Original Article

Phospholamban Antisense RNA Improves SR Ca^{2+}-ATPase Activity and Left Ventricular Function in STZ-induced Diabetic Rats*

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Abstract

Objective To study the effect of phospholamban antisense RNA (asPLB) on sarcoplasmic reticulum Ca^{2+}-ATPase activity and cardiac function in rats with diabetes mellitus (DM) mediated by recombinant adeno-associated virus (rAAV) vector.

Methods Six weeks after the induction of DM by streptozotocin injected intraperitoneally, the rats were divided into three groups, namely: DM-rAAV-asPLB group, DM-saline group and DM group (control group). The rats in the DM-rAAV-asPLB group were intramyocardially injected with rAAV-asPLB, the rats in the DM-saline group were injected with saline, and those in the control group did not receive any treatment. Six weeks after gene transfer, the expressions of PLB protein and PLB phosphorylation were detected by Western-blot, while the activity of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase and left ventricular function were measured.

Results The PLB protein expression level was significantly higher whereas the PLB phosphorylation, SR Ca^{2+}-ATPase activity and left ventricular function were significantly lower in the DM-saline group than in the control group. No significant difference was found in PLB protein expression level, PLB phosphorylation or SR Ca^{2+}-ATPase activity between the DM-rAAV-asPLB group and the control group. The left ventricular function in the DM-rAAV-asPLB group was poorer than in the control group and was better than in the DM-saline group.

Conclusion rAAV-asPLB can down-regulate PLB protein expression and up-regulate PLB phosphorylation and SR Ca^{2+}-ATPase activity, thus contributing to the improvement of *in vivo* left ventricular function.

Key words: Diabetes mellitus; Phospholamban; Sarcoplasmic reticulum Ca^{2+}-ATPase; Gene therapy

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INTRODUCTION

Diabetes mellitus (DM) represents an important risk factor for cardiac diseases. The Framingham study has revealed that the incidence of congestive heart failure in diabetics is independent of age, arterial hypertension, and coronary artery disease[1]. One of the vital features of heart failure in diabetics is the development of special cardiomyopathy in the absence of vascular complications. Diabetic cardiomyopathy is characterized by ventricular

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systolic/diastolic dysfunction which results from alterations in calcium-handling proteins, such as Na^+/Ca^{2+} exchange and sarcoplasmic reticulum (SR) Ca^{2+}-ATPase[2].

Phospholamban (PLB) is a pivotal regulator for Ca^{2+} affinity in cardiac SR Ca^{2+}-ATPase. Ablation of PLB in mice results in stimulated SR Ca^{2+} uptake and enhanced contractile performance. It has been reported that adenoviral gene transfer of antisense PLB improves contractile function of myocardial cells from human failing hearts[3]. PLB is shown to be a potential target for improving the function of failing hearts[4].

Recently, we have constructed a recombinant adeno-associated virus vector containing gene encoding phospholamban antisense RNA (rAAV-asPLB), demonstrating that rAAV-asPLB is able to disrupt the expression of PLB, enhance the activity of SR Ca^{2+}-ATPase and reduce the resting $[Ca^{2+}]$ in cardiomyocytes of neonatal rats[5].

The above studies have failed to elucidate whether PLB is able to improve the SR Ca^{2+}-ATPase activity and left ventricular function in diabetic rats. Therefore, the present study is aimed to assess the effect of rAAV-asPLB on diabetes-induced cardiomyopathy.

MATERIALS AND METHODS

Reagents

Streptozotocin (STZ) was purchased from Sigma. The AAV Helper-Free System was obtained from Stratagene. The system consists of pAAV-MCS containing inverted terminal repeat (ITR) of adeno-associated virus, multiple clone site (MCS), cytomegalovirus enhance/promoter and SV 40 poly-adenylation. pAAV-RC contains AAV replication and capsid genes. pHelper contains adenovirus-derived genes (i.e. E2A, E4, and VA RNA gene). M-MLV reverse transcriptase was obtained from Promega. Pyrobest DNA polymerase was purchased from Takara. Mouse monoclonal antibodies of PLB and goat anti-mouse immunoglobulin G (IgG) were from ABR. Enhanced chemiluminescence (ECL) kit was obtained from Amersham Crop (USA).

Construction of Recombinant Adeno-Associated Virus

Construction of the rAAV-asPLB has been described elsewhere[5]. In brief, rat PLB cDNA fragment was synthesized by RT-PCR from total RNA isolated from the heart of Wistar rats. The pAAV-asPLB was generated by cloning the PLB cDNA in reversed orientation relative to the promoter of pAAV-MCS. pAAV-asPLB, pAAV-RC, and pHelper were co-transfected into AAV293 cells.

Induction of Diabetic Mellitus (DM) in Rats

Male Wistar rats (8 weeks old) weighing 220-250 g were randomly divided into model group (n=24) and normal control group (n=6). Rats in the model group received a single intraperitoneal injection with 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). Rats in the normal control group received an equivalent volume of citrate buffer alone. DM was verified by overt physical signs including lack of weight gain, polydipsia and polyuria, and confirmed by urine analysis and serum glucose level tests. All rats were housed under similar conditions on a 12 h light/dark cycle at a temperature of 21±1 °C and a humidity of 55%±5%, and fed with the same diet and water *ad libitum*. The Institutional Animal Care and Use Committee at Zhejiang University approved all experimental procedures.

Intramyocardial rAAV-asPLB Injection

The rats with DM were randomized into DM-rAAV-asPLB group, DM-saline group, and DM group (control group), 8 in each group. Rats in the DM-rAAV-asPLB group received rAAV-asPLB gene transfer, rats in the DM-saline group were injected with saline, and those in the control group did not receive any treatment. Six weeks later, the rats were anesthetized with chloral hydrate (400 mg/kg), endotracheally intubated, and mechanically ventilated with room air (55-65 breaths per minute, tidal volume of 2.5 mL). Left anterior thoracotomy was performed under sterile conditions to expose the heart and pericardium. One hundred Ml (1×10^9 IU) rAAV-asPLB was injected into the left ventricle apex and free wall in the DM-rAAVV-asPLB group with a 30-gauge needle. Saline equal to that of the virus solution was injected into the DM-saline group with the same procedures as used in the DM-rAAV-asPLB group. The lungs were then reinflated and the chest incision was closed. Five rats were sacrificed in each of the control and DM-saline groups and 6 rats were sacrificed in the DM-rAAV-asPLB group. Activity of sarcoplasmic

reticulum (SR) Ca^{2+}-ATPase and the left ventricular function were measured.

Hemodynamic Measurements

Six weeks after gene transfer, hemodynamic measurements were performed. The rats were anesthetized with chloral hydrate (400 mg/kg). A 20-gauge catheter-tip pressure transducer was introduced into the left ventricle through the right carotid artery for measurements of left ventricular pressure (LVSP), maximal rates of pressure development and decline. ($\pm dP/dt_{max}$) and LV end-diastolic pressure (LVEDP).

Western Blot Analysis

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. An equal amount of protein (40 μg/lysate) was fractionated on 12% sodium dodecyl sulfate-polyacrylamide 1-dimensional gel electrophoresis (SDS-PAGE) and electrophoretically transferred on to a nitrocellulose membrane. Non-fat milk (5%) in Tris-buffered saline (25 mmol/L Tris and 150 mmol/L NaCl, containing 0.05% Tween 20) was used to block non-specific sites of the membrane for 2 h at room temperature. Then the membrane was incubated with mouse monoclonal antibodies of PLB (1:1000) at 4 °C over night, and incubated with a dilution of peroxidase-conjugated goat anti-mouse IgG (1:500) for 1 h. The immune complexes were visualized by the ECL chemiluminescence method. Each protein band was analyzed on Kodak Digital Science Image Analysis System. The protein expression level was determined by calculating the ratio of density metric value from each group versus the normal control.

Phospholamban Phosphorylation

Left ventricles were homogenized in extracting buffer at 4 °C. The concentration of the protein in each lysate was determined with Coomassie brilliant blue G-250. An equal amount of protein (40 μg/ lysate) was warmed at 37 °C for 30 min prior to electrophoresis to dissociate fully PLB into its monomeric form. Following electrophoresis, proteins were transferred to a nitrocellulose membrane, which was probed with the Anti-phospho-Phospholamban (Ser16) antibody (1:500, upstate company, USA). The nitrocellulose membrane was incubated with peroxidase-conjugated goat anti-mouse IgG (1:5000). The measurement of PLB phosphorylation was the same as described for total PLB level.

SR Ca^{2+}-ATPase Activity

SR Ca^{2+}-ATPase activity was determined following the methods of Larsen[6], with some modification. 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 °C. 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‰ TritonX-100, 100 mmol/L KCl, 0.8 mmol/L $CaCl_2$) and pre-incubated at 37 °C for 10 min. The reaction was initiated by adding 10 mmol/L p-Npp, incubated at 37 °C 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 LSD method. A *P* value less than 0.05 was defined as statistically significant.

RESULTS

Construction of rAAV-asPLB and Characteristics of Rats with DM

The rAAV-asPLB was constructed with AAV Helper-Free System. The rAAV-asPLB was propagated and purified. The titer of rAAV-asPLB used in the present experiments was 1×10^{10} infectious units/mL[5].

After streptozotocin injection for one week, the DM rats were verified by overt physical signs including lack of weight gain, polydipsia and polyuria, and confirmed by blood sugar detection. Body weight were lower in the model group rats than that in the normal control group (219.0±7.71 *vs* 405.17± 11.69 g) ($P<0.05$). The plasma glucose level was elevated in the model group rats (27.4±1.4 mmol/L) compared with that in the normal control group rats (7.6±.0.2 mmol/L) ($P<0.05$) (Table 1).

Protein Content of PLB

Western-blot was performed to investigate the

expression of PLB protein. Six weeks after treatment, the PLB protein content in the DM rats and DM-saline rats was higher than that in the NC group and the DM-rAAV-asPLB group. However, the PLB protein level in the DM-rAAV-asPLB group was similar to that in the NC group (Figure 1).

Protein Content of PLB Phosphorylation

The levels of phosphorylated PLB protein were decreased in the DM group and the DM-saline group compared with those in the DM-rAAV-asPLB group and the NC group. There was no significant difference between the DM-rAAV-asPLB group and the NC group (Figure 2).

SR Ca^{2+}-ATPase Activity

Compared with the NC group, SR Ca^{2+}-ATPase activity was decreased in the DM and DM-saline groups. However, Ca^{2+}-ATPase activity was similar as that in the DM-rAAV-asPLB rats after gene transfer for six weeks (Figure 3).

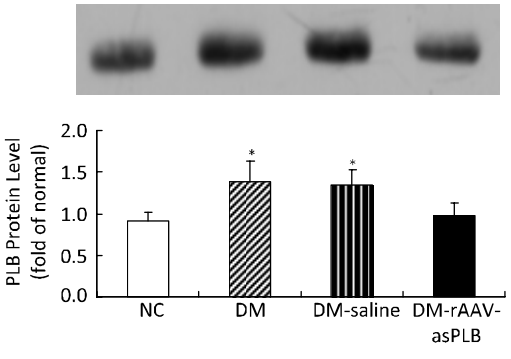

Figure 1. The PLB protein content was analyzed by western blot. The results are mean±SD. The upper panel shows protein bands from a typical experiment. NC: normal control group; DM: diabetes mellitus group; DM-saline: diabetes mellitus with saline injection group; DM-rAAV-asPLB: diabetes mellitus rats with rAAV-asPLB gene transfer. $^{*}P$<0.05 *vs.* normal control.

Left Ventricular Function

Assessed by LVSP, LVEDP and ±d*P*/d*t*, left ventricular dysfunction developed in all the DM groups, and a tendency of left ventricular function improvement was observed in six weeks after rAAV-asPLB treatment. The depression of LVSP, ±d*P*/d*t* with an elevation of LVEDP was detected in

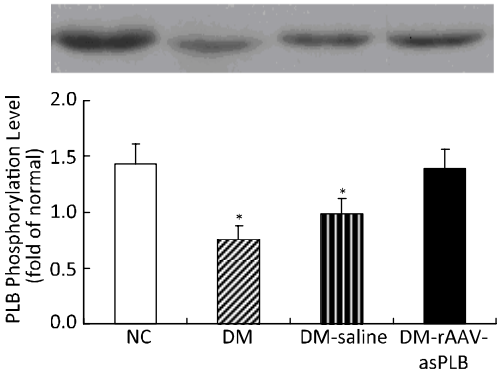

Figure 2. The phosphorylated PLB (PS) protein content was analyzed by western blot. The upper panel shows protein bands from a typical experiment. NC: normal control group; DM: diabetes mellitus group; DM-saline: diabetes mellitus with saline injection group; DM-rAAV-asPLB: diabetes mellitus rats with rAAV-asPLB gene transfer. $^{*}P$<0.05 *vs.* normal control.

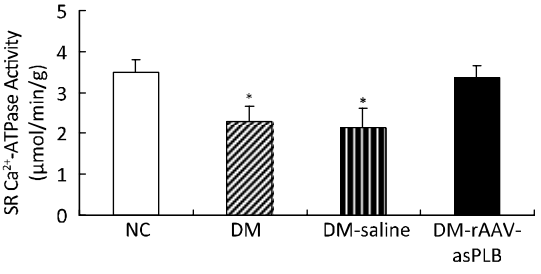

Figure 3. SR Ca^{2+}-ATPase activity from the left ventricles of each group. Data are mean±SD and expressed as $\mu mol \cdot min^{-1} \cdot g^{-1}$ wet weight. $^{*}P$<0.05 , each group *vs.* control.

Table 1. Characteristics of Normal Group and Diabetic Rat Model Group

Groups	Blood Sugar (mmol/L)	Heart Weight (mg)	Body Weight (g)	Cardiosomatic Ratio
Normal Group	7.6±0.2	1007.67±16.38	405.17±11.69	2.49±0.06
Model Group	27.4±1.4*	625.80±21.37*	219.00±7.71*	2.86±0.05*

Note. $^{*}P$<0.05 *vs.* normal group.

the DM group, DM-saline group and DM-rAAV-asPLB group compared with that in the NC group. However, LVSP and ±d*P*/d*t* were ascended, the LVEDP was descend in the DM-rAAV-asPLB group compared with those in the DM and DM-saline groups. At the same time, LVSP, LVEDP and ±d*P*/d*t* were similar as compared with those in the DM and DM-saline groups (Figure 4).

DISCUSSION

Cardiovascular mortality was about 2- to 4-fold higher in diabetics than in non-diabetics. Furthermore, diabetics were 2.5 times more likely to develop congestive heart failure as compared to non-diabetics[7]. Therefore, it was pertinent to identify novel drugs which might alleviate cardiac dysfunction in diabetics. Our previous work had demonstrated that rAAV-asPLB infection could decrease the PLB protein expression in cardiomyocytes of neonatal rats. In the present study, we delivered rAAV-asPLB to explore the potential therapeutic effects in STZ-induced diabetic cardiomyopathy. To our knowledge, this was the first report on the study of using rAAV-asPLB as a gene therapy strategy for diabetic cardiomyopathy, though other different gene therapy strategies for diabetic cardiomyopathy had been performed[8].

However, there were several limitations to the application of gene transfer for heart failure. The approach to direct injection of plasmid DNA vectors into the left ventricular myocardium was limited by the lower efficiency of cardiomyocyte transduction. While, the adenovirus-mediated gene transfer was been restricted by immune responses to viral and foreign transgene protein. Adeno-associated viruses (AAV) featured versatility in host range, long-term gene transfer potential, and minimum immunoreaction. Iwanaga Y, et al. examined the effects of a pseudophosphorylated form of phospholamban mutant using recombinant AAV (rAAV) vector in rats with heart failure after myocardial infarction, and found that PLB inhibition by rAAV gene transfer was an effective strategy for the chronic treatment of this acquired form of heart failure[9].

Several evidences depicted an essential role of intracellular Ca^{2+} dysregulation, reduced contractility, prolonged duration of contraction and relaxation in the onset and progression of diabetic cardiomyopathy[10]. Cardiomyocyte relaxation and contraction were tightly controlled by the activity of the cardiac SR Ca^{2+} pump. Correcting SR Ca^{2+}-ATPase activity would constitute a therapeutic approach to improve the contractility of the failing heart.

PLB was a transmembrane protein that interacted with and inhibited SR Ca^{2+}-ATPase activity by lowering its apparent Ca^{2+} affinity. Ca^{2+}-ATPases

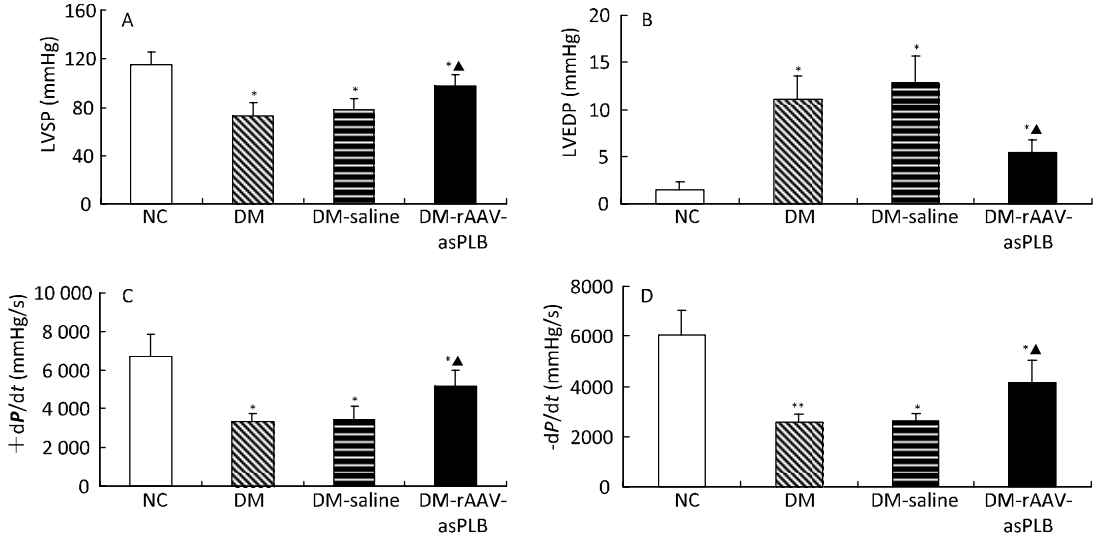

Figure 4. The alterations of hemodynamics parameters of each group. A: LVSP, left ventriclar pressure; B: LVEDP, left ventriclar end-diastolic pressure; C and D: $\pm dp/dt_{max}$, maximal rates of pressure development and decline. NC: normal control group; DM: diabetes mellitus group; DM-saline: diabetes mellitus with saline injection group; DM-rAAV-asPLB: diabetes mellitus rats with rAAV-asPLB gene transfer. $^{*}P<0.05$ *vs.* NC group $^{\blacktriangle}P<0.05$ *vs.* DM group and DM-saline group.

functioned to maintain low cytoplasmic calcium levels, acting to resequester calcium into the SR lumen after each contractile event, thus controlling relaxation rates in the heart[11]. Increased SR Ca^{2+}-ATPase activity might also be caused by an alteration of phosphorylated state of PLB. The unphosphorylated PLB inhibited SR Ca^{2+}-ATPase activity, whereas the phosphorylated PLB led to enhanced pumping activity. PLB phosphorylation disrupted the inhibition on SR Ca^{2+}-ATPase activity and subsequently activated Ca^{2+} uptake into the SR.

Consistent with previous studies[12], we also found that diabetes increased expression of PLB and decreased phosphorylated PLB protein, resulting in loss of SR Ca^{2+}-ATPase activity in the DM group and the DM-saline group. PLB protein and phosphorylated PLB protein were normalized, and the same effect was observed of SR Ca^{2+}-ATPase activity after rAAV-asPLB was injected for six weeks in the DM-rAAV-asPLB group. The data suggested that PLB was increased while phosphorylated PLB decreased in STZ-induced diabetic cardiomyopathy and meanwhile a decreased SR Ca^{2+}-ATPase activity could significantly affect cardiac function in diabetic cardiomyopathy[13].

Prolongation of relaxation and reduction in cardiac contractions force were root causes of the increased incidence of morbidity and mortality among diabetic patients. The cardiac function of STZ-induced diabetic rats was determined by hemodynamic measurements. Compared with the NC group, a reduced LVSP and $\pm dP/dt$ with an elevated LVEDP was observed in the DM, DM-saline and DM-rAAV-asPLB groups. The LVSP and $\pm dP/dt$ were increased, accompanying decrease of LVEDP in the DM-rAAV-asPLB group as compare with those in the DM and DM-saline groups (Figure 4). The results indicated that the diabetic cardiomyopathy was characterized with impaired left ventricular systolic/diastolic function. The heart failure was developed in all the DM rats. However, after rAAV-asPLB transfer, the heart failure was partly rescued and the left ventricular function was improved[14-15].

Similarly, rAAV-asPLB reduced the inhibitive effect of PLB on SR Ca^{2+}-ATPase, which contributed to enhanced SR Ca^{2+}-ATPase activity and thus resulted in improved left ventricular function in DM rats. rAAV-asPLB might serve as a promising target for the treatment of diabetic cardiac mechanical dysfunction.

In order to achieve better results, the following aspects need to be addressed as a follow up to the current study. First, it is necessary to explore more efficient approach to gene transferring, such as by intravenous or intracoronary injection. Since direct intra-myocardium injection was used in the present study because of rather small rats, further efforts should be made to ascertain whether the gene could be administered into the myocardium. Second, due to the fact that the depression of PLB expression and the restored phosphorylated PLB protein resulted in limited LV function improvement, further studies would be warranted for a gene therapy strategy in diabetic cardiomyopathy, for example, an exploration of other more effective gene targets or combined gene targets.

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