Decrease in calcium-sensing receptor in the progress of diabetic cardiomyopathy

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1. Introduction

Diabetes mellitus (DM) is characterized by elevated blood glucose levels resulting from the body’s inability to produce insulin or resistance to insulin action, or both [1]. The most prevalent form (90-95%) of DM is type 2 diabetes (non-insulin-dependent) [2]. Diabetic cardiomyopathy (DCM), a serious DM complication, can cause heart failure, arrhythmia, cardiac shock and sudden death [3]. However, the underlying molecular mechanisms on DCM largely remain unclear [4].

The extracellular calcium-sensing receptor (CaSR) is a member of the G protein-coupled receptors superfamily, whose primary function is to regulate parathyroid hormone...
secretion and renal Ca\(^{2+}\) reabsorption, thus it is crucial to the control of extracellular Ca\(^{2+}\) homeostasis [5]. In 2003, our team first found that CaSR exist functionally in rat cardiac tissue [6], and sequentially demonstrated that CaSRs are involved in cardiac ischemia/reperfusion injury, apoptosis, calcium overload, endoplasmic reticulum stress, ischemic post-conditioning, cardiac hypertrophy and atherosclerosis, etc. [7–10]. However, the potential role of CaSR in the progress of DCM has not been examined.

The activation of CaSR can cause intracellular Ca\(^{2+}\) increase by inducing calcium release from sarcoplasmic reticulum (SR) and opening of storage operated calcium channel (SOCC).

In addition, other players in storage operated calcium entry/calcium release activated calcium currents such as STIM1/Orai1 in the myocardium [11].

With this background, the aim of this study is to observe changes in cardiac CaSR expression in type 2 diabetic rats, and will discuss potential mechanisms.

2. Materials and methods

2.1 Establishment of type 2 diabetes rats

Forty male Wistar rats, 180–200 g, were provided by the Experimental Animal Center of Harbin Medical University. Type 2 diabetic models were prepared as previously described [12]. All rats were housed in a temperature-controlled room (22–24 °C) and kept on a 12-h:12-h light–dark cycles in quiet environment. All rats received humane care in accordance with the principles of the Chinese Council on Animal Care. After one week’s adaptation, the rats were randomly divided into control, diabetic-4 weeks (dia-4w), diabetic-8 weeks (dia-8w) and spermine treatment (dia + sper) groups. Control rats were maintained on standard rat chow and tap water ad libitum. Diabetic rats were fed with high-fat and high-sugar chow (ingredients: 20% sucrose, 10% refined lard, 2% cholesterol, 1% sodiumcholate and 67% common food). One month later, experimental rats were induced by an intraperitoneal injection of a low dose of streptozotocin (STZ, Biosharp, Japan, 30 mg/kg), dissolved in 0.1 M citrate buffer (pH 4.4). The blood glucose level was detected after 3 days, only the rats with blood glucose level ≥16.7 mM were considered to be diabetic and used in our experiments. Age-matched control rats were injected with a vehicle alone. The diabetic rats maintained on high-fat and high-sugar and tap water ad libitum for 4 or 8 weeks. The rats in dia + sper group received 240 μM of spermine (lower than toxic dose) [13] in their drinking water for 4 weeks. Blood glucose, food and water intake levels were determined on a weekly basis. After the end of experiment, all animals were sacrificed and their hearts were stored at −80 °C for further studies.

2.2 Serum measurements

The blood samples were centrifuged and sera were stored at −80 °C until assay. Random serum insulin levels were determined by a commercially available ultrasensitive ELISA kit (Morinage Institute of Biological Science Inc., Kanagawa, Japan). Triacylglycerol (TG) and cholesterol (CHE) in serum were analyzed using a standard biochemistry panel (Senlo, Zuhuai, Guangdong). Random blood glucose in blood samples from tail vein was measured using a blood glucose analyzer (ACCU CHEK, Roche, Germany).

2.3 Echocardiographic analysis of cardiac structure and function

Cardiac function and dimensions were assessed using an echocardiography system (GE VIVID7 10S, USA). Echocardiography was performed on self-breathing rats under anesthesia (intraperitoneal injection of 10% chloral hydrate at 0.3 ml/100 g body weight) as described previously [14]. Heart rate was kept relatively constant throughout the procedure. The following left ventricular (LV) parameters were measured: left ventricle end-systolic lumen diameter (ESLD), fractional shortening (FS), ejection fraction (EF), the early transmitial peak diastolic flow velocity (E), peak early diastolic tissue velocity (E'). The ratio of E to E' (E/E' ratio) was calculated for analyzing cardiac diastolic function. All parameters represent the mean of 3 consecutive cardiac cycles.

2.4 Morphologic study

After echocardiography assessment, the rats were anesthetized, hearts were rapidly removed and washed with phosphate buffered saline solution. The cardiac tissues fixed in 10% buffered formalin were embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin (HE) for light microscopic morphologic study.

2.5 Transmission electron microscopy

For transmission electron microscopy, rat left ventricular tissue was fixed in 2.5% glutaraldehyde, cut into small blocks (1 mm\(^3\)), and fixed at 4 °C for 8 h. Images of the longitudinal sections were obtained after fixation, soaking, stepwise alcohol dehydration, displacement, embedding, polymerization, sectioning, and staining and observed with an electron microscope (H-7650, Japan). Random sections were taken and analyzed by two technicians blinded to the treatments.

2.6 Isolation of cardiomyocytes from diabetic rats

Ventricular myocytes were isolated from normal and diabetic rats according to previously described techniques [15]. Hearts were removed rapidly after anesthetization, connected to the Langendorff apparatus and perfused at a constant flow and at 37 °C with a Tyrode’s solution containing (mM): 140 NaCl, 5.4 KCl, 0.6 MgCl\(_2\), 0.6 Na\(_2\)HPO\(_4\), 1 NaHCO\(_3\), 10 HEPES, 1.8 CaCl\(_2\), and 5.55 glucose (pH 7.4), bubbled with O\(_2\) at 37 °C for 5 min. After stabilization, the hearts were perfused with Ca\(^{2+}\)-free Tyrode’s solution for 5 min, then with Ca\(^{2+}\)-free isolation solution containing 1 mg/ml collagenase II (Sigma, St. Louis, MO) for 30 min. Isolated myocytes were kept in modified Kraft-Brühe solution containing (mM) 30 KCl, 10 KH\(_2\)PO\(_4\), 0.5 MgCl\(_2\), 70 glutamic acid, 10 glucose, 10 HEPES, 15 taurine and 0.5 EGTA (pH 7.4 with KOH). The percentage of viable cells in control and diabetic groups were >70%. Subsequently, cells were kept at 4 °C until used for following experiments.
The intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{i}\)]) was determined according to the manufacturer’s instructions (Molecular Probes) and previously described method [9]. In brief, the isolated cells were stained using 5 \(\mu\)M fluo-3 AM (Invitrogen, Burlington, ON) for [Ca\(^{2+}\)\(_{i}\)], for 30 min at 37 °C in the dark. Then the cells were rinsed with Ca\(^{2+}\)-free Tyrode’s solution to remove the remaining dye. Fluorescence measurement of Ca\(^{2+}\) was performed using a laser confocal scanning microscope (Olympus, LSM, Japan) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm for [Ca\(^{2+}\)\(_{i}\)]. Following a 60 s baseline recording, cells were exposed to Ca\(^{2+}\) (3 mM) or Gd\(^{3+}\) (300 \(\mu\)M), then intracellular fluo-3 fluorescence measurements were recorded for 300 s at 3 s intervals. In some experiments, the cardiomyocytes preincubated with different specific inhibitors, such as NiCl\(_{2}\) (1 mM, inhibitor of Na\(^{+}-\)Ca\(^{2+}\) exchanger), CdCl\(_{2}\) (0.2 mM, inhibitor of L-type calcium channel), NPS2390 (10 \(\mu\)M, antagonist of CaSR) (Sigma, St. Louis, MO) [9], for 20 min before Ca\(^{2+}\) (3 mM) or Gd\(^{3+}\) (300 \(\mu\)M) challenge. Image analysis was performed off-line using FluoView-FV300 (Olympus, Japan) to select cell regions from which FI was extracted, and further analysis was conducted with Excel (Microsoft) and Origin Version 7.5 software (OriginLab Corporation). [Ca\(^{2+}\)\(_{i}\)] changes were expressed as fluorescence intensity representing FI and normalized to initial fluorescence intensity (FI).

### 2.7. Fluo-3/AM measurements of [Ca\(^{2+}\)\(_{i}\)]

<table>
<thead>
<tr>
<th>Water intake (ml/day)</th>
<th>Food intake (g/day)</th>
<th>Glucose (mM)</th>
<th>CHE (mM)</th>
<th>TG (mM)</th>
<th>Insulin (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28 ± 2.1</td>
<td>12.5 ± 1.1</td>
<td>5.2 ± 0.2</td>
<td>1.15 ± 0.13</td>
<td>0.34 ± 0.05</td>
</tr>
<tr>
<td>Dia-4w</td>
<td>180 ± 5.6</td>
<td>38.5 ± 2.3</td>
<td>29.6 ± 2.8</td>
<td>21.07 ± 2.61</td>
<td>4.09 ± 0.37</td>
</tr>
<tr>
<td>Dia-8w</td>
<td>230 ± 6.2</td>
<td>45 ± 2.9</td>
<td>26.8 ± 2.5</td>
<td>19.87 ± 1.31</td>
<td>3.79 ± 0.57</td>
</tr>
</tbody>
</table>

CHE, cholesterol; TG, triglyceride.
- *p < 0.05 vs. control.
- **p < 0.05 vs. dia-4w, n = 10.

### 3. Results

#### 3.1. General features of experimental animals

Type 2 diabetic rats were induced by high-fat and high-sugar diet and additional intraperitoneal injection of STZ in this study. Compared with control group, the water and food intake, the concentration of glucose, CHE, TG, and insulin were significantly increased in diabetes groups (Table 1). These results suggested that our type 2 diabetic models were established successfully.

#### 3.2. Histological changes in myocardium of diabetic rats

HE staining on heart tissues showed that the myocardial cells were well-arranged, with clear cellular nucleus and the cytoplasm stained in uniformity in control group; while in dia-4w group, the myocardial cells lined up in disorder, the sizes of cellular nucleus were irregular, and the myocardial fibrils were broken and disordered; the heart tissues appeared severe disruption of contractile apparatus: hypercontracted muscle and thickened fibers in dia-8w group; the mentioned cardiac injuries were lightened in dia + sper group (Fig. 1A).

#### 3.3. Transmission electron microscopy

Using transmission electron microscopy, we observed that cardiac fibers with regular arrays and clear Z-lines were abundant, and the mitochondria were typically intact in control group; however, the mitochondria and the myofibrils were swollen and disrupted slightly in dia-4w group, these change were more severe in dia-8w group; and structural abnormalities were significantly ameliorated by spermine treatment (Fig. 1B).

#### 3.4. Echocardiographic assessment of diastolic and systolic function

Echocardiographic assessment showed that in diabetic hearts, left ventricle ESLD and E/E’ were increased, FS and EF were reduced significantly, compared with the control group, suggesting impaired contractile and diastolic function of the left ventricle in diabetic rats. Compared with dia-4w group, treatment with spermine could relieve the above-mentioned changes (Fig. 2).

#### 3.5. Protein expression of the CaSR in diabetic rats’ hearts

To observe the CaSR expression in diabetic heart, immuno-blotting was used in this study. Compared with control group,
3.6. **Mediation of intracellular calcium concentration ([Ca\textsuperscript{2+}]i)**

The [Ca\textsuperscript{2+}]i was detected by laser-scanning confocal microscope (LSCM). The results showed that the fluorescence intensity (FI) of [Ca\textsuperscript{2+}]i in both the NiCl\textsubscript{2} + CdCl\textsubscript{2} + CaCl\textsubscript{2} (or GdCl\textsubscript{3}) group and the NPS2390 + CaCl\textsubscript{2} (or GdCl\textsubscript{3}) group was decreased than CaCl\textsubscript{2} (or GdCl\textsubscript{3}) group (p < 0.05), but higher than that in control group (p < 0.01), and the FI of [Ca\textsuperscript{2+}]i was decreased significantly in the NiCl\textsubscript{2} + CdCl\textsubscript{2} + NPS2390 + CaCl\textsubscript{2} (or GdCl\textsubscript{3}) group (p < 0.01 vs. CaCl\textsubscript{2} or GdCl\textsubscript{3} group).

Fig. 3 was a representative record using LSCM for CaCl\textsubscript{2}. The experimental results for GdCl\textsubscript{3} were similar with the experiments for CaCl\textsubscript{2}, therefore they did not be placed in this paper.

3.7. **Protein expressions of PKC-α, PLN, SERCA and RyR in cardiac tissues of type 2 DM rats**

The protein expression of PKC-α and PLN were increased, while the protein expression of SERCA and RyR were reduced in cardiac tissues of type 2 DM rats, compared with control group. Moreover, above-mentioned the changes of protein expression were more obvious in dia-8w group in comparison with dia-4w group (Fig. 4C–F).

4. **Discussion**

DCM is a serious DM complication, which can cause heart failure and sudden death. To explore molecular mechanisms underlying DCM, the establishment of a reliable animal model of DCM is critical. In this study, high-fat, high-sugar diet and intraperitoneal injection of low dose of STZ were used to build rat diabetes models. Compared with control group, the water and food intake, the serum concentration of glucose, CHE, TG and insulin were increased significantly in diabetic model groups (see Table 1), indicating the successful establishment of type 2 DM model.

Doppler echocardiography is an excellent non-invasive and practical imaging tool for defining cardiac structure and function [16]. Ventricular systolic function in vivo is determined by ESLD, EF and FS, and the ratio of E/E’ is used as a parameter of LV diastolic dysfunction [17,18]. In present study, we observed that ESLD was increased, EF and FS were decreased, while E/E’ was elevated in diabetic groups. Furthermore, obvious swelling, vacuolating and disruption of mitochondria and myofibrils were observed in cardiac tissues from type 2 DM rats. All systolic and diastolic dysfunction, and ultrastructural injuries in diabetic rats were well corresponds to the characteristics of DCM.

The mechanism for DCM is very complicated. CaSR is a G protein-coupled receptor. Our team previously found that hyperlipidemia and atherosclerosis could up-regulate myocardial CaSR expression, promote myocardial apoptosis, aggravate oxidative stress and myocardial ischemia [7,9], pointing to the potential role of CaSR in heart dysfunction.

Present study first found that the CaSR protein expression of myocardium in diabetic rats was decreased with a time-dependent manner. Other groups also observed that the CaSR expression were reduced in kidney of type 1 diabetic rats [19] and in mesenteric artery of Zucker diabetic fatty rats [20]. The blood glucose is a potent stimulator for synthesis of diacylglycerol (DAG) and free fatty acid (FFA). Thus hyperglycemia could increase the synthesis of FFA and DAG, which further activate PKC, which acts as a negative regulator for CaSR expression [21]. Therefore increase of PKC could induce decrease of CaSR expression. The decrease of cardiac CaSR expression in diabetic rats suggests that CaSR maybe involve in DCM.
Fig. 2 – Echocardiographic assessment of left ventricular diastolic and systolic function in diabetic rats. ESLD, end-systolic lumen diameter; EF, ejection fraction; FS, fractional shortening; E, early transmitral peak diastolic flow velocity; E', Peak early diastolic tissue velocity. ★ p < 0.05 vs. control; ▲ p < 0.05 vs. dia-4w; n = 6.

Fig. 3 – The [Ca\textsuperscript{2+}]\textsubscript{i} was detected by a laser-scanning confocal microscope. The cardiomyocytes in the experimental group were pretreated with CaCl\textsubscript{2} (3 mM), NiCl\textsubscript{2} (1 mM), CdCl\textsubscript{2} (0.2 mM) or NPS2390 (10 \mu M) for 5 min. The cells in control group were incubated in Ca\textsuperscript{2+}-free Tyrode’s solution.
As we know, Ca\(^{2+}\) is an excitation–contraction coupling factor, and the increase of [Ca\(^{2+}\)]\(_{\text{cyt}}\), is dependent on extracellular Ca\(^{2+}\) entry and Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores, such as sarcoplasmic reticulum (SR) or mitochondria. In addition to L type of calcium channel, store-operated Ca\(^{2+}\) entry (SOCE) also is an important Ca\(^{2+}\) influx pathway, which is regulated by the filling state of intracellular Ca\(^{2+}\) stores, notably SR. Reduction in [Ca\(^{2+}\)]\(_{\text{cyt}}\) results in activation of plasma membrane Ca\(^{2+}\) release activated Ca\(^{2+}\) (CRAC) channels that mediate sustained Ca\(^{2+}\) influx which is required for many cell functions as well as refilling of Ca\(^{2+}\) stores. ORAI1 acts as the pore-forming subunit of the CRAC channel in the plasma membrane. STIM1 is localized in the ER, senses [Ca\(^{2+}\)]\(_{\text{ER}}\), and activates the CRAC channel upon store depletion by binding to ORAI1 [22].

To investigate whether decrease of CaSR expression could cause disbalance of intracellular Ca\(^{2+}\), [Ca\(^{2+}\)]\(_{\text{cyt}}\), changes in control and diabetic groups under different treatment were detected. We found that FL of [Ca\(^{2+}\)]\(_{\text{cyt}}\), was decreased in both the NiCl\(_2\) (an inhibitor of Na\(^{+}\)–Ca\(^{2+}\) exchanger) + CdCl\(_2\) (a specific blocker of L type of calcium channel) + CaCl\(_2\) (or GdCl\(_3\)) group and the NPS2390 (an antagonist of CaSR) + CaCl\(_2\) (or GdCl\(_3\)) group, and NiCl\(_2\) + CdCl\(_2\) + NPS2390 could abolish the increase of [Ca\(^{2+}\)]\(_{\text{cyt}}\), induced by 3 mM Ca\(^{2+}\) (or 300 \(\mu\)M GdCl\(_3\)). These data indicated that Na\(^{+}\)–Ca\(^{2+}\) exchanger, L type of calcium channel and CaSR are involved in the increase of [Ca\(^{2+}\)]\(_{\text{cyt}}\), induced by extracellular Ca\(^{2+}\) or Gd\(^{3+}\) increase.

During systole, the extracellular Ca\(^{2+}\) enters into cardiac cell through L type of calcium channel. The resulting increase in intracellular Ca\(^{2+}\) triggers further Ca\(^{2+}\) release from the SR through the RyR, raising Ca\(^{2+}\) levels around the sarcomere. During diastole, intracellular Ca\(^{2+}\) be removed by sarcoplasmic Ca\(^{2+}\)–ATPase pump and Na\(^{+}\)–Ca\(^{2+}\) exchanger or Ca\(^{2+}\) reuptake through the SERCA. In this study, we found that the response of diabetic myocardium to extracellular 3 mM Ca\(^{2+}\) or 300 \(\mu\)M Gd\(^{3+}\) was decreased, which possible reason was the decrease of CaSR expression in cardiac tissues of type 2 DM rats.

Precise control of intracellular Ca\(^{2+}\) homeostasis is central for regulating myocardial function. Ca\(^{2+}\) homeostasis is directly regulated by the Ca\(^{2+}\)-cycling proteins, such as RyR, IP\(_3\) receptor, the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase pump (SERCA) [23]. The decrease of SERCA pump activity usually is
the result of either a decreased expression of SERCA itself or an increase of its inhibitory PLN. During each heartbeat, Ca$^{2+}$ enters the cardiomyocyte through L-type channels, triggers further Ca$^{2+}$ release from the SR through the RyR, transiently induced cytosolic Ca$^{2+}$ binds to the thin filament troponin C and activates the contractile machinery. Ca$^{2+}$ uptake through the SERCA pump is negatively regulated by PLN [24]. Ca$^{2+}$ reuptake into the SR by SERCA, and consequent declines in cytoplasmic Ca$^{2+}$ allows for cardiac relaxation.

CaSR activation could induce increase of [Ca$^{2+}$], via G protein-PLC-IP$_3$ pathway [6,25], and PKC exerts negative effects on CaSR’s function [26]. A recent study reported that PKC-α-deficient mice exhibit increased cardiac contractility and are less susceptible to heart failure following long-term pressure-overload stimulation [27]. To further explore the mechanism for calcium disorder in DCM, we detected the expression of PKC-α, RyR, SERCA and PLN using Western blot. The results showed that the expression of PKC-α and PLN were elevated, whereas the expression of SERCA (SERCA/PLN) and RyR were reduced in the diabetic rats. These changes, which are consistent with echocardiographic findings in other murine models of type 2 DM [28,29], can partially explain, at least, the cause of systolic and diastolic dysfunction in DCM rats. The increase of PKC-α, as a negative regulator of CaSR expression, certainly results in decrease of CaSR expression. Decrease of SERCA/PLN and RyR could cause disorder of Ca$^{2+}$ release and reuptake for SR. All alteration could induce intracellular calcium disbalance and following cardiac disfunction for DCM.

To demonstrate the role of CaSR in development of DCM, spermine (an agonist of CaSR) was used in this study. We found that CaSR agonist could improve cardiac function and prevent or slow the progression of DCM. It indicates that CaSR is both cause and effect (to say in other words, is an important component element) in progression of DCM.

Certainly, our findings in rodents cannot directly extrapolate to humans, because rodents are different from humans in species.

In conclusion, the present study found for the first time that CaSR expression is decreased in myocardium of type 2 DM rats and possibly related with disbalance of calcium homeostasis, which could induce systolic and diastolic dysfunction. Therefore increasing CaSR expression and recovering calcium homeostasis possibly become a new therapeutic target for DCM.

**REFERENCES**


