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# Decreased cardiac sarcoplasmic reticulum $Ca^{2+}$-ATPase activity contributes to cardiac dysfunction in streptozotocin-induced diabetic rats

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XIAO-YAN ZHAO, SHEN-JIANG HU, JIANG LI, YUN MOU, BAO-PING CHEN and QIANG XIA. *Decreased cardiac sarcoplasmic reticulum $Ca^{2+}$-ATPase activity contributes to cardiac dysfunction in streptozotocin-induced diabetic rats.* J. Physiol. Biochem., **62** (1), 1-8, 2006.

Diabetic cardiomyopathy is characterized by reduced cardiac contractility due to direct changes in myocardium function independent of vascular disease. This study is to investigate the alterations of cardiac sarcoplasmic reticulum $Ca^{2+}$-ATPase activity and cardiac function in streptozotocin-induced diabetic rats. Diabetes mellitus (DM) was induced in male Wistar rats by intraperitoneal injection of streptozotocin. The activity of myocardium sarcoplasmic reticulum $Ca^{2+}$-ATPase and the left ventricular hemodynamic parameters were measured in DM rats 4 weeks, 6 weeks and 8 weeks after streptozotocin was administered. Phospholamban mRNA expression was detected by reverse transcription-polymerase chain reaction, and the protein levels of phospholamban and sarcoplasmic reticulum $Ca^{2+}$-ATPase were determined by Western blot. Normal rats served as control group. It was found that in DM rats 4 weeks after streptozotocin injection, the cardiac function, myocardium sarcoplasmic reticulum $Ca^{2+}$-ATPase activity, phospholamban mRNA and phospholamban protein were not significantly changed compared with those in the control rats. At 6 and 8 weeks after the streptozotocin injection, DM rats showed a significant decrease in sarcoplasmic reticulum $Ca^{2+}$-ATPase activity and cardiac function, as indicated by an increase of LVEDP and a marked depression in LVSP and $\pm dP/dt_{max}$. At the same time points, increases in phospholamban mRNA and protein levels were observed in DM rats. Sarcoplasmic reticulum $Ca^{2+}$-ATPase protein level showed no significant alterations in all DM rats compared with that in control rats. Our work confirms that sarcoplasmic reticulum $Ca^{2+}$-ATPase activity is depressed in rats with streptozotocin-induced DM, which is accompanied by elevated phospholamban protein level thus contribute to the pathogenesis of cardiac dysfunction in diabetic rats.

**Key words:** Diabetes, Phospholamban, Sarcoplasmic reticulum $Ca^{2+}$-ATPase, Cardiac function.

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Diabetic cardiomyopathy is one of the most important complications of diabetes mellitus (DM), and is characterized by reduced cardiac contractility due to direct changes in heart muscle function independent of vascular disease. Though it is well known that several mechanisms (3), such as defective glucose transport, fatty acid overload, abnormalities in cellular biochemistry and structure, may be involved in the pathogenesis of diabetic cardiomyopathy, the gene expression alterations involved in the occurrence and progression of diabetic cardiomyopathy remain controversial to date. Contractility and dilation of myocardium are related to sarcoplasmic reticulum calcium-ATPase (SERCA) activity, which is regulated by phospholamban (PLB). Previous studies found that, in idiopathic dilated cardiomyopathy, chronic PLB-SERCA interaction is the critical calcium cycling defect (11), and expression of additional PLB molecules resulted in inhibition of sarcoplasmic reticulum (SR) $Ca^{2+}$ transport, decrease of systolic $Ca^{2+}$ levels in ventricular myocytes, and depression of basal left ventricular systolic function *in vivo* (7).

As to diabetic cardiomyopathy, it was found that altered SR protein expression was associated with contractile dysfunction in diabetic rat hearts, and insulin treatment normalized SR protein expression and cardiac function (22). It was shown (14, 18) that in cardiomyocytes isolated from diabetic rats, systolic and diastolic function were markedly depressed, which were related with altered SR functions of uptake and/or release of $Ca^{2+}$. Some *in vivo* studies (12, 15, 22) have observed the altered expression of $Ca^{2+}$-handling proteins in diabetic rats and obtained conflicting results. In this study, using a DM rat model, we investigated the alterations of SERCA protein and activity, the expressions of PLB mRNA and protein in diabetic myocardium, and their relationship with cardiac function.

## Material and Methods

*Reagents.*– Streptozotocin (STZ) was purchased from Sigma (USA). M-MLV reverse transcriptase was obtained from Promega (USA). Pyrobest DNA polymerase was purchased from Takara (Japan). Primary monoclonal antibodies of PLB and SERCA, and secondary IgG antibodies were from ABR (USA). ECL chemiluminescence kit was obtained from Amersham Corp (USA).

*Induction of diabetes in rats.*– DM was induced as described elsewhere (10, 16). In brief, 28 male adult Wistar rats (8 weeks old) weighting about 220-250g were randomly divided into two groups. One (DM group) received a single intraperitoneal injection of STZ (65 mg/kg), which was freshly dissolved in a sterile citrate solution (0.1 mmol/L citric acid and 0.2 mmol/L sodium phosphate, pH 4.5). The other group (control group) received an equivalent volume of citrate buffer alone. The development of DM was verified by overt physical signs including lack of weight gain, polydipsia and polyuria, and confirmed by urine analysis and serum glucose level determination (≥16.7mmol /L) on the 7th day of streptozotocin injection. DM rats were then randomly subdivided into 3 groups according to the time they were detected and sacrificed: 4 weeks (4-wk-DM), 6 weeks (6-wk-DM) and 8 weeks (8-wk-DM) after streptozotocin injection. All rats were housed under similar conditions with 12-hour light/dark cycles with the temperature maintained at

21±1 °C and humidity at 55±5%, and free access to the same food and water. The study was approved by the Institutional Animal Care and Use Committee of Zhejiang University.

*Hemodynamic measurement*.– 4, 6, or 8 weeks after STZ injection, hemodynamic measurement was performed to determine left ventricular function in the rats. Briefly, after rats were anesthetized with 10% chloral hydrate (300 mg/kg) injection intraperitoneally, a 20-gauge catheter-tip pressure transducer was introduced into the left ventricle through the right carotid artery for measuring of left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP) and the maximum velocity of ascending or descending in intraventricular pressure ($\pm dp/dt_{max}$). All data were recorded in computer and analyzed with MedLab biosoftware.

*Western blot analysis*.– Western blot was performed to analyze the protein levels of PLB and SERCA in rat myocardium. After hemodynamic measurement, rats were sacrificed with 10% KCl injected into the left ventricle. Left ventricles were homogenized in extracting buffer (10 mmol/L Tris-HCl, pH 7.4, 0.32 mmol/L sucrose) at 4 °C. The concentration of the protein in each lysate was determined with Coomassie brilliant blue G-250. Equal amounts of protein (40 μg) were fractionated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a nitrocellulose membrane. Non-fat milk (5%) was used to block the membrane in Tris-buffered saline (25 mmol/L Tris and 150 mmol/L NaCl, containing 0.05% Tween 20) for 2 h at room temperature. The membrane was then incubated with monoclonal antibody of PLB or SERCA (1:1000) at 4 °C over night, and incubated with a dilution of peroxidase-conjugated goat anti-rabbit IgG (1:1000) for 1 h. The immune complex was visualized by the ECL chemiluminescence method. Protein bands were analyzed with Kodak Digital Science Image Analysis System. To ensure equal loading among the lanes, values were normalized to the expression of "housekeeping" protein GAPDH. The protein expression level was determined by calculating the ratio of density metric value from each lane versus corresponding lane of GAPDH.

*Semi-quantitative RT-PCR analysis*.– Total RNA was extracted from left ventricles using TRIZOL reagent according to the manufacturer's instruction. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to analyze the level of PLB mRNA expression. GAPDH mRNA level was determined as an endogenous control. The primers for GAPDH (305bp) were: 5'-TGGTGAAGGTCGGTGTGAAC-3', and 5'-GGTGGTGAAGACGCCAGTAG-3'. The primers for PLB (141bp) were: 5'-TACCTTACTCGCTCGGCTATC-3', and 5'-CAGAAGCATCACAATGATGCAG-3'. PCR products were resolved on a 1.5% agarose gel and the bands were visualized by ethidium bromide staining. Bands were analyzed with Kodak Digital Science Image Analysis System. The gene expression level was determined semi-quantitatively by calculating the ratio of density metric value of each band versus corresponding endogenous control.

*Determination of SERCA activity*.– SR $Ca^{2+}$-ATPase activity was determined following the method of LARSEN *et al.* (9),

with some modifications. In brief, the rat left ventricular myocardium was homogenized in tissue buffer (20 mmol/L Hepes, 2 mmol/L EDTA, 250 mmol/L sucrose, pH 7.4) at 4 ºC. The myocardial tissue homogenate
(1 mg wet weight/mL) was added to reaction medium (20 mmol/L Hepes, pH 7.4, 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 0.01 ‰ Triton X-100, 100 mmol/L KCl, 0.8 mmol/L $CaCl_2$ ) and preincubated at 37 ºC for 10 min. The reaction was initiated by adding 10 mmol/L p-Npp, incubated at 37 ºC for 30 min, stopped by using double volume cold buffer (500 mmol/L Tris, 55 mmol/L EDTA), and quantified by absorption spectrophotometry at a wavelength of 410 nm.

*Statistical analysis.*– Data were expressed as mean ± SD and statistically compared by one-way analysis of variance (ANOVA) with Tukey's method. *P* values less than 0.05 were defined as statistically significant.

## Results

SERCA protein level and activity was shown in Fig. 1. Compared with control, we observed no significant alterations of myocardium SERCA protein level and SERCA activity in 4-week-DM rats. Although there was a trend toward suppression of SERCA protein expression in 6-wk-DM and 8-wk-DM rats, quantitative analysis did not reach statistical significance in both of the groups. However, compared with control, myocardium SERCA activity in 6-wk-DM rats and 8-wk-DM rats had a 29% and 32.7% decrease, respectively.

Compared with control rats, myocardium PLB mRNA level and PLB protein level were not significantly changed in

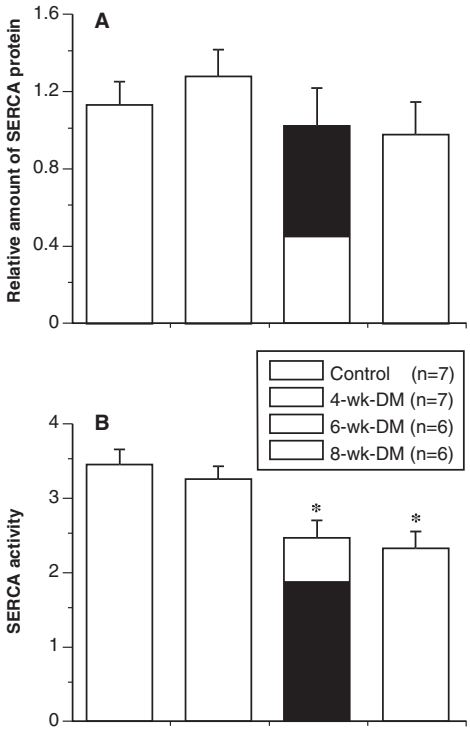


Fig 1. *Myocardium SERCA protein level analyzed by Western blot (A) and SERCA activity (B).* To ensure equal loading among the lanes, values of SERCA protein were normalized for the expression of GAPDH. SERCA activity values are in μmol/min•g. All values are mean±SD. *P< 0.05 *vs.* control.

4-wk-DM rats, but were increased in 6-wk-DM rats and 8-wk-DM rats. Moreover, there was no significant difference between the latter two groups (Fig. 2).

Table I shows the hemodynamic parameters of each group. In 4-wk-DM rats, LVSP, LVEDP and $\pm dp/dt_{max}$ were at the same level as the control group. Compared with the control rats, LVSP and $\pm dp/dt_{max}$ were markedly decreased and LVEDP increased in 6-wk-DM rats and 8-wk-DM rats. The results indicated that diabetic cardiomyopathy involved both diastolic and systolic dysfunction.

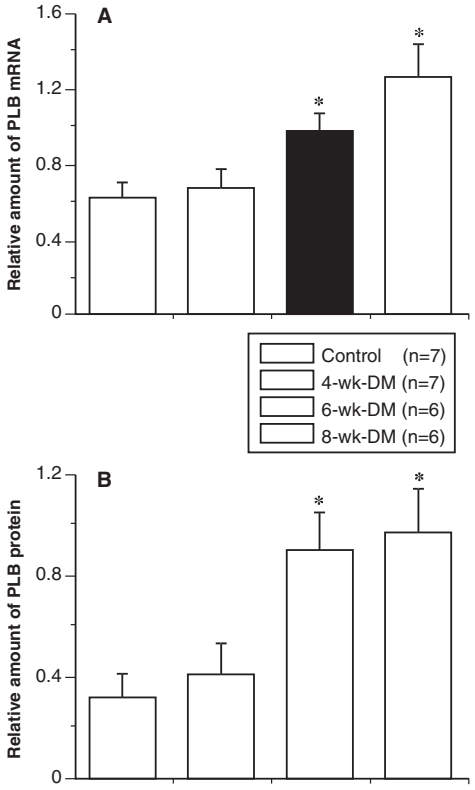


Fig 2. *Detection of myocardium phospholamban (PLB) mRNA by RT-PCR (A) and Western blot analysis for myocardium phospholamban protein expression (B).*

Gene expression level was determined semi-quantitatively by calculating the ratio of density metric value between each band and corresponding endogenous standard (GAPDH, 305bp). Values of protein expression were normalized for the expression of GAPDH. Results are expressed as mean ± SD. *P < 0.05 *vs.* control.

## Discussion

The major findings of this study demonstrated that STZ-induced diabetic rats developed cardiac dysfunction after 6 weeks. At the same time point, myocardium SERCA activity was depressed, and both myocardium PLB mRNA and protein expression level were upregulated.

One of the most important complications of DM is cardiovascular disease, and sclerosis of blood vessels was once thought as the only mechanism. However, HAMBY *et al.* (5) in 1974 demonstrated that myocardium disorder, named diabetic cardiomyopathy, contributed more to the cardiac dysfunction. But the mechanism of diabetic cardiomyopathy is unclear to date. Failing heart muscle generally exhibits distinct changes in intracellular $Ca^{2+}$ handling, including impaired removal of cytosolic $Ca^{2+}$, reduced $Ca^{2+}$ loading of the cardiac SR, and defects in SR $Ca^{2+}$ release accompanied by impairment of cardiac relaxation and systolic function. Consistent findings of a diminished $Ca^{2+}$ dependent SERCA activation were found, and increasing evidence of PLB quantity alteration, one of the underlying mechanisms for a decreased activation of SERCA, has been provided. It was found (17) that in terminally failing human myocardium from dilated cardiomyopathy, SERCA function was

Table I. *Hemodynamic measurements for control and STZ-induced diabetic rats at 4weeks, 6 weeks and 8 weeks after STZ injection*.

| Group | LVSP(mmHg) | LVEDP(mmHg) | +dp/dt(mmHg.s$^{-1}$) | –dp/dt(mmHg.s$^{-1}$) |
|---|---|---|---|---|
| Control (7) | 145.96 ± 17.62 | -0.32 ± 1.11 | 7672.46 ± 1006.88 | 7373.18 ± 664.02 |
| 4-wk-DM (7) | 139.81 ± 8.59 | 2.87 ± 2.59 | 7309.10 ± 632.35 | 5991.32 ± 574.06 |
| 6-wk-DM (6) | 84.81 ± 12.77* | 9.29 ± 3.79* | 4138.94 ± 899.41* | 3892.75 ± 488.31* |
| 8-wk-DM (6) | 78.76 ± 7.30* | 12.20 ± 4.37* | 3616.08 ± 766.38* | 3089.21 ± 706.67* |

LVSP: left ventricular systolic pressure, LVEDP: left ventricular end diastolic pressure, +dp/dt$_{max}$: the maximum velocity of ascending in intraventricular pressure. -dp/dt$_{max}$: the maximum velocity of descending in intraventricular pressure. In parenthesis, number of animals. *P< 0.05 *vs.* control.

reduced, protein levels of SERCA and PLB were unchanged even though mRNA levels of SERCA and PLB were decreased. A similar result about the myocardium SERCA and PLB protein levels was also found in human heart failure from idiopathic dilated cardiomyopathy (13).

As to diabetic rat models, several studies (2,20) have confirmed that defective intracellular $Ca^{2+}$ signaling contributed to cardiomyopathy, and transgenic overexpression of SERCA protein improved reticular $Ca^{2+}$ handling in normal and diabetic rat hearts. Considering that SERCA is the most important channel to handle $Ca^{2+}$ in cardiomyocytes and PLB is its main regulator, we detected myocardium SERCA activity, SERCA protein expression and PLB expression in DM rats at different time points. It was shown that although SERCA protein level didn't change much in diabetic rats, its activity was markedly depressed in 6-wk-DM rats and 8-wk-DM rats. In this study, unchanged PLB mRNA and protein levels were observed at 4 weeks after the initiation of DM, but increased PLB mRNA and protein were shown up 6 and 8 weeks later. The result that the decrease of SERCA activity and increase of PLB expression manifested at the same time point indicated that elevated PLB protein level inhibited SERCA activity in DM rats.

Several studies had also observed the myocardium SERCA and PLB expression in diabetic rat models and obtained discrepant results. Most reported decreased myocardium SERCA protein and increased PLB protein level in untreated diabetic rats (1, 8, 21, 22). Transgenic diabetic mouse model also demonstrated similar results (4). Other authors (12) revealed downregulated SERCA protein and unchanged PLB protein in diabetic hearts 3 weeks after STZ injection, while VASANJI *et al.* (19) found the protein levels of both SERCA and total PLB were decreased in STZ-induced diabetic rats. In the present study, we observed a tendency of myocardium SERCA protein decrease in diabetic rats compared with control, but got no statistical significance. Discrepant SERCA and PLB protein levels of diabetic myocardium in different laboratories may result from different experimental protocols, such as different time period when the animals were killed and detected. Another explanation may be the different protein extracting procedure. In the present study, as well as some others', total cellular protein was extracted and used for Western blot. But some researchers isolated membrane protein or crude membrane SR preparations or isolated SR vesicles for the detection of SERCA and PLB protein.

The most reliable method to assess cardiac function is invasive hemodynamic measurement, which showed that cardiac function was markedly depressed at 6 weeks and 8 weeks after STZ administration in this study. Compared with control, 4-wk-DM rats had unchanged LVSP, LVEDP, $\pm dp/dt_{max}$, but both 6-wk-DM rats and 8-wk-DM rats had a significant increase in LVEDP and marked decrease in LVSP and $\pm dp/dt_{max}$, which was in company with significant decrease of SERCA activity in our experiment. Therefore, it can be inferred that although SERCA protein level didn't change much, the marked decrease of its activity is more important in the occurrence and progression of cardiac dysfunction in diabetic cardiomyopathy.

Except for routine treatment for chronic heart failure, no specific therapeutics for diabetic cardiomyopathy is available

nowadays. Therefore, it is necessary to explore new forms of treatment. As a potential therapeutic strategy for heart failure, enhancing the reuptake of $Ca^{2+}$ into the cardiac SR has obtained much attention in recent years. This study warranted the supposition that inhibiting PLB expression may enhance SERCA activity, and so may improve cardiac function in DM rats.

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XIAO-YAN ZHAO, SHEN-JIANG HU, JIANG LI, YUN MOU, BAO-PING CHEN y QIANG XIA. *Disfunción cardíaca en ratas diabéticas y reducción de la actividad $Ca^{2+}$-ATPasa en retículo sarcoplásmico.* J. Physiol. Biochem., 62 (1), 1-8, 2006.

La cardiopatía diabética se caracteriza por reducción de la contractilidad cardiaca debida a cambios directos de la función del miocardio. En este trabajo se investiga acerca de las alteraciones de la actividad $Ca^{2+}$-ATPasa en retículo sarcoplásmico en miocardio y de la función cardíaca en ratas diabéticas. Se utilizaron ratas macho Wistar a las que se provocó diabetes mellitus (DM) mediante administración intraperitoneal de estreptozotocina y a un grupo control sólo se administró el vehículo. La actividad $Ca^{2+}$-ATPasa en retículo sarcoplásmico del miocardio y los parámetros hemodinámicos del ventrículo izquierdo se midieron en ratas diabéticas tras 4, 6 y 8 semanas de administración de estreptozotocina. La expresión del RNAm de fosfolamban se detectó por RT-PCR y los niveles de fosfolamban y de $Ca^{2+}$-ATPasa de retículo sarcoplásmico por Western-blot. Los resultados indican que en ratas DM, tras 4 semanas de administración de estreptozotocina, no se detectan cambios significativos en la función cardíaca, la actividad $Ca^{2+}$-ATPasa y los niveles de RNAm y de fosfolamban respecto de las ratas control. Tras 6 y 8 semanas, las ratas DM presentan disminución de la función cardíaca y la actividad $Ca^{2+}$-ATPasa con aumento de los niveles del RNAm y de la cantidad de fosfolamban. El nivel de la proteína $Ca^{2+}$-ATPasa de retículo sarcoplásmico (SERCA) no se modifica en las ratas DM respecto de las control. Los resultados de este trabajo confirman que la actividad $Ca^{2+}$-ATPasa de retículo sarcoplásmico del miocardio se reduce en las ratas con DM inducida por estreptozotocina con aumento del nivel de fosfolamban, lo que contribuye a la patogénesis de la disfunción cardíaca en ratas diabéticas.

**Palabras clave:** Diabetes, Fosfolamban, $Ca^{2+}$-ATPasa, Retículo sarcoplásmico, Función cardíaca.

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