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CARDIAC SARCOPLASMIC RETICULUM Ca^{2+}-ATPase EXPRESSION IN STREPTOZOTOCIN-INDUCED DIABETIC RAT HEART

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SUMMARY: Chronic diabetes due to streptozotocin administration has been shown to induce heart dysfunction characterized by prolonged relaxation time as well as decreased Ca^{2+} transport and Ca^{2+}-ATPase (SERCA2) activities of the cardiac sarcoplasmic reticulum (SR). Rats made diabetic with 65 mg/kg of streptozotocin for 3 and 5 weeks exhibited decreased SR Ca^{2+}-pump activities; these were normalized upon treatment with insulin. Northern blot and slot blot analyses did not show statistically significant reduction in the relative level of SERCA2 mRNA expression in diabetic or insulin treated rats. Quantitation of SERCA2 protein by Western blot did not reveal any change in diabetic and insulin treated animals. These results suggest that the defect in SR Ca^{2+}-pump may not be due to changes at the transcriptional or translational levels in the diabetic heart.

The sarcoplasmic reticulum (SR) is the major intracellular organelle that sequesters intracellular Ca^{2+} and regulates the relaxation and the tension development of the myocardium. Calcium uptake by the SR in cardiac myocytes is driven by the Ca^{2+}-ATPase (SERCA2) (1). The cardiac SERCA2 gene encodes two alternatively spliced transcripts, one expressed in cardiac and slow-twitch muscle (SERCA2a), and the other expressed in smooth muscle and non-muscle tissues (SERCA2b) (2-6). In the cardiac myocyte only the SERCA2a isoform is expressed during development, neonatal and adult hearts, as well as in various physiologic and pathologic forms of cardiac hypertrophy (1,3,6,7). Several investigators have provided evidence that the Ca^{2+} transport function of SR is altered in myocardial hypertrophy in experimental animals as well as in humans (6,8). Such alterations in SR function are considered

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primarily to be due to changes in the expression of mRNAs encoding SR Ca^{2+} transport proteins. It has been demonstrated that the relative mRNA level for SERCA2 is decreased by about 60% in experimental animal models of pressure overload cardiac hypertrophy (7,8).

Insulin-dependent diabetes invariably results in heart dysfunction characterized by decreased velocity of contraction, prolonged diastolic relaxation time, decreased cardiac output and high filling pressure (9). Chronic diabetes due to streptozotocin administration in rats has been shown to induce cardiomyopathy characterized with defects in cardiac function, ultrastructure, and metabolism similar to those observed in diabetes mellitus (*10,11*). Studies from different laboratories have shown that prolonged relaxation time of the diabetic myocardium is correlated with decreased SR Ca^{2+} uptake and Ca^{2+}-ATPase (Ca^{2+} stimulated ATPase) activities (10,12,13); however, the molecular mechanisms for these changes are poorly understood. In fact the results reported for changes in SERCA2 mRNA levels in diabetic heart are controversial (14,15). Therefore the object of the present study was to determine whether alterations in SR Ca^{2+}-pump activities in the diabetic heart are associated with a decrease in the SERCA2 mRNA level or a decrease in the amount of corresponding SERCA2 protein.

MATERIALS AND METHODS

Male Sprague-Dawley rats (175-200 g) were made diabetic with a single injection of citrate-buffered streptozotocin (Sigma) at a dosage of 65 mg/kg body wt via the femoral vein as described previously (10,11). All animals were maintained on normal rat chow and water *ad libitum*. Diabetic or control (citrate buffer injected) rats were sacrificed 3 and 5 weeks after the injection. Insulin treatment in diabetic rats was started 3 weeks after the induction of diabetes at a doses of 3 U PZI (Lilly)/100g body wt/day and was carried out for a period of 2 weeks. All the animals were sacrificed by decapitation and the hearts were quickly removed. The left ventricular tissue was weighed and frozen immediately in liquid nitrogen.

SR vesicles were isolated from the left ventricular tissue of control and experimental animals as described elsewhere (10). Basal Mg^{2+}-ATPase activity was determined at 37°C in a reaction medium containing 100 mM KCl, 20 mM Tris-HCl, 5 mM $MgCl_2$ and 5 mM Tris-ATP (pH 6.8), and 5 mM Na-azide. For total (Ca^{2+}+Mg^{2+})-ATPase the reaction medium was adjusted to 10 μM [Ca^{2+}] using a Ca^{2+}/EGTA buffer. The Ca^{2+}-ATPase activity was calculated as the difference between the total (Ca^{2+}+Mg^{2+})-ATPase and basal Mg^{2+}-ATPase activities. SR Ca^{2+}-uptake was determined using the Millipore filtration technique (10) in a medium containing 100 mM KCl, 20 mM Tris-HCl (pH 6.8), 5 mM $MgCl_2$, 5 mM Tris-ATP, 5 mM K-oxalate and 5

mM Na-azide. The reaction was started by the addition of Ca^{2+}/EGTA buffer to give a final concentration of 10 μM [$^{45}Ca^{2+}$]. The reaction was terminated after 2 min by rapid filtration through 0.45 μm Millipore filters and washed with 5 ml of 100 mM KCl, 20 mM Tris-HCl (pH 6.8), then filters were dried and incorporation of [$^{45}Ca^{2+}$] was measured by scintillation counting technique.

Total cellular RNA was isolated from the left ventricular tissue using the guanidine thiocyanate-phenol-chloroform extraction procedure (16). RNA gel electrophoresis and analysis were performed as described previously (7,17). Briefly, for Northern blots, 20 μg of total RNA was size fractionated on 1% agarose gel containing 1 M formaldehyde and blotted onto a nitrocellulose membrane (Schleicher & Schuell). For slot blots 3 and 6 μg of total RNA from each sample were loaded onto the nitrocellulose membranes of a slot blot apparatus. The same amount of tRNA was also loaded to detect non-specific binding of probes. The following cDNA fragments were used: for SERCA2a, a 1.7-kb BamH1-Pst1 cDNA fragment containing the 3' protein coding region and the full 3' untranslated region of a rabbit SERCA2a cDNA was used (2,7); for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 1.63-kb chicken GAPDH cDNA was used (18); for the 28S rRNA, a 4.8 kb mouse 28S rRNA EcoRI-SalI fragment was used (19). The cDNA fragments were labeled with [^{32}P]-αdCTP using random hexamer labeling kit (Life Technologies). After hybridization with [^{32}P]-labeled cDNA probes and washing as described (8,18). Membranes were exposed to Kodak X-Omat-AR film. Radiolabeled mRNA bands were quantitated by densitometric scanning. The mRNA values for SERCA2a were divided by that for GAPDH and/or 28S rRNA to obtain normalized relative SERCA2 mRNA values.

Fifty μg of SR membrane proteins were separated in 7.5% SDS-PAGE (20). Gels were electroblotted onto PVDF membranes (Bio-Rad) as described previously (21) and the membranes were blocked with 5% skim milk and then incubated with a rabbit antibody (PAb87) prepared against the 5'-coding sequence of the rabbit SERCA2a isoform (kindly provided by Dr. A. Grover, Univ. Western Ontario) (22). The membranes were washed, incubated with [^{125}I]-labeled protein A, exposed to X-ray film, and quantitated by densitometric scanning. The results are presented as mean $\pm$ SE. Unpaired "t" test was used to compare the control and experimental groups. A Bonferroni/Dun correction for multiple comparisons was used to evaluate the significance of the data.

RESULTS

As reported earlier (10,11) the diabetic rats exhibited decreased body weight and heart weight to 71% and 77% of control animals after 3 or 5 weeks of injecting streptozotocin, respectively. The level of plasma glucose in diabetic animals was increased 5-6 fold whereas plasma insulin level was decreased after 3 or 5 weeks of streptozotocin injection (Table 1). The treatment of 3 weeks diabetic animals with insulin for 2 weeks was found to normalize body weight and heart weight to 90% of control animals with a concomitant decrease in the circulating plasma glucose and increase in plasma insulin levels (Table 1).

TABLE 1. General characteristics of control and experimental rats, as well as their cardiac SR Ca^{2+}-ATPase and Ca^{2+} uptake activities

	Age-matched Control	3-wk DM	5-wk DM	3-wk DM + 2-wk insulin
Plasma glucose (mg/ 100 ml)	117 ± 120	563 ± 15*	658 ± 25*	77 ± 16*
Plasma Insulin (μU/ml)	30 ± 1.42	11.2 ± 0.94*	11.4 ± 0.87*	34.2 ± 1.71
SR protein yield (mg/g heart)	1.64 ± 0.32	1.58 ± 0.29	1.71 ± 0.41	1.59 ± 0.37
SR Ca^{2+} uptake (nmol/mg protein/min)	54.6 ± 4.50	29.2 ± 3.80*	26.4 ± 2.9*	51.3 ± 4.10
SR Ca^{2+}-stimulated ATPase (μmol Pi/mg protein/5 min)	0.96 ± 0.07	0.61 ± 0.05*	0.54 ± 0.06*	0.84 ± 0.06*

*Values are mean ± SE (n= 3-6 per group). Diabetes (DM) was induced by an injection of streptozocin (65 mg/kg) and the animals were sacrificed 3 and 5 wk later. For the insulin-treated group, 3-wk diabetic rats were given 3 U insulin per day subcutaneously, and the animals were sacrificed 2 wk later. * Significantly different from control ($p < 0.05$).*

The Ca^{2+}-stimulated ATPase and Ca^{2+}-uptake activities were determined in SR vesicles from the left ventricular tissue of diabetic and insulin treated rats. While no changes in the SR protein yield were apparent, a marked reduction in SR Ca^{2+}-uptake activity was observed in diabetic heart (Table 1). The results in Table 1 also show that in 3 and 5 weeks diabetic rats the Ca^{2+}-stimulated ATPase was decreased 63% and 56% compared to age-matched control animals, respectively. After 2 weeks treatment with insulin of the 3 weeks diabetic rats, the cardiac SR Ca^{2+}-stimulated ATPase and Ca^{2+}-uptake activities were restored to values observed in control rats.

The expression of the relative level of mRNA for SERCA2 in the left ventricle was examined using Northern blot and slot blot techniques (Figure 1 and Table 2). A small (less than 20%), but not statistically significant, decrease in the relative level of expression of SERCA2 mRNA normalized with 28S rRNA was observed in 3 or 5 weeks diabetic left ventricle compared to the control tissue. Insulin treatment of diabetic animals for 2 weeks did not significantly alter the SERCA2 mRNA level in the left ventricular myocardium compared to the age-matched control or diabetic animals. Table 2 also shows that similar results were obtained from slot blot analysis. In addition, there was no significant difference between the results obtained by normalizing the SERCA2 mRNA levels with those for 28S rRNA or GAPDH mRNA (data not shown).

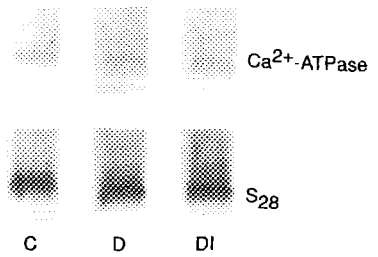

Figure 1. Representative Northern blot of Control, Diabetic and Insulin treated diabetic hearts. Twenty μg of total RNA isolated from the left ventricular tissue was electrophoretically separated in 1% agarose/formaldehyde gel, transferred onto nitrocellulose and hybridized with SERCA2 cDNA and 28S rRNA [^{32}P]-labeled probes. The membrane was washed and exposed to X-ray film as mentioned under "Materials and Methods".C- control; D- 5 wk diabetic; DI- 3 wk diabetic and 2 wk insulin treated.

Figure 2 shows the results obtained from the Western blot analysis using a polyclonal antibody that specifically recognizes the SERCA2 protein. The steady state level of the SERCA2 protein in the SR membrane fraction was not altered in 5 weeks diabetic animals. Furthermore, treatment of 3 weeks diabetic rats with insulin for additional 2 weeks did not modify the level of the SERCA2 protein.

DISCUSSION

Several reports indicate that handling of Ca^{2+} by cardiac myocytes is altered in diabetes (10,13). In the insulin-dependent

TABLE 2. SERCA2 mRNA levels in control, diabetic and insulin treated rat hearts

	n	SERCA2 mRNA/28S rRNA			
		Northern blot		Slot blot	
3-wk control	3	1.00 ± 0.050		0.987 ± 0.281	
3-wk diabetic	5	1.01 ± 0.240	(0.761)[a]	0.642 ± 0.094	(0.201)[a]
5-wk control	6	1.00 ± 0.132		0.967 ± 0.128	
5-wk diabetic	6	0.83 ± 0.180	(0.529)[b]	0.728 ± 0.149	(0.190)[b]
3-wk diabetic + 2-wk Insulin	5	1.27 ± 0.265	(0.347)[c],(0.134)[d]	0.788 ± 0.071	(0.740)[c],(0.340)[d]

Values are mean ± SE. Unpaired *t* test was used to compare the control and treated animals. A Bonferroni/Dunn correction for multiple comparisons was performed to evaluate the significance of the data using Statview 4.0 software. The p values shown in parentheses were obtained by comparing: a =3-wk control vs 3-wk diabetic; b= 5-wk control vs 5-wk diabetic; c= 5-wk control vs 5-wk diabetic + Insulin; and d= 5-wk diabetic vs 3-wk diabetic + 2-wk insulin.

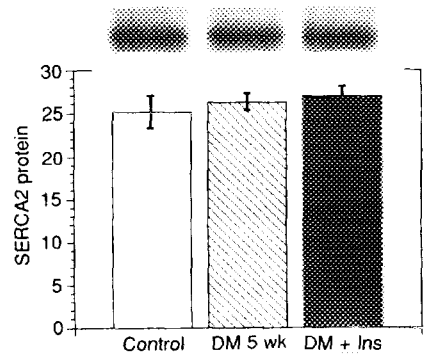

Figure 2. Western blot analysis of SR vesicles using anti-SERCA2 antibody. Bars show levels of SERCA2 in control (▭) n=4; 5 wk diabetic (▨) n=5; 3 wk diabetic and 2 wk insulin treated (▬) n=5. Inset shows the SERCA2 band from a representative Western blot autoradiography. Results are mean ± SE.

diabetic myocardium the defect in calcium homeostasis involves significant impairment in the SR and sarcolemma (SL) calcium pump activities (10,13,14,23) as well as the SL Na^+/Ca^{2+} exchanger (24,25); however, the molecular mechanisms (transcriptional and translational) are poorly understood. In this study we have found a decreased Ca^{2+}-stimulated ATPase and Ca^{2+}-uptake activities in the SR of diabetic rats; these observations are in agreement with previous studies (10,12,13). Such a defect in SR Ca^{2+}-pump activities can be explained on the basis of a decreased SERCA2 mRNA levels. In fact one study has reported 50% reduction in SERCA2 mRNA level in rat hearts after 4 weeks of streptozotocin injection (14). Although, in our study a trend of decrease in the level for SERCA2 was observed in 3 and 5 weeks diabetic rats, this change was not statistically significant when compared to control animals. No changes in cardiac SERCA2 mRNA levels were also reported in 4 weeks streptozotocin-diabetic rats (15). Furthermore, in the present study no alterations in the steady state SERCA2 content in SR membrane were seen in the diabetic heart. Thus it appears that the decreased SR Ca^{2+}-ATPase and Ca^{2+} transport activities observed in diabetic cardiomyopathy may not be due to a change in the expression levels of the SERCA2 mRNA and/or SERCA2 protein.

The decrease in SERCA2 mRNA level reported in the previous study (14) as well as the small decrease observed in the present study may in part be a consequence of a decrease in the circulating levels of thyroid hormone in the streptozotocin-induced diabetic rat (10). It has been shown that in 4 weeks

propylthyouracil induced hypothyroid rabbits, the level of SERCA2 mRNA in the heart is decreased approximately by 35% compared to euthyroid animals (7). Alternative explanations for the SERCA2 activity defect in the diabetic myocardium include a generalized alteration in phospholipid composition as well as phosphatidylethanolamine *N*-methylation in SR membranes (10,26).

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