanism of cardiomyopathy in diabetic patients. Therefore, one of the objectives of this study is to examine the hypothesis that cardiac diastolic dysfunction develops before systolic dysfunction in STZ-induced diabetic rats.

Sarcoplasmic reticulum (SR) is one of the critical elements in cardiac contractility. It is the major regulator of cytosolic free Ca2+ concentration ([Ca2+]i) in beat-to-beat contraction and relaxation of cardiac myocytes (18). The ryanodine receptor (RyR)-linked Ca2+ release from sarco(endo)plasmic reticulum contributes about 90% of the free Ca2+ for contraction, and the SR Ca2+-ATPase or Ca2+ pump (SERCA2) sequesters this fraction of Ca2+ during relaxation of rat cardiac myocytes (2). Phospholamban (PLB) is a key regulator of SERCA2 function (12). In its nonphosphorylated form, PLB inhibits SERCA2 function by decreasing its affinity for Ca2+, whereas phosphorylation of PLB enhances SERCA2 function by increasing its affinity for Ca2+. Transgenic ablation of cardiac PLB has been found to increase the affinity of SERCA2 for Ca2+, resulting in a reduced rate of Ca2+ sequestration into SR and increased rates of contraction and relaxation (17). On the other hand, transgenic overexpression of cardiac PLB in mice has been shown to decrease the affinity of SERCA2 for Ca2+, resulting in depression of SERCA2 function and contraction and relaxation (11). The importance of SERCA2-PLB interaction in cardiomyopa-
SR PROTEIN EXPRESSION IN DIABETIC CARDIOMYOPATHY

The objective of this study is to determine whether there is a sequential or a gradual alteration of expression and function of SR proteins associated with sequential development of diastolic and systolic dysfunction in STZ-induced diabetic rats. This concept of cardiac SR dysfunction underlying sequential development of diastolic and systolic dysfunction has not been examined previously in any diabetic or nondiabetic animal model of cardiomyopathy.

The materials and methods section describes the experimental procedures used in the study. The study involved the injection of streptozotocin (STZ) to diabetic rats and the use of various buffers, such as citrate and LIM protein, to achieve specific pH levels. The rats were monitored for blood glucose levels and cardiac function over a period of time.

The results demonstrated that there is a sequential or a gradual alteration of expression and function of SR proteins associated with the development of diastolic and systolic dysfunction in STZ-induced diabetic rats. The study also showed that the diabetic rats had a slower cardiac contraction at the late stage of STZ-induced diabetes in rats. This concept of cardiac SR dysfunction was confirmed through the use of different buffers, such as citrate, to achieve specific pH levels.
Lowry et al. (15), using bovine serum albumin for standard curve.

The homogenates were diluted to 0.1 mg/ml with a medium containing 62.5 mM Tris-HCl buffer (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 2.5% bromophenol blue. Aliquots containing 0.25, 0.5, 0.75, and 1.0 μg of protein for PLB, casein (CSQ), or α-actin and 8, 16, 32, and 64 μg for SERCA2 were loaded on the SDS-polyacrylamide gel and separated at 4°C by electrophoresis in 4% acrylamide stacking gel and 12% acrylamide separating gel (13) initially at 120 V for 15 min and then at 180 V for 30 min. In each gel, identical protein concentration range of control and diabetic rat heart homogenate proteins were loaded on the gel as described above. The separated proteins were transferred electrophoretically from the gel onto nitrocellulose membranes (0.2 μm pore size, Bioread Hercules, CA) at 200 mA for 90 min (30) in a buffer containing 25 mM Tris base, 192 mM glycine, and 20% methanol and by using the Bio-Rad Trans-Blot electrophoretic transfer system (Bio-Rad). The nitrocellulose membranes were washed for 5 min with 100 mM Tris-HCl buffer (pH 7.4) containing 0.9% NaCl solution (TBS) and blocked with 5% Carnation instant milk in TBS for 1 h at room temperature.

The membranes were then washed three times with TBS and were incubated at 4°C overnight with mouse PLB monoclonal antibody (Affinity Bioreagents; Golden, CO) or mouse α-actin monoclonal antibody (Sigma) or rabbit CSQ monoclonal antibody (a gift from Dr. Larry R. Jones, Indiana University School of Medicine, Indianapolis, IN). The primary antibody dilution was 1:1,000 for PLB, 1:2,000 for α-actin, and 1:5,000 for CSQ in 2% Carnation instant milk-TBS. The solution was decanted, the membranes were washed in TBS for 30 min at room temperature with agitation, and the TBS was changed every 10 min. The membranes were then incubated for 5 h with a secondary antibody conjugated to horseradish peroxidase after dilution in 2% Carnation instant milk-TBS. The secondary antibodies and the dilutions were donkey anti-mouse at 1:500 for PLB, goat anti-mouse at 1:5,000 for α-actin, and goat anti-rabbit at 1:5,000 for CSQ. The secondary antibody solution was decanted, and the membranes were washed for 30 min with TBS alone.

For SERCA2 protein, the membranes were washed three times with TBS plus 0.05% Tween 20 and incubated at room temperature for 2 h with a goat polyclonal anti-rat SERCA2 (Santa Cruz Biotechnology; Santa Cruz, CA) as a primary antibody at 1:400 dilution in 2% Carnation instant milk-TBS-Tween 20. The solution was decanted and TBS-Tween 20 solution was added. The membranes were washed for 30 min with agitation, and the TBS-Tween 20 solution was changed every 10 min. The membranes were incubated for 2 h with a secondary anti-goat antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) at a 1:4,000 dilution in 2% Carnation instant milk-TBS-Tween 20. The solution was decanted and washed for 30 min with TBS-Tween 20 solution.

Quantitative immunoblotting of ryanodine receptor. The preparation of cardiac homogenate proteins for RyR immunoblot was the same as that described above. Aliquots of SDS-digested homogenate proteins were loaded on the SDS-polyacrylamide gel at 4, 8, 16, and 32 μg of protein and separated by electrophoresis (4% acrylamide stacking gel and 6% acrylamide separating gel) at 120 V for 15 min and then at 180 V for 30 min. In each gel, homogenates from an age-matched control and a diabetic rat heart homogenate were loaded on the gel. For quantitation, bands were scanned, and the intensity of each band was determined using NIH Image software. The density of pixels versus the amount of homogenate protein. The slope of the regression line was calculated.

Analysis of immunoblots. The bands representing the pentameric PLB (25 kDa), SERCA2 (110 kDa), RyR (565 kDa), and α-actin (42 kDa) proteins were visualized using ECL system (Amersham Pharmacia Biotech; Piscataway, NJ). The protein levels were determined by using linear regression analysis with the linear lines of the number of pixels versus the amount of homogenate protein. The slope ($r^2 > 0.90$) of the lines (pixels/μg) of a control and a diabetic rat separated in the same gel was compared. The levels of each of the proteins in diabetic rat hearts were expressed as a percentage of those of the control rat hearts separated in the same gel. This procedure eliminates false results due to errors during loading and separation of proteins in the gel and transfer of protein bands to membrane and experiment-to-experiment variations in measurement of density of the bands.

Determination of phosphorylated PLB by immunoblotting. Preparation of cardiac homogenate and immunoblotting were the same as described for total PLB level. For measurement of phosphorylated PLB, a 1:10 dilution of antibodies (Fluorescence; Leeds, UK) were used as described previously (17). The dilution of the antibodies used in this study was 1:5,000.

Analysis of immunoblots. The bands representing the pentameric PLB (25 kDa), SERCA2 (110 kDa), RyR (565 kDa), and α-actin (42 kDa) proteins were visualized using ECL system (Amersham Pharmacia Biotech; Piscataway, NJ). The protein levels were determined by using linear regression analysis with the linear lines of the number of pixels versus the amount of homogenate protein. The slope ($r^2 > 0.90$) of the lines (pixels/μg) of a control and a diabetic rat separated in the same gel was compared. The levels of each of the proteins in diabetic rat hearts were expressed as a percentage of those of the control rat hearts separated in the same gel. This procedure eliminates false results due to errors during loading and separation of proteins in the gel and transfer of protein bands to membrane and experiment-to-experiment variations in measurement of density of the bands.

Determination of $\text{Ca}^{2+}$ uptake into SR. Frozen ventricles were powdered by stainless steel mortar and pestle cooled with liquid N2. The powdered tissue was suspended in a medium containing 300 mM sucrose, 50 mM K+-phosphate buffer (pH 7.0), 10 mM NaF to inhibit phosphatases, 0.3 mM PMSF to inhibit proteases, and 0.5 mM DT to prevent oxidation and breakdown of proteins containing a sulfur-sulfur bond. The suspension was homogenized four times each time with 10 passes with a Teflon pestle in a glass Potter-Elvehjem tissue homogenizer attached to a drill driven at an output of 50 W (120 V). During homogenization, the temperature was maintained at 2–4°C by submerging the homogenizer in a plastic bottle packed with saline-soaked crushed ice. The homogenate was centrifuged at 35,000 g for 30 min, and the supernatant was discarded. The pellet was resuspended in the homogenization buffer in a ratio of 1 g tissue to 15 ml of buffer. The homogenate for $\text{Ca}^{2+}$ uptake study was used within 2 h after preparation.

Initial rate of $\text{Ca}^{2+}$ uptake into SR as a function of time and free $\text{Ca}^{2+}$ in the assay medium was measured using $^{45}$Ca as a tracer by Millipore filtration technique (16). The rate of $\text{Ca}^{2+}$ uptake was measured in a 1.5-ml reaction medium containing (in mM) 40 imidazole-HCl buffer (pH 7.0), 95 KCl, 0.5 EGTA, 5 potassium oxalate, 5 MgCl2, 5 Na3VO4, and 0.001 ruthenium red, and 120–150 μg of protein and free $\text{Ca}^{2+}$ concentration varying from 0.001 to 10 μM achieved by adding varying volume of 10 mM CaCl2 with soaked in methanol just before use, Bio-Rad) in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol and using the Bio-Rad Trans-Blot electrophoretic transfer system at 250 mA at 4°C for 3 h, and then at 50 V for about 12 h. The membranes were treated with 5% Carnation instant milk in TBS with 0.2% Tween 20 for 1 h at room temperature and then for 24 h with anti-ryanodine receptor (Affinity Bioreagents), at 1:700 dilution in 0.5% Carnation instant milk/TBS with 0.2% Tween 20. The primary antibody solution was decanted, and the membranes were washed three times with TBS-0.2% Tween 20 solution each time for 10 min. The blots were then incubated for 2 h with a secondary anti-mouse antibody conjugated to horseradish peroxidase (Affinity Bioreagents) at 1:500 dilution in 0.5% Carnation instant milk/TBS with 0.2% Tween 20. The secondary antibody solution was decanted, and the membranes were washed for 30 min with TBS-0.2% Tween 20.

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pared with control rats. The VW-to-BW ratio in diabetic rats was significantly lower ($P < 0.05$) compared with that of age-matched control rat hearts. The left ventricular +dP/dt, time to peak pressure (TPP), and intraventricular peak pressure (IVP) were not significantly different in 4-wk diabetic rat hearts and age-matched control rat hearts. However, the +dP/dt, TPP, and IVP were significantly ($P < 0.05$) decreased in 6-wk diabetic rat hearts compared with those of age-matched control rat hearts. The rate of relaxation as assessed by the $-dP/dt$ was significantly ($P < 0.05$) decreased, and both the time to 50% relaxation (RT50) and 90% relaxation (RT90) were significantly ($P < 0.05$) prolonged in 4- and 6-wk diabetic rat hearts compared with those of age-matched control rat hearts. The contractile dysfunction in diabetic rat hearts is not due to decreased HR because similar changes in diabetic rat hearts were observed when the HR of control and diabetic rats was equalized to 300 beats/min by electrical pacing (data not shown here). Coronary resistance in 4-wk diabetic rat hearts was increased by about 20% compared with that of control rat hearts, but it was decreased by about 20% in 6-wk diabetic rat hearts. The reasons for this pattern of change in coronary resistance could not be determined in this study.

To determine whether replacement of insulin in diabetic rats prevents the development of delayed relaxation and depressed contraction in diabetic rat hearts, 4-wk diabetic rats were treated with insulin for 2 wk and examined at 6 wk after STZ injection. Daily insulin treatment of diabetic rats for 2 wk normalized the blood glucose and serum insulin levels (Table 3). The slow relaxation and slow and depressed contraction in diabetic rat hearts observed after 6 wk of diabetes were also normalized to the levels of the control rat hearts (Table 2).

The results of cardiac function study demonstrate slow rate of relaxation in 4-wk diabetic rat hearts and slow rates of contraction and relaxation and depressed magnitude of contraction in 6-wk diabetic rat hearts and that these changes are reversible or preventable with insulin replacement.

Table 1. Characteristics of STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>4 Week Control</th>
<th>4 Week Diabetic</th>
<th>6 Week Control</th>
<th>6 Week Diabetic</th>
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<tr>
<td>Body wt, g</td>
<td>347 ± 8</td>
<td>185 ± 16*</td>
<td>412 ± 8</td>
<td>223 ± 13*</td>
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<tr>
<td>Food intake, g/day</td>
<td>30 ± 2</td>
<td>46 ± 3*</td>
<td>28 ± 2</td>
<td>55 ± 4*</td>
</tr>
<tr>
<td>Water intake, ml/day</td>
<td>42 ± 2</td>
<td>211 ± 5*</td>
<td>47 ± 2</td>
<td>227 ± 26*</td>
</tr>
<tr>
<td>Serum insulin, nM</td>
<td>5.71 ± 0.80</td>
<td>0.56 ± 0.13*</td>
<td>4.01 ± 0.65</td>
<td>0.48 ± 0.08*</td>
</tr>
<tr>
<td>Blood glucose, mM</td>
<td>7.4 ± 0.2</td>
<td>28.6 ± 1.4*</td>
<td>7.4 ± 0.3</td>
<td>28.9 ± 1.9*</td>
</tr>
<tr>
<td>Ventricular wt, g</td>
<td>0.92 ± 0.03</td>
<td>0.55 ± 0.04*</td>
<td>1.02 ± 0.02</td>
<td>0.62 ± 0.05*</td>
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<tr>
<td>VW/BW ratio, mg/g</td>
<td>2.65 ± 0.06</td>
<td>2.97 ± 0.07*</td>
<td>2.49 ± 0.04</td>
<td>2.78 ± 0.06*</td>
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</table>

Values are means ± SE of 4 rats in each group. *Significantly different from control rats ($P < 0.05$).
**SR Ca\(^{2+}\)-cycling protein expression.** The immunoblots indicated the presence of pentameric form (mol mass 25 kDa) of PLB illustrated with a typical experiment in Fig. 1A. No detectable trace of the monomeric (mol mass 5 kDa) form of the PLB in the immunoblots of the control or diabetic rat heart homogenates was observed (not illustrated here). Homogenates from both control and diabetic rat hearts were run in the same gel. The pentameric PLB level in the diabetic rat hearts was determined relative to the density of the 25-kDa bands in the age-matched control rat hearts. Cumulative data revealed a significant increase in PLB level in diabetic rat hearts by 31% at 4 wk and 60% at 6 wk of diabetes compared with age-matched control rat hearts (Fig. 1A, bar graphs).

The immunoblot of the SERCA2 protein is also illustrated in Fig. 1B. SERCA2 protein level as determined by the density of the 110-kDa bands relative to that of the age-matched control rat hearts was not significantly altered in 4-wk diabetic rat hearts compared with 1.00 ± 0.3 in age-matched control rat hearts. The basal phosphorylation level at the serine-16 site of the PLB (P-Ser16) was significantly increased by 10.2 ± 0.3 in 4-wk diabetic rat hearts compared with 1.00 ± 0.19 in 6-wk control rat hearts.

The immunoblot of the RyR protein is also illustrated in Fig. 1C. The RyR protein level in 4-wk diabetic rat hearts as determined by the density of the 565-kDa bands relative to that of the age-matched control rat hearts was not significantly changed in diabetic rat hearts (Fig. 1C, bar graphs). However, RyR was significantly decreased by 31% in 6-wk diabetic rat hearts compared with the age-matched control rat hearts.

CSQ is a SR luminal Ca\(^{2+}\)-buffering protein (19). To determine whether CSQ was altered in diabetic rat hearts, immunoblots of homogenates of 4- and 6-wk diabetic rat hearts and age-matched control rat hearts were performed. The results presented in Fig. 2A demonstrate no significant change in CSQ protein level in 4- or 6-wk diabetic rat hearts.

α-Actin is a myofilament protein (28). To determine whether α-actin was altered in diabetic rat hearts, immunoblots of the cardiac homogenates of control and diabetic rat hearts were performed. The results presented in Fig. 2B demonstrate no change in α-actin level in 4- or 6-wk diabetic rat hearts relative to that of the age-matched control rat hearts.

The results of SR Ca\(^{2+}\)-cycling protein expression study demonstrate a sequential alteration of SR Ca\(^{2+}\)-cycling proteins as indicated by increase in PLB level in 4-wk diabetic rat hearts and decrease in SERCA2 and RyR levels along with increased PLB level in 6-wk diabetic rat hearts without any change in SR luminal Ca\(^{2+}\)-buffering protein CSQ or myofilament protein α-actin.

**Basal phosphorylated levels and reversibility with insulin treatment.** Phosphorylation level of PLB determines its ability to inhibit SERCA2 by decreasing its affinity for Ca\(^{2+}\). Therefore, phosphorylated PLB level was determined in diabetic and age-matched control rat hearts. The basal phosphorylation level at the serine-16 site of the PLB (P-Ser16) was significantly increased by 36% in 6-wk diabetic rat hearts.

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**Table 2. Hemodynamics and contractile function of isolated hearts**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Diabetic</th>
<th>Insulin-treated diabetic</th>
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</thead>
<tbody>
<tr>
<td>Hemodynamics</td>
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<tr>
<td>Heart rate, beats/min</td>
<td>279 ± 11</td>
<td>214 ± 13*</td>
<td>273 ± 5</td>
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<tr>
<td>Coronary resistance (AoP/CF)</td>
<td>5.4 ± 0.2</td>
<td>6.5 ± 0.3*</td>
<td>6.3 ± 0.3</td>
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<tr>
<td>Contraction</td>
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<tr>
<td>−dP/dt, mmHg/s</td>
<td>2,789 ± 96</td>
<td>2,480 ± 190</td>
<td>2,440 ± 200</td>
</tr>
<tr>
<td>IVP, mmHg</td>
<td>80 ± 2</td>
<td>77 ± 3</td>
<td>72 ± 2</td>
</tr>
<tr>
<td>TPP, ms</td>
<td>42.4 ± 0.8</td>
<td>45.4 ± 1.7</td>
<td>42.2 ± 1.2</td>
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<tr>
<td>Relaxation</td>
<td></td>
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</tr>
<tr>
<td>−dP/dt, mmHg/s</td>
<td>1,930 ± 126</td>
<td>1,504 ± 104*</td>
<td>1,974 ± 64</td>
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<tr>
<td>RT50, ms</td>
<td>33.5 ± 2.5</td>
<td>53.2 ± 3.9*</td>
<td>46.6 ± 2.5</td>
</tr>
<tr>
<td>RT90, ms</td>
<td>54.8 ± 2.7</td>
<td>85.6 ± 10.1*</td>
<td>69.3 ± 4.3</td>
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</table>

Values are means ± SE of 6 rats in each group. *Significantly different from control rats (P < 0.05); †significantly different from diabetic rats (P < 0.05); AoP, aortic pressure (mmHg); CF, coronary flow (ml·min\(^{-1}\)·g\(^{-1}\)); −dP/dt, rate of pressure decline; IVP, intraventricular pressure; TPP, time to peak pressure; −dP/dt, rate of pressure decline; RT50, time to 50% relaxation; RT90, time to 90% relaxation.

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**Table 3. Characteristics of insulin-treated diabetic rats**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Diabetic</th>
<th>Insulin-treated diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>429 ± 3</td>
<td>228 ± 9*</td>
<td>287 ± 18†</td>
</tr>
<tr>
<td>Serum insulin, nmol/l</td>
<td>3.82 ± 0.47</td>
<td>0.43 ± 0.07*</td>
<td>4.32 ± 0.50†</td>
</tr>
<tr>
<td>Blood glucose, mmol/l</td>
<td>7.1 ± 0.5</td>
<td>27.7 ± 0.8*</td>
<td>8.1 ± 1.1†</td>
</tr>
<tr>
<td>Ventricular wt, g</td>
<td>1.12 ± 0.03</td>
<td>0.67 ± 0.03*</td>
<td>0.84 ± 0.05†</td>
</tr>
<tr>
<td>VW/BW ratio, mg/g</td>
<td>2.60 ± 0.05</td>
<td>2.93 ± 0.06*</td>
<td>2.97 ± 0.07†</td>
</tr>
</tbody>
</table>

Values are means ± SE of 4 rats in each group. *Significantly different from control rats (P < 0.05); †significantly different from diabetic rats (P < 0.05).
decreased in 4-wk and 6-wk diabetic rat hearts compared with age-matched control rat hearts (Fig. 3A). The basal phosphorylation at the threonine-17 site of the PLB (P-Thr17) was also significantly decreased in 4-wk and 6-wk diabetic rat hearts (Fig. 3B). Insulin treatment of the diabetic rats completely prevented the decreased levels of P-Ser16 (Fig. 3A) and P-Thr17 (Fig. 3B). These results indicate that the increased PLB in diabetic rat hearts is predominantly unphosphorylated form.

**SR Ca\(^{2+}\) uptake.** The initial rate of Ca\(^{2+}\) uptake into SR was increased with increasing free Ca\(^{2+}\) concentration from 100 nM and above, approaching the maximum rate at about 1 μM Ca\(^{2+}\) of 4- and 6-wk diabetic and age-matched control rat hearts (Fig. 4A). The concentration of free Ca\(^{2+}\) that produced half of the maximum rate (EC\(_{50}\)) of Ca\(^{2+}\) uptake into the SR of control rat heart membrane homogenates was 0.195 ± 0.02 μM and did not significantly change with the increasing age of the rats (Fig. 4B). However, the EC\(_{50}\) of Ca\(^{2+}\) uptake into SR of 4- and 6-wk diabetic rat hearts was significantly increased, respectively, by 31% and 56% compared with that of age-matched control rat hearts (Fig. 4B). The data indicate a decrease in the affinity of SR Ca\(^{2+}\) pump for Ca\(^{2+}\) in diabetic rat hearts, consistent with the increase in PLB level (see Fig. 1A). The maximum velocity (V\(_{\text{max}}\)) of Ca\(^{2+}\) uptake into SR of 4- and 6-wk control rats was 11.75 ± 0.50 and 11.67 ± 0.72 nmol·min\(^{-1}·mg\) protein\(^{-1}\), respectively, indicating no significant change in V\(_{\text{max}}\) with increasing age of the control rats (Fig. 4C). The V\(_{\text{max}}\) of Ca\(^{2+}\) uptake into SR of 4-wk diabetic rat hearts was not significantly decreased in 4-wk and 6-wk diabetic rat hearts compared with age-matched control rat hearts (Fig. 3A).

![Fig. 1. Quantitative immunoblots demonstrating increased level of PLB protein (A) in 4 wk (4W) and 6 wk (6W) diabetic rat hearts and decreased levels of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2) (B), and ryanodine receptor (RyR) (C) proteins in 6 W diabetic rat hearts compared with age-matched control rat hearts. Bands are quantitative immunoblots of a representative control (C) and diabetic (D) rat heart run in the same gel. Slope of linear regression line (r\(^2\) = 0.90) of control rat heart was taken as 100% to determine the change in diabetic rat hearts. Bars represent cumulative data of means ± SE of 4 hearts in each group. *Significantly different from control rat hearts (P < 0.05).](image)

![Fig. 2. Quantitative immunoblots demonstrating no significant change in the levels of calsequestrin (CSQ; A) and α-actin proteins (B) in 4W and 6W diabetic rat hearts compared with age-matched control rat hearts. Same hearts as in Fig. 1 were studied. Format of data presentation is the same as in Fig. 1.](image)
different from that of age-matched control rat hearts. However, it was significantly decreased by about 27% in 6-wk diabetic rat hearts (Fig. 4C) consistent with the decrease in SERCA2 protein level (see Fig. 1B).

The results of Ca\(^{2+}\) uptake into the SR study demonstrate decreased apparent affinity of SR Ca\(^{2+}\)-pump for Ca\(^{2+}\) in 4-wk diabetic rat hearts and decreased apparent affinity for Ca\(^{2+}\) and maximum velocity of
Ca\textsuperscript{2+} uptake into the SR in 6-wk diabetic rat hearts. Thus a sequential change in Ca\textsuperscript{2+} uptake into the SR of diabetic rat hearts was also observed.

Reversal of change in PLB and prevention of the changes in SERCA2 and RyR protein levels and SR Ca\textsuperscript{2+}-pump activity with insulin treatment. To determine whether changes in the SR protein expression and function were preventable or reversible with insulin replacement, rats diabetic for 4 wk were treated with insulin for 2 wk and SR protein expression and Ca\textsuperscript{2+} uptake into SR were examined. Four-week diabetic rats were selected because PLB level was increased but SERCA2 and RyR levels were unchanged at this stage. This time point was selected because treatment of diabetic patients with insulin begins shortly after diagnosis of the disease, most likely after some cellular changes have already occurred and before long-term changes have begun. The goal of the treatment is to reverse any changes that may have occurred and prevent further changes and complications. Thus the objective of our study was to determine whether the early onset increase in PLB is reversible and the decreases in SERCA2 and RyR levels are preventable. Insulin treatment of the diabetic rats completely normalized blood glucose and serum insulin levels (Table 3). The insulin-treated rats gained body weight significantly but not to the level of that of control rats. The VW-to-BW ratio was not decreased to that of control rats. Instead it was significantly increased in the insulin-treated rats.

Insulin treatment of diabetic rats resulted in complete normalization of PLB, SERCA2, and RyR protein levels to that of control rat hearts (Fig. 5). The results of Ca\textsuperscript{2+} uptake into the SR of 6-wk diabetic rat hearts, insulin-treated diabetic rat hearts, and control rat hearts are presented in Fig. 6. The EC\textsubscript{50} of the Ca\textsuperscript{2+} uptake into the SR was normalized to that of control rat hearts, and the V\textsubscript{max} was normalized almost to the level of control rat hearts. The results demonstrate that changes in SR protein expression and dysfunction are associated with insulin deficiency in STZ-induced diabetic rat hearts.

**DISCUSSION**

This is the first report demonstrating an increase in PLB protein level associated with slow rate of relaxation in an animal model of cardiomyopathy. Although depression of SR Ca\textsuperscript{2+}-pump and Ca\textsuperscript{2+}-ATPase activity in STZ-induced diabetic rat hearts have been reported (8, 14, 23), this is the first report of a sequential alteration of expression and function of SR proteins associated with early-onset slow rate of relaxation and late onset slow rates of contraction and relaxation and depressed magnitude of contraction in this model of diabetic cardiomyopathy. The sequential and antithetic changes in expression of the SR Ca\textsuperscript{2+}-cycling proteins accompanying sequential alteration of contractile function of diabetic rat hearts, to our knowledge, have not been observed in any other animal models of cardiomyopathy. In this respect, our discovery is novel, indicating that diabetic cardiomyopathy may be a unique type of cardiomyopathy.

Early-onset diastolic dysfunction preceding systolic dysfunction in diabetic patients has been observed (for a review, see Ref. 6), but the underlying mechanism is currently unknown. The observation in the present...
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Fig. 6. Ca\(^{2+}\) uptake (A) into SR demonstrating reversal of the decrease in apparent affinity (increased EC\(_{50}\)) of Ca\(^{2+}\) and prevention of the decrease in \(V_{\text{max}}\) (C) after 2 wk of insulin treatment of 4W rats. Same hearts as in Fig. 5 were studied. Same experimental conditions and data analysis were used as in Fig. 4. *Significantly different from control rat hearts (P < 0.05).

study of sequential alteration of expression and function of SR proteins associated with sequential alteration of contractile function in STZ-induced diabetic rats underscores the potential role of a similar mechanism in human diabetic patients. However, the levels of SR Ca\(^{2+}\)-cycling proteins and Ca\(^{2+}\)-pump activity in diabetic human heart muscle have not yet been determined. Thus it remains to be seen whether a sequential alteration of expression of SR proteins underlies early-onset diastolic dysfunction and late-onset systolic dysfunction in diabetic human hearts. Alteration of PLB level in idiopathic failing human hearts has been controversial; decreased or unchanged level of this protein has been reported (for a review see Ref. 12), but increased PLB level has not been reported. Decreased SERCA2 and RyR levels have been observed in failing human hearts and in nondiabetic animal models of cardiomyopathy and heart failure (1, 20), but sequential change in the expression or function of SR proteins has not been observed. Thus it also remains to be seen whether diabetic cardiomyopathy is different from idiopathic cardiomyopathy.

The increase of PLB protein level observed in diabetic rat hearts is moderate compared with the twofold transgenic overexpression of PLB in mouse hearts, which was accompanied with about 80% decrease in apparent affinity (increase in EC\(_{50}\)) of SR Ca\(^{2+}\)-pump for Ca\(^{2+}\) (11). Twofold increase of PLB in mice was found to accompany depression of both contraction and relaxation of the heart (11). The increase in PLB in diabetic rat hearts was predominantly a nonphosphorylated form indicating that it was interacting with SERCA2 to decrease its apparent affinity for Ca\(^{2+}\). In 4-wk diabetic rats, we observed a 30% increase in PLB level accompanied with a 30% increase in EC\(_{50}\) of Ca\(^{2+}\) uptake into the SR. Thus the moderate increase in nonphosphorylated PLB and reduction in apparent affinity of SR Ca\(^{2+}\)-pump for Ca\(^{2+}\) may slow the rate of Ca\(^{2+}\) sequestration into the SR and delay relaxation without affecting contraction of the heart at the early stages of diabetes. The rate of decrease in Ca\(^{2+}\) sequestration at the early stages of diabetes may not be to the extent that decreases the SR Ca\(^{2+}\) store and the rate of Ca\(^{2+}\) release from SR to depress the rate and magnitude of contraction of the heart. This could be the underlying cause for slow relaxation without any change in contraction of the heart observed at the early stage of diabetes. On the other hand, during increased cardiac workload even this moderate increase in PLB may more drastically depress SR function and thus cause both systolic and diastolic dysfunction. The decreased SERCA2 protein level at the late stage was accompanied by a 27% decrease in maximum velocity of Ca\(^{2+}\) uptake into SR. The decrease in SERCA2 and RyR protein levels along with a further increase (to \(\sim 60\%\)) in PLB protein level at the late stage of diabetes may have further a decreased the SR function resulting in a decreased SR Ca\(^{2+}\) store and rate of Ca\(^{2+}\) release from the SR. These changes not only can slow relaxation but also can slow contraction of the heart. Our data demonstrate that 1) moderate increase in PLB protein level and decrease in apparent affinity of SR Ca\(^{2+}\)-pump for Ca\(^{2+}\) are associated with slow relaxation, and 2) decreases in SERCA2 and RyR protein levels further increase in PLB protein level, and decrease in maximum velocity and apparent affinity for Ca\(^{2+}\) of SR Ca\(^{2+}\)-pump are associated with slow contraction and relaxation in diabetic rat hearts. The results are consistent with the indications that abnormal cellular calcium handling is probably linked to abnormal mechanical function in diabetes (27). However, contribution of other processes, such as isoform shift in myosin heavy or light chain proteins, slow repolarization of membrane potential, or altered energy metabolism on early-onset slow relaxation or late-onset slow and depressed contraction cannot be excluded. Nevertheless, the results of this study strongly
implicate altered SR Ca\(^{2+}\) cycling as an important contributing factor underlying the sequential development of contractile dysfunction in diabetes.

The mechanism of alteration of SR protein expression in STZ-induced diabetic rat hearts is unclear at present. The data presented in this study demonstrate that PLB overexpression and decreased SR Ca\(^{2+}\)-pump affinity for Ca\(^{2+}\) were completely reversed, and SERCA2 and RyR proteins underexpression and decreased SR Ca\(^{2+}\)-pump maximum velocity were prevented with insulin replacement. The results indicate that alteration of cardiac SR protein expression and depression of the SR Ca\(^{2+}\) pump are associated with insulin deficiency in STZ-induced diabetic rats. The acute effect of insulin receptor signaling in muscle, adipose cells, and liver is to mobilize glucose transporter (Glut-4) from a cytosolic compartment to cell membrane and regulate substrate metabolism in the cell (33). However, insulin receptor signaling also chronically regulates gene transcription and translation (22). Insulin receptor and its signaling process have been demonstrated in cardiac myocytes (32). Insulin has been shown to regulate protein synthesis in cardiac myocytes (4). Therefore, it is likely that insulin receptor signaling could be involved in SR gene or protein expression. Nevertheless, it remains to be seen whether insulin deficiency and downregulation of its signaling or indirect effects of insulin deficiency, such as alteration of growth hormones or hyperglycemic stress, underlie alteration of transcription or translation of SR proteins in insulin-deficient (type 1) diabetic rat hearts. It is possible that alteration of SR protein expression may also underlie cardiac contractile dysfunction in type 2 diabetes because insulin signaling is downregulated in insulin-resistant (type 2) diabetes.

In conclusion, this study demonstrates a novel increase in PLB protein at the early stage and decrease in SERCA2 and RyR proteins at a later stage of STZ-induced diabetic rats hearts. The early increase in nonphosphorylated PLB level is accompanied with decrease in the apparent affinity of SR Ca\(^{2+}\) pump for Ca\(^{2+}\) and slow rate of relaxation of the heart. The decrease in SERCA2 and RyR protein levels and the further increase in PLB protein level are accompanied with decreased maximum velocity of SR Ca\(^{2+}\) pump and slow rates of contraction and relaxation and depressed magnitude of contraction of the heart. The results of the study strongly indicate a contribution of a sequential alteration of SR Ca\(^{2+}\) protein expression and function underlying development of early onset slow relaxation and late onset slow and depression of contraction in diabetic cardiomyopathy.

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