

Original Article

Enhanced store-operated Ca²⁺ entry in high glucose-cultured neonatal and adult diabetic rat cardiomyocytes

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Abstract: The imbalance of intracellular Ca²⁺ homeostasis is a major problem in diabetic cardiomyopathy. Store-operated Ca²⁺ entry (SOCE) is an important Ca²⁺ signalling pathway in excitable and non-excitable cells, and mediates Ca²⁺ influx contributing to the maintenance of intracellular Ca²⁺ homeostasis. Our study aimed to explore the SOCE changes and its contribution on Ca²⁺ overloading in the ventricular muscle of diabetic rats. We used thapsigargin, an endoplasmic reticulum Ca²⁺ pump inhibitor, to deplete Ca²⁺ store. SOCE activities were significantly enhanced in streptozotocin-induced adult diabetic rat left ventricular muscle and high glucose-cultured neonatal cardiomyocytes. In addition, store-operated currents were significantly increased in neonatal left ventricular muscle cells cultured in high glucose medium. We further used immunoblotting to identify that the expression levels of several crucial SOCE components were dramatically upregulated in the high glucose-cultured neonatal left ventricular muscle and streptozotocin-induced adult diabetic rat left ventricular muscle. We conclude that SOCE activity was enhanced in the high glucose-cultured neonatal left ventricular muscle and streptozotocin-induced adult diabetic rat left ventricular muscle. Our finding may shed new light on the mechanism of intracellular Ca²⁺ overloading in diabetic cardiomyocytes and potential value of SOCE for clinical treatment of diabetic cardiomyopathy.

Keywords: Diabetic cardiomyopathy, ventricular muscle, store-operated Ca²⁺ entry, orai, stromal interaction molecule

Introduction

The endocrine disease diabetes mellitus has become a serious health problem in recent years. With the development of diabetes mellitus, various organ complications may occur, including those involving the aorta, kidney, retina and the heart [1]. Diabetic cardiomyopathy, initially identified by Rubler, is defined as a distinctive cardiomyopathy resulting from hyperglycaemia rather than hypertension, coronary disease and other aetiologies [2]. Recent studies have shown that the hyperglycaemia affects the electrophysiological properties of the heart through decreased energy usage in the cardiomyocytes and by destroying the function of the ion channels in the plasma membrane [3-5].

Eventually, the dysfunction of the ion channels causes a decrease in the depolarisation amplitude, and the prolongation of the action potential duration and QT interval, which induces arrhythmia, heart failure, cardiogenic shock, and even sudden death [6, 7]. As a critical secondary messenger, intracellular Ca²⁺ ([Ca²⁺]_i) is closely involved in various cellular processes, including muscle contraction, cell excitability, motility, apoptosis, proliferation and others [8]. Major causative reasons for the left ventricular dysfunction in diabetic cardiomyopathy are the imbalance of [Ca²⁺]_i homeostasis and the impaired endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) function in the cardiomyocytes [9].

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Store-operated Ca^{2+} entry (SOCE) is a common signalling pathway that regulates intracellular Ca^{2+} homeostasis in living cells [10]. Both in excitable and non-excitable cells, SOCE is involved in numerous critical functions, including proliferation, gene expression, contraction and secretion [11-13]. As the two key components of SOCE, STIM (stromal interaction molecule) located in the membrane of ER/SR is the Ca^{2+} sensor to sense Ca^{2+} concentration in the ER/SR, whereas Orai in the plasma membrane forms Ca^{2+} channels mediating Ca^{2+} entry [14]. To date, three Orai isoforms, including Orai1, Orai2 and Orai3, and two STIM isoforms, STIM1 and STIM2 have been identified [15]. According to the literature, all three Orai isoforms have positive effects on SOCE. Among the isoforms, Orai1 may play a more important role in SOCE and is well documented in a variety of cells [16]. Additionally, STIM1 may interact with Orai channels to initiate SOCE [14]. However, the function of STIM2 in SOCE has not yet been adequately clarified. Some studies have shown that STIM2 inhibits STIM1-initiated SOCE. However, in cases of STIM1 impairment, STIM2 will assume the role of STIM1 to initiate SOCE [17-20]. Accumulating evidence suggests that STIM1 and Orai1 are the two key components of SOCE [21, 22]. Recently, several studies demonstrated that Orai1 and STIM1 had a pivotal role in the maintenance of cardiomyocyte $[\text{Ca}^{2+}]_i$ homeostasis [23-25] and in the development of cardiomyocyte hypertrophy [26-28]. However, these results indicated that Orai1 and STIM1 had contrary effects on these processes. Orai1 might have a compensatory effect in slowing dilated cardiomyopathy; however, the knock-down of STIM1 was able to reduce cardiomyocyte hypertrophy. Therefore, the function of SOCE in cardiomyocytes remains incompletely understood, particularly in disease states.

In the present study, we used various techniques to investigate the alteration of SOCE and the underlying mechanisms in neonatal or adult cardiomyocytes in high glucose-cultured condition or diabetic rats.

Materials and methods

Animals

Male Sprague-Dawley (SD) rats (200-250 g) were obtained from the Animal Centre, Anhui Medical University. All of the rat experiments were conducted in accordance with NIH publi-

cation no. 8523 and approved by the Animal Experimentation Ethics Committee of Anhui Medical University. Diabetic rats were induced by an intraperitoneal injection of 60-mg/kg streptozotocin (STZ, Biosharp company) [29]. STZ was dissolved in 10 mM citrate buffer (sodium citrate/citric acid) at a pH of 4.5 immediately prior to its use. The diabetic rats were considered to be successfully induced when the caudal vein blood glucose levels were ≥ 20 mmol/L. The control male SD rats were treated with an equivalent volume of sterile normal saline. All of the animals were supplied ample food and water. All experiments were performed after 8 weeks from the time when the animals received the STZ injection. Every rat blood glucose was monitored continuously every week.

Primary culture of neonatal left ventricular muscle cell

Left ventricular muscle cells were isolated from neonatal SD rat according to previous study [30]. Briefly, the rat left ventricle was excised and minced in phosphate-buffered saline (PBS) (140 mM NaCl, 3 mM KCl, 25 mM Tris pH 7.4) at 4°C. Minced ventricular tissues were incubated in 0.09% trypsin at 37°C for 10 min. The supernatants were collected and centrifuged at 900 g for 5 min. The pellet was then re-suspended in DMEM supplemented with 20% fetal bovine serum, 100 $\mu\text{g}/\text{mL}$ penicillin, and 100 units/mL streptomycin. The cells were cultured at 37°C in a humidity-controlled incubator with 5% CO_2 . In high glucose (HG) treatment, the concentration of D-glucose was 25 mM. In normal glucose (NG) group, 20 mM α -mannitol was added into NG medium containing 5 mM D-glucose for an osmotic control. Electrophysiological recording was performed after the cardiomyocytes were cultured for 7 days in NG or HG medium.

Electrophysiological recording

Whole-cell patch-clamp recording was performed at room temperature (22-24°C) using an EPC-10 amplifier and Patchmaster software (HEKA). Patch pipette had a resistance of 2-5 M Ω . To record SOC, the follow solutions and setting were used [31]. The standard bath solution contained (in mM): 135 NaCl, 5 KCl, 2 CaCl_2 , 2 MgCl_2 , 10 glucose, 10 HEPES, pH 7.4 adjusted with NaOH. The pipette solution consisted of (in mM): 140 CsCl, 8 NaCl, 10 EGTA, 10 MgCl_2 , 10 HEPES, pH 7.2 adjusted with

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CsOH. The membrane potential was clamped at 0 mV during recording and a voltage ramp from -80 mV to +80 mV in 500 ms duration was applied every 2 s. Data analysis was performed using Patchmaster software. Series resistance compensation was used and set to 60%-80%.

Histochemical analysis

The rats were sacrificed via an overdose of CO₂. The left ventricular muscle tissues were excised and fixed for 24 h in 4% paraformaldehyde, and then washed for 24 h in PBS (pH 7.4). The samples were processed by dehydration, wax infiltration and embedding. Subsequently, the tissues sectioned at a thickness of 5 µm for staining with haematoxylin and eosin (HE). Histological images were obtained using a digital camera on an Olympus BX51 microscope (Olympus).

Measurement of intracellular Ca²⁺ concentration ([Ca²⁺]_i)

Measurement of [Ca²⁺]_i was performed as described in our previous study [32]. Fresh left ventricular muscle tissue was cut into small pieces about 10 × 5 × 1 mm³ in volume. The tissue was pinned on to a rubber block. The tissue block or primary cultured-cells were loaded with 10 µM Fluo-8/AM and 0.02% pluronic F-127 (Invitrogen) for 1 h at 37°C in Krebs-Henseleit solution contained 1 µM verapamil to remove Ca²⁺ influx by voltage-gated Ca²⁺ channels. The Ca²⁺ stores of the cardiomyocytes were depleted by treatment with 4 µM thapsigargin (TG) for 10 min in Ca²⁺-free saline, which contained the following in mM: NaCl 118, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, and glucose 11.1, at pH 7.4. Ca²⁺ influx was evoked by the subsequent application of 2.5 mM extracellular Ca²⁺. The fluorescence signals were recorded by a Ca²⁺ image system (Nikon T200 fluorescence microscopy). The excitation and emission wavelengths were at 488 nm and 518 nm. The changes of [Ca²⁺]_i were analysed as a ratio of the fluorescence relative to the intensity before the application of extracellular Ca²⁺ (F1/F0).

Western blotting

Western blotting has been described elsewhere [32]. The rat heart muscle was quickly isolated and placed into ice-cold Krebs-Henseleit solution. The left ventricular muscle

tissue was resected and weighed. The proteins from the left ventricular muscle tissue or primary cultured cells were extracted using a detergent extraction buffer, which contained 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, and 2.5 mM sodium pyrophosphate, supplanted with protease inhibitor cocktail tablets. A total of 100 µg of protein was loaded onto each lane and separated on a SDS/PAGE gel. The PVDF membrane carrying transferred proteins was blocked in 5% non-fat dry milk in TBST (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween-20 at pH 7.5) for 1 h at room temperature and incubated at 4°C overnight with anti-Orai1, anti-Orai2, anti-Orai3, anti-STIM1 or anti-STIM2 primary antibody (1:200) alone. Immunodetection was performed by the treatment with horseradish peroxidase-conjugated secondary antibody (1:5000) (Irritant NA934V), followed by processing through an ECL detection system. Autoradiography was detected using an enhanced chemiluminescence development system (Bio-Rad Laboratories, Richmond, CA, USA). The optical density of each blot was normalised to that of β-tubulin analysed within the same lane and represented as relative optical density.

Statistical analysis

The data were expressed as the means ± SEM of the indicated number of samples. Statistical analysis for Student's t-tests was performed routinely using SigmaPlot software. The value of *P* < 0.05 was considered as statistical significance.

Results

Effect of high glucose environment on SOCE and SOC in neonatal rat ventricular muscle cells

Many studies have demonstrated that abnormal Ca²⁺ homeostasis occurred in the cardiomyocytes of diabetic rats [33-35]. Here, we investigated the SOCE signalling pathway in the left ventricular muscle. First, we used HG medium containing 25 mM D-glucose to mimic diabetic hyperglycemia in vitro and NG medium containing 5 mM D-glucose as control, and chose neonatal rat ventricular muscle cell as our study object. Literature showed that HG medium treatment for 7 days might markedly

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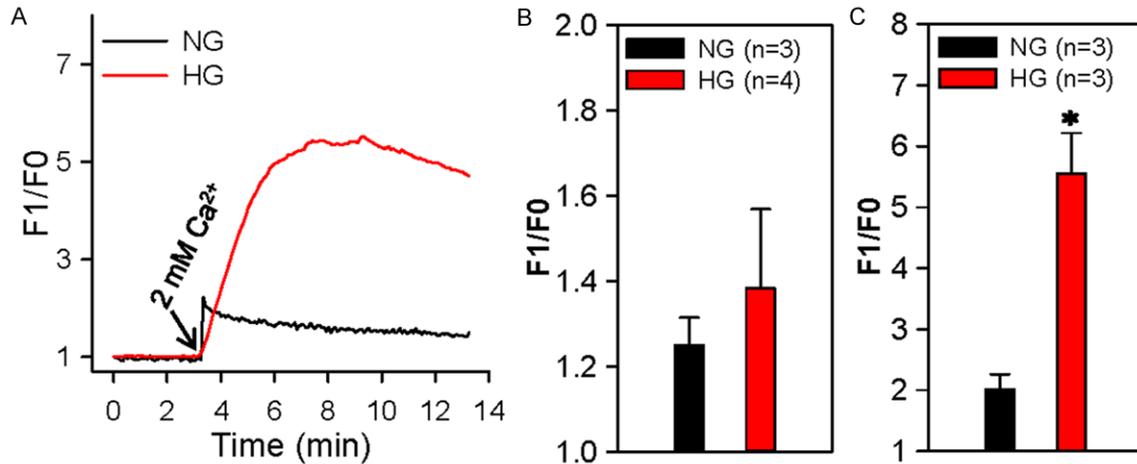


Figure 1. Changes of store-operated Ca²⁺ entry in high glucose (HG)-medium cultured neonatal rat left ventricular muscle cells. (A-C) Representative traces (A) and summarized data (B, C) showing 4 μ M thapsigargin (TG)-induced intracellular Ca²⁺ ([Ca²⁺]_i) increase (B) in HG (25 mM)-cultured neonatal left ventricular muscle cells. (A and C) After TG treatment for 10 min, the application of 2 mM Ca²⁺ induced an [Ca²⁺]_i increase known as store-operated Ca²⁺ entry. The representative traces (A) and summarized data (C) showing store-operated Ca²⁺ entry in HG-cultured neonatal left ventricular muscle cells. Values are means \pm SEM (n = 3-4 samples). *P < 0.05 compared to the values of normal glucose (NG, 5 mM) cultured cells.

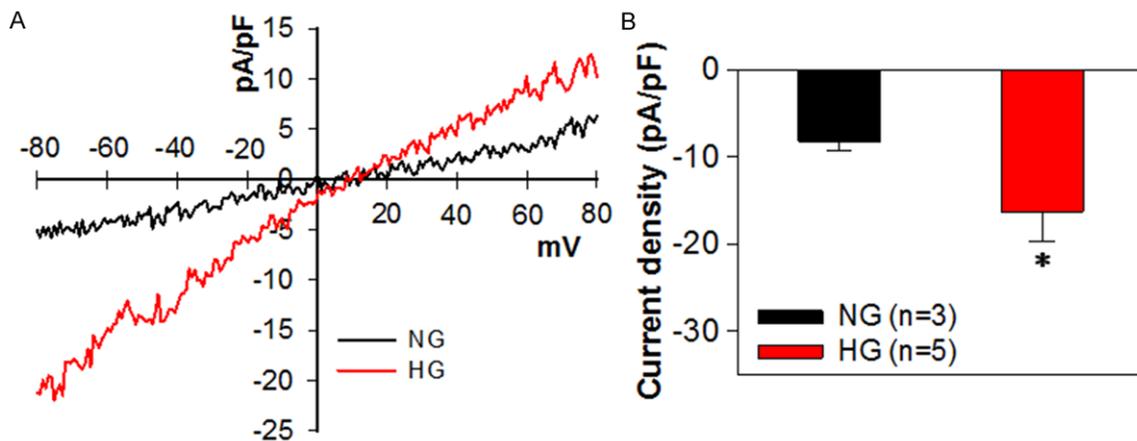


Figure 2. Changes of store-operated current (SOC) in high glucose (HG)-medium cultured neonatal rat left ventricular muscle cells. A. The I-V relationships of the SOC at the maximal current after TG application. B. Summarized data showing the current density at -80 mV in NG and HG groups. Values are means \pm SEM (n = 3-5 cells). *P < 0.05 compared the values of NG cultured cells.

enhance SOCE in mesangial cells [36]. Therefore, we also treated the neonatal rat ventricular muscle cells for 7 days in NG and HG medium respectively. After the cells were primarily cultured in the NG and HG medium for 7 days, the Ca²⁺ store was depleted by the application of 4 μ M TG, which is an endoplasmic reticulum Ca²⁺ pump inhibitor in Ca²⁺-free solution. Then SOCE was evoked by the subsequent application of 2 mM extracellular Ca²⁺. Our [Ca²⁺]_i measurement data showed that SOCE was dramatically increased in HG-cultured cells, but TG-

induced transient [Ca²⁺]_i was not increased compared to that in NG-cultured cells (Figure 1).

To confirm the effect of HG culture on SOCE, we further employed patch clamp to verify the alteration of current evoked by store depletion in HG-cultured neonatal rat left ventricular muscle cells. TG (4 μ M) application induced store operated current (SOC) (Figure 2). The ramp model of whole-cell patch-clamp recording was utilized to measure SOC. The data

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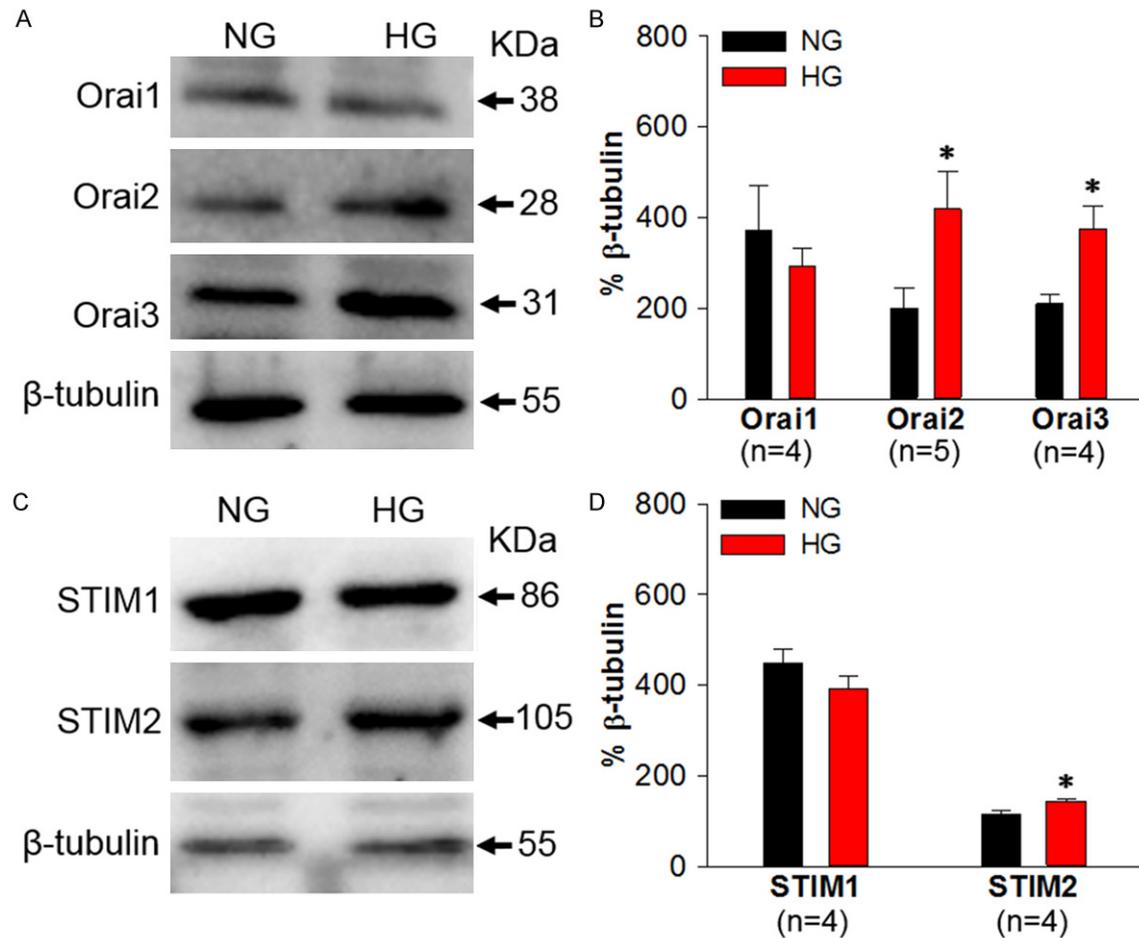


Figure 3. Expression changes of Orai and STIM proteins in high glucose (HG)-medium cultured neonatal rat left ventricular muscle cells. Representative western blotting images (A and C) and summarized data (B and D) of Orai (A and B: Orai1, Orai2, Orai3) and STIM (C and D: STIM1, STIM2) proteins in the neonatal left ventricular muscle cultured in normal glucose (NG, 5 mM) and HG (25 mM) mediums. β -tubulin was used as the loading control. Values are means \pm SEM (n = 4-5 samples). *P < 0.05 compared to the values of NG cultured cells.

showed that TG-induced whole-cell ramp current was significantly augmented in HG-cultured cells compared to that in NG-cultured cells (**Figure 2A**). At -80 mV, TG-induced SOC density in HG-cultured neonatal rat left ventricular muscle cells was also significantly increased compared to that in NG-cultured cells (**Figure 2B**). These results indicate that HG environment is able to amplify SOCE of neonatal rat ventricular muscle cells.

Effect of HG environment on the expression of Orai and STIM proteins in neonatal rat left ventricular muscle

Orai and STIM proteins are crucial components involving SOCE process. Therefore, the changes of Orai and STIM protein expression levels may affect the intensity of SOCE. We then used

western blotting to investigate the expression changes of all isoforms of Orai and STIM in HG-cultured neonatal rat left ventricular muscle. Our data showed that the expression levels of Orai2, Orai3 and STIM2 were significantly increased, but Orai1 and STIM1 protein expression levels did not altered in HG-cultured cells compared to those in NG-cultured cells (**Figure 3**). The results suggest that HG environment may enhance SOCE by increasing Orai2 and Orai3 protein expression in neonatal rat left ventricular muscle.

Changes of body weight, heart weight, blood glucose, heart/body weight ratio and heart rate in diabetic rats

To further verify our finding obtained from primary cultured cell, we next used STZ to gener-

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Table 1. Changes in body weight, heart weight, blood glucose, heart rate and heart/body weight ratio at the 8th week

	Control rats (n = 19)	Diabetic rats (n = 18)
Body weight (g)	433.4±69.5	284.7±48.2*
Heart weight (g)	1.19±1.52	0.96±0.14
Blood glucose (mmol/L)	7.9±3.3	24.4±3.6*
Heart rate (bpm)	347.5±58.4	310.2±54.1
Heart/body weight (%)	0.28±0.03	0.34±0.03*

Values are expressed as the means ± SEM for each group of animals. *P < 0.05 level of significance, compared to the values in the control group.

ate type 1 diabetic rats. After STZ treatment for 8 weeks, rat body weight was significantly reduced as we had expected. However, the blood glucose levels and heart/body weight ratios were significantly increased compared to the control rats (**Table 1**). In contrast, the heart rates were decreased in the STZ-treated rats, although the change was not significant (**Table 1**). The data suggested that we had successfully generated diabetes via the STZ injection.

Changes in left ventricular muscle histology of diabetic rats

We employed HE staining to observe the histological changes of the left ventricular muscle. The images revealed that the muscle cells in diabetic rats were swollen and hypertrophic (**Figure 4**).

Changes of SOCE in diabetic rat left ventricular muscle

The result of above cultured cell indicated that SOCE was enhanced in HG environment. Hence, we also examined the alteration of SOCE in the left ventricular muscle cells of the diabetic rats. Similarly, we used TG to deplete Ca^{2+} store and evoke SOCE. We observed that 4 μ M TG induced a transient $[Ca^{2+}]_i$ rise in the left ventricular muscle cells in both control and diabetic rats (**Figure 5A**); however, the TG-induced $[Ca^{2+}]_i$ increase was significantly reduced in the diabetic rats (**Figure 5A** and **5B**). Afterwards, SOCE was evoked by the subsequent application of 2.5 mM extracellular Ca^{2+} . Interestingly, the $[Ca^{2+}]_i$ increase via SOCE was strongly enhanced in the left ventricular muscle cells of the diabetic rats compared with the control rats (**Figure 5C** and **5D**). Additionally, if the tissues

were pretreated with 10 μ M SKF-96365 which is a SOCE channel inhibitor, TG-induced $[Ca^{2+}]_i$ increase was not affected but SOCE was dramatically inhibited both in the control and diabetic rats (**Figure 5B** and **5D**).

Expression profile of Orai and STIM proteins in diabetic rat left ventricular muscle

To uncover the reason of increased intensity of SOCE, we utilized western blotting to examine the changes of Orai and STIM protein expression levels. The results demonstrated that the expression levels of SOCE components, including Orai1, Orai2 and STIM1 were significantly increased in the left ventricular muscle of the diabetic rats (**Figure 6**). However, the Orai3 expression level was only slightly increased (**Figure 6A** and **6B**). In contrast, the STIM2 expression level was markedly decreased (**Figure 6C** and **6D**).

Several studies have reported that Ca^{2+} release channel inositol 1, 4, 5-triphosphate receptor (IP_3R) expression levels were decreased in kidney, pancreatic acini and aortic smooth muscle cells in STZ-induced diabetic animals [37-39]. Here, we also investigated type I IP_3R (IP_3R-I) protein expression. Our data indicated that IP_3R-I expression was notably reduced in the left ventricular muscle of the diabetic rats (**Figure 7**).

Discussion

In the present study, we investigated the changes in SOCE activity and the expression levels of SOCE components of the HG-cultured neonatal left ventricular muscle and STZ-induced adult diabetic rat left ventricular muscle. Compared with the control group, the diabetic rats had higher heart/body weight ratios but lower heart rates. By HE staining, the diabetic rats exhibited swollen and hypertrophic left ventricular muscle cells. The data regarding $[Ca^{2+}]_i$ measurements indicated that the activity of SOCE was significantly enhanced in the HG-cultured neonatal left ventricular muscle and STZ-induced adult diabetic rat left ventricular muscle. Meanwhile, electrophysiological results showed that SOC was significantly increased in the neonatal left ventricular muscle cells cultured in HG medium. Moreover, the protein expression results in the diabetic rats provided evidence that the SOCE components, including

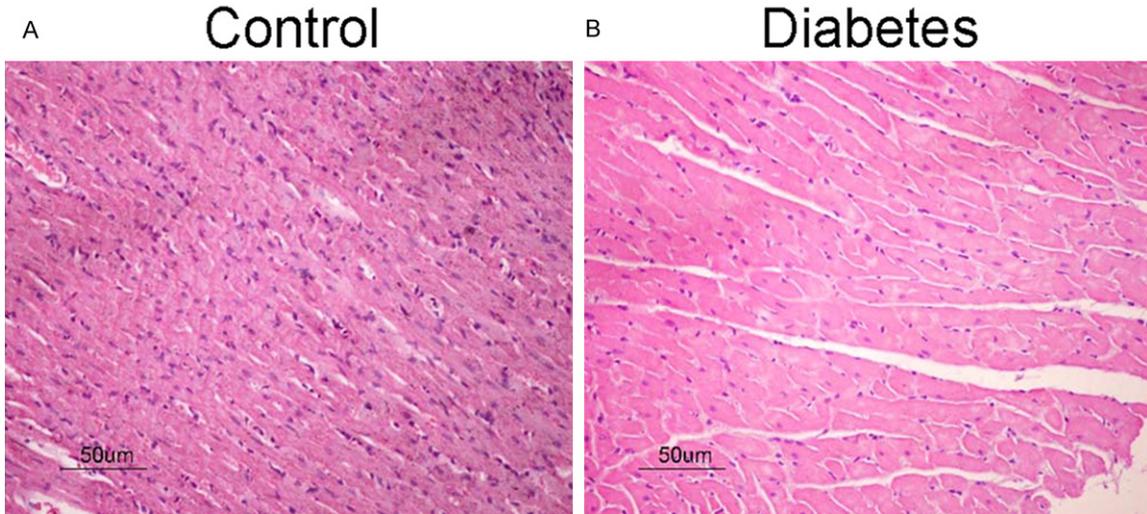
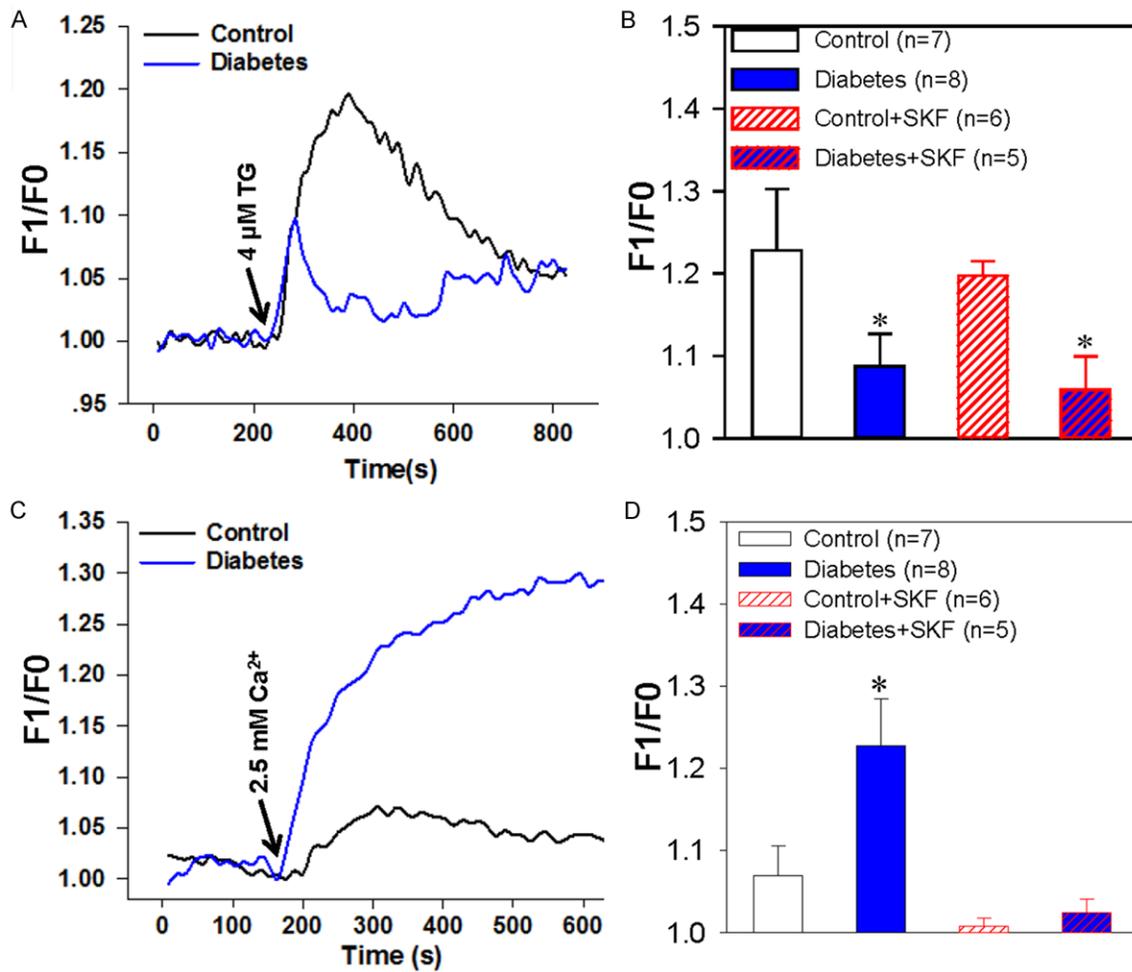


Figure 4. Changes in left ventricular muscle histology of diabetic rats. (A and B) Representative HE staining images showing the left ventricular muscle tissues from control (A) and diabetic (B) rats. The experiments were repeated in four control and diabetic rats.



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Figure 5. Changes of store-operated Ca^{2+} entry in diabetic rat left ventricular muscle cells. (A and B) Representative traces (A) and summarized data (B) showing 4 μM thapsigargin (TG)-induced intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) increase in diabetic and control rat cardiomyocytes with or without 10 μM SKF-96365. (C and D) After TG treatment for 10 min, the application of 2.5 mM Ca^{2+} induced an $[\text{Ca}^{2+}]_i$ increase known as store-operated Ca^{2+} entry. The representative traces (C) and summarized data (D) showing store-operated Ca^{2+} entry in diabetic and control rat cardiomyocytes with or without 10 μM SKF-96365. Values are means \pm SEM (n = 5-8 rats). *P < 0.05 compared to the values of age control rats.

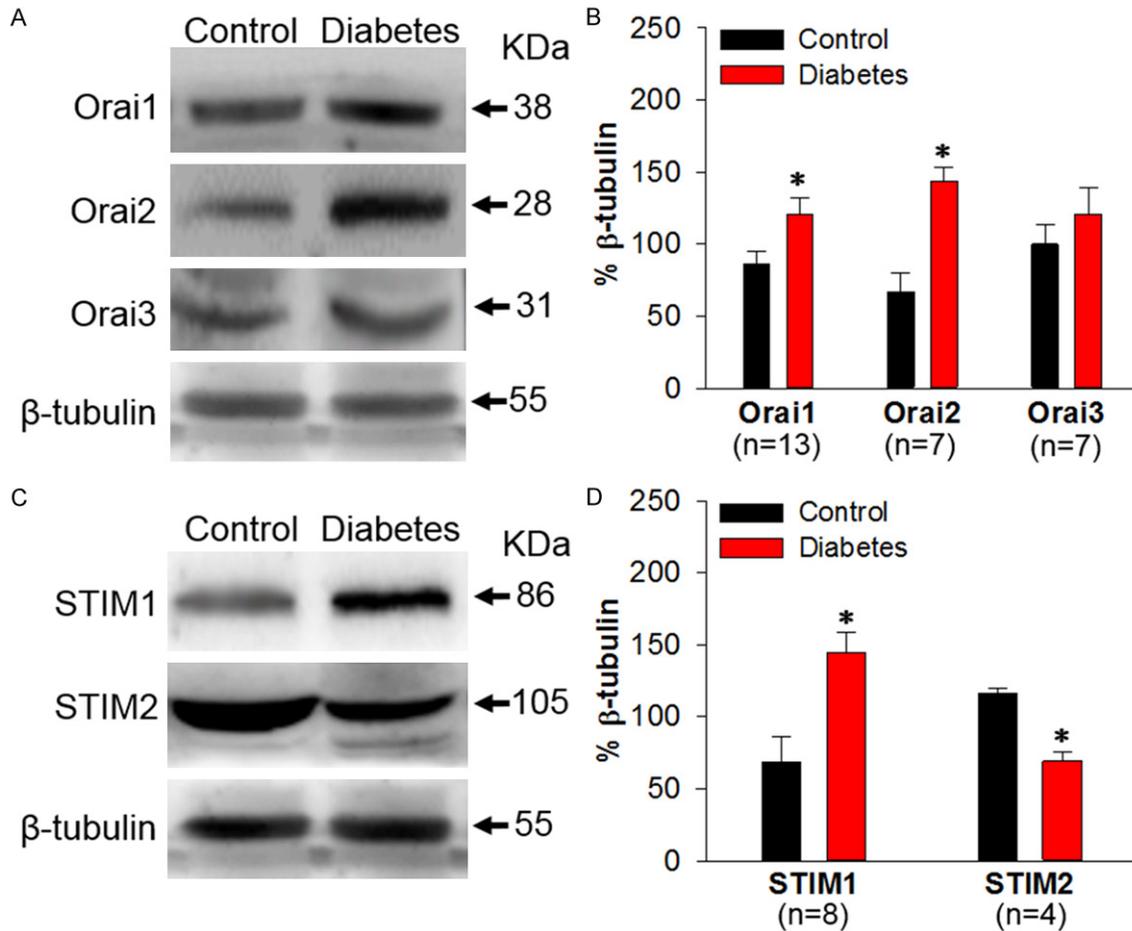


Figure 6. Expression changes of Orai and STIM proteins in diabetic rat left ventricular muscle. Representative western blotting images (A and C) and summarized data (B and D) of Orai (A and B: Orai1, Orai2, Orai3) and STIM (C and D: STIM1, STIM2) proteins in the left ventricular muscle from diabetic and control rats. β -tubulin was used as the loading control. Values are means \pm SEM (n = 4-13 rats). *P < 0.05 compared to the values of age control rats.

Orai1, Orai2 and STIM1 were significantly up-regulated in the left ventricular muscle of the diabetic rats, but Orai3 expression levels were only slightly increased. Contrarily, STIM2 and $\text{IP}_3\text{R-I}$ expression levels were decreased. In HG-cultured neonatal cells, the expression levels of Orai2, Orai3 and STIM2 were significantly increased, but Orai1 and STIM1 protein expression levels were not altered. Taken together, SOCE was significantly enhanced in HG-cultured neonatal and diabetic left ventricular muscle

cells, which may be caused by the increased expression of SOCE-related proteins.

It is well known that the continuous accumulation of $[\text{Ca}^{2+}]_i$ will cause Ca^{2+} overload in cardiomyocytes, which induces the impairment of energy production and the further promotion of oxidative stress in diabetes. Precise control of $[\text{Ca}^{2+}]_i$ homeostasis is crucial for the regulation of myocardial function and growth [40]. At present, SOCE is considered to be the unique mech-

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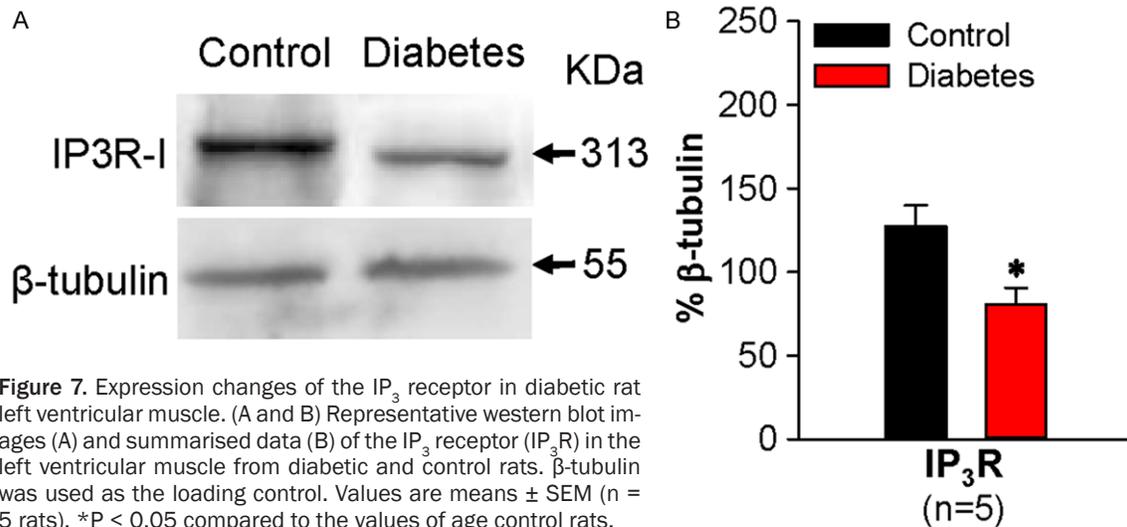


Figure 7. Expression changes of the IP₃ receptor in diabetic rat left ventricular muscle. (A and B) Representative western blot images (A) and summarised data (B) of the IP₃ receptor (IP₃R) in the left ventricular muscle from diabetic and control rats. β-tubulin was used as the loading control. Values are means ± SEM (n = 5 rats). *P < 0.05 compared to the values of age control rats.

anism for cellular Ca²⁺ entry triggered by Ca²⁺ store depletion and participates in numerous biological processes. Commonly, SOCE is known as an important pathway for Ca²⁺ entry to replenish intracellular Ca²⁺ stores [41]. Depletion of Ca²⁺ stores can occur physiologically as part of signalling events, as for example in the activation of G protein-coupled receptors on the plasma membrane, leading to the IP₃-mediated release of Ca²⁺ from the ER/SR via IP₃R [42]. Long-term hyperglycaemia may generate Ca²⁺ overload and impair the ER/SR in the cardiomyocytes [43]. According to our results, we observed that SOCE was enhanced in the neonatal left ventricular muscle cultured in HG medium or the left ventricular muscle from the adult diabetic rats. Moreover, SOC was significantly increased in the primary cultured neonatal left ventricular muscle cells in HG medium. Enhancement in SOCE and SOC may be due to the upregulated Orai2 and Orai3 expression levels. Two studies also support HG culture and the diabetes as capable of enhancing SOCE in mesangial cells and platelets [36, 44]. However, several other studies showed that advanced glycation end product which is accumulated in a HG environment, HG culture and hyperglycemia suppressed SOCE in the mesangial cells and neonatal cardiomyocytes [45-47]. Therefore, the effect of the HG culture and diabetes on SOCE may be various in different condition. Our results also showed that Ca²⁺ release evoked by TG from the ER/SR was reduced in the adult diabetic rats. Moreover, the expression level of IP₃R in the diabetic rat

ventricular muscle was significantly decreased. Hence, impaired IP₃-mediated signalling possibly causes the reduction of Ca²⁺ release. The original purpose of SOCE is to replenish the depleted Ca²⁺ stores in the ER/SR. In the diabetic rats, the ventricular muscle ER/SR was impaired because of the reduction of TG-induced Ca²⁺ release but the expression levels of STIM1 and the Orai1 channel were dramatically increased. Thus, our findings lead us speculate that this increased SOCE may occur to compensate for the impairment of the ER/SR. These results are consistent with the findings from other group in dilated cardiomyopathy [28]. Previous studies have shown that STIM2 may inhibit STIM1-evoked SOCE and may also initiate SOCE when STIM1 is absent [17-20]. We found that STIM2 protein expression levels were decreased in the neonatal left ventricular muscle of the diabetic rats. The lower STIM2 expression levels might alleviate the inhibition of SOCE, which is coincident with the enhanced SOCE activity in the diabetic left ventricular muscle. However, the alteration of the expression profile of Orai and STIM proteins is different in HG-cultured neonatal left ventricular muscle cells from that in the diabetic rats. We think that it may be because (1) our results of cultured cells are from neonatal rat left ventricular muscle cells, some properties of which may be different from those of the left ventricular muscle cells in adult rat; (2) HG culture is a relative simple strategy to only mimic diabetic hyperglycaemia in vitro, but the real situation of the diabetes in vivo should be much complex.

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Therefore, the cardiomyocytes may be affected by many diabetes-related factors in animals, but not only stimulated by the hyperglycaemia.

Several studies clearly indicated that SOCE mediated by Orai1 was found in neonatal rat ventricular muscle cells as well as our finding [26, 27, 48]. However, literature also shows an opposite view on the exist of Orai1 and SOCE in adult ventricular muscle cells. Recently, a study reported that Orai1 and SOCE were not existed in adult SD rat ventricular muscle cells, but on the contrary two other studies reported that Orai1 were found in the adult SD rat ventricular muscle cells by western blotting and immunofluorescence methods and might be involved in ventricular arrhythmia and Ca^{2+} overloading [24, 49, 50]. Beside in SD rat ventricular cardiomyocytes, Orai1 was also found in adult mouse ventricular cardiomyocytes to mediate Ca^{2+} overloading [23]. Here, we used western blotting method to demonstrate that all three OraIs, including Orai1, Orai2 and Orai3, were expressed in the adult SD rat ventricular muscle cells. Moreover, Orai1 and Orai2 were two main isoforms involving diabetes-induced SOCE increase in the adult SD rat left ventricular muscle. Therefore, the further profound study should be benefit for clarifying this issue in future. At this stage, we think that it is possible that the sensitivity and specificity of Orai1 antibody to recognize Orai1 protein in different tissues and species may be various.

Notwithstanding Orai channels, numerous studies have suggested that other Ca^{2+} channels and ion transporters such as L-type Ca^{2+} channel, TRPC1, TRPC4, TRPC6, SERCA2 and CaMKII also participate in the development of the diabetes [51-55]. Therefore, many components linked to the maintenance of $[Ca^{2+}]_i$ homeostasis may also be altered in the cardiomyocytes of the diabetes. SOCE appears to be one of the components to affect $[Ca^{2+}]_i$ homeostasis in diabetic cardiomyocytes. Besides Orai channels, TRPC families are also expressed in ventricular muscle cells and implicated in the SOCE [56, 57]. Hence, TRPCs may be other potential components involving in the change of SOCE in the diabetes. Here, we only provide certain preliminary data regarding the change of SOCE in the diabetic left ventricular muscle. Further studies in electrophysiological relevance of SOCE and the disorder of other cellu-

lar components regulating $[Ca^{2+}]_i$ homeostasis in the future should help for clarifying these issues.

In summary, we conclude that SOCE is largely enhanced in the HG-cultured neonatal left ventricular muscle and STZ-induced adult diabetic rat left ventricular muscle because of the upregulated SOCE-related proteins. However, the ER/SR of left ventricular muscle is impaired with the reduction of IP_3 -R-I expression in the diabetic rats. The present study may provide a new clue for understanding the mechanism of intracellular Ca^{2+} overloading in diabetic cardiomyocytes and potential therapeutic targets for diabetic cardiomyopathy. Additionally, we used the neonatal rat left ventricular muscle to do the present study in cell level. Some properties of the neonatal rat left ventricular muscle may be different from the adult cells. It is hard to match the results from the neonatal rat left ventricular muscle with those from the adult diabetic cells. Therefore, the present result in cell level is limited and only can provide a clue for future further study.

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Disclosure of conflict of interest

None.

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