Calhex$_{231}$ Alleviates High Glucose-Induced Myocardial Fibrosis via Inhibiting Itch-Ubiquitin Proteasome Pathway in Vitro

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Diabetic cardiomyopathy (DCM) is a major complication of diabetes, and features myocardial fibrosis as its main pathological feature. Calcium sensing receptor (CaSR) is a G protein-coupled receptor, which involves in myocardial fibrosis by regulation of calcium homeostasis. Calhex$_{231}$, the CaSR inhibitor, is not clear whether it regulates myocardial fibrosis in DCM. In the present study, type 1 diabetic (TID) rats and primary neonatal rat cardiac fibroblasts were used to observe the role of Calhex$_{231}$. In vivo experiments showed that in the TID group, contractile dysfunction and the deposition of collagen I and III were obvious after 12 weeks. In vitro experiments, we found that high glucose (HG) could increase the expression of CaSR, α-smooth muscle actin (α-SMA), transforming growth factor-$β_1$ (TGF-$β_1$) collagen I/III, matrix metalloproteinase-2 (MMP-2), MMP9, along with cardiac fibroblast migration and proliferation. We further demonstrated that CaSR activation increased intracellular Ca$^{2+}$ concentration and upregulated the expression of Itch (atrophin-1 interacting protein 4), which resulted in increasing the ubiquitination levels of Smad7 and upregulating the expression of p-Smad2, p-Smad3. However, treatment with Calhex$_{231}$ clearly inhibited the above-mentioned changes. Collectively these results suggest that Calhex$_{231}$ could inhibit Itch-ubiquitin proteasome and TGF-$β_1$/Smads pathways, and then depress the proliferation of cardiac fibroblasts, along with the reduction deposition of collagen, alleviate glucose-induced myocardial fibrosis. Our findings indicate an important new mechanism for myocardial fibrosis, and suggest Calhex$_{231}$ would be a new therapeutic agent for the treatment of DCM.

Key words diabetic cardiomyopathy; myocardial fibrosis; calcium sensitive receptor; Calhex$_{231}$; transforming growth factor-$β_1$ (TGF-$β_1$); itch

INTRODUCTION

In recent years, the incidence and mortality of diabetes have shown a rapid growth trend worldwide.$^3$ Diabetic cardiomyopathy (DCM) is a major complication of diabetes. The main pathological changes involved are myocardial hypertrophy, apoptosis and myocardial interstitial fibrosis. Of these, myocardial fibrosis is the main pathological feature, and can eventually induce cardiac remodeling, cardiac dilatation, cardiac dysfunction, arrhythmia and congestive heart failure.$^2$ According to recent data,$^3$ cardiac fibroblasts are highly activated during diabetes, which results in dynamic balance disorder of cardiac extracellular matrix synthesis and deposition, along with the excessive deposition of collagen, thus leading to myocardial fibrosis and cardiac dysfunction.$^3$/$^5$ Calcium sensing receptor (CaSR) is a member of the C family of the G protein coupling receptor superfamily and is widely expressed in both prokaryotic and eukaryotic cells. CaSR is involved in regulating the homeostasis of calcium and other metal ions, cell secretion, proliferation, differentiation, chemotaxis, apoptosis, gene expression, membrane potential, ion channel switching and aging.$^6$/$^7$ Our team, and others, have demonstrated that Calhex$_{231}$ ameliorates myocardial hypertrophy induced by pressure-overload or angiotensin II (Ang II) via inhibiting CaSR expression.$^3$ Recently, we found that the expression of CaSR in the myocardium tissues of diabetic rats,$^8$ and cardiac fibroblasts treated with high concentrations of glucose, were significantly increased. However, whether Calhex$_{231}$ alleviates high glucose-induced myocardial fibrosis has not yet been elucidated.

In this study, type 1 diabetic rats, and cardiac fibroblasts undergoing high glucose, Calhex$_{231}$, Itch-small interfering RNA (siRNA) or the CaSR agonist treatment, were used to explore the functional role of Calhex$_{231}$ in diabetic myocardial fibrosis.

MATERIALS AND METHODS

Animal Experimental Protocol Eight weeks old male Wistar rats (250 ± 50g) were provided by the Animal Research Institute of Harbin Medical University (HMU), and the study was approved by the HMU Medical Science Ethics Committee. All rats were maintained on a 12-h light/dark cycle and fed with a standard chow and clean water ad libitum. The rats were randomly divided into three groups (n = 8 per group): (1) control group: age-matched non-diabetic Wistar rats were injected with citric acid-citrate sodium buffer; (2) type 1 diabetes (TID) group: intraperitoneal injection of streptozotocin$^{10}$ (STZ; Sigma-Aldrich, Munich, Germany; 60 mg/kg); (3) TID + Calhex$_{231}$ group: the rats were intraperitoneally injected with Calhex$_{231}$ until 12 weeks (10 μmol/kg/d in saline). Rats in the three groups were sacrificed after 12 weeks, and a range of indices were measured.

Isolation and Incubation of Neonatal Rat Cardiac Fibroblasts (CFs) Rat CFs were isolated from the hearts of 1–3-d old Wistar rats. In brief, the heart was quickly re-
moved, immediately placed in D-hanks solution, cut into pieces (0.5 mm³), digested with trypsin for 8 min; the digestion was then terminated by adding Dulbecco’s modified Eagle’s medium (DMEM) culture medium. After the same process had been repeated 8 times, cells were collected by 10 min centrifugation at 800 × g and a temperature of 4°C. After two hours of incubation, the unattached cells were discarded; the attached cells (CFs) were plated in a petri dish and maintained at 37°C in a 5% CO₂ humidified incubator in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin or streptomycin. The media was changed two times per week.

To ensure the purity of CFs, the cells were passaged for three generations and then CFs were treated with HG, R568 (the CaSR agonist), or Calhex231, Iitch-siRNA according to the experiment protocol.

**Serum and Culture Media Measurements** Blood samples which taken from the aorta were centrifuged and serum stored at −80°C until assayed. Random serum insulin levels were determined by a commercially-available ultrasensitive enzyme-linked immunosorbent assay (ELISA) kit (Beyotime, Nantong, China). Serum levels of triglyceride (TG), total cholesterol (TC) were then analyzed using a standard biochemistry panel (Beyotime, Shanghai, China). Blood glucose in blood samples from the tail vein was measured using a chemistry panel (Beyotime, Shanghai, China). Serum levels of triacylglycerol (TG), total cholesterol (TC) were then analyzed using a standard biochemistry panel (Beyotime, Shanghai, China). Blood glucose in blood samples from the tail vein was measured using a chemistry panel (Beyotime, Shanghai, China). Blood glucose in blood samples from the tail vein was measured using a chemistry panel (Beyotime, Shanghai, China).

**Immunohistochemistry** The hearts were removed, post-fixed with 4% paraformaldehyde, embedded routinely in paraffin, and sections. The sections were dewaxed, rehydrated, and pretreated by high-pressure heating for 3 min in 10 mM citrate buffer, pH 6.0. Subsequently, the sections were incubated with 3% H₂O₂ for 10 min and with a rabbit/mouse polyclonal antibody against CaSR, α-smooth muscle actin (α-SMA), Itch, Col-I/III (diluted 1:300, Proteintech, China) overnight at 4°C. Negative controls were created replacing primary antibodies with phosphate-buffered saline (PBS) or non-immune isotype immunoglobulin G (IgG). Subsequently, the sections were treated with a horseradish peroxidase-conjugated goat anti-rabbit/mouse secondary antibody (Zhongshan Golden Bridge, China) for 30 min at room temperature, followed by development with 3,3-diaminobenzidine–H₂O₂ solution (Zhongshan Golden Bridge). Finally, the sections were further counterstained with hematoxylin. Five nonoverlapping images of the area of interest were captured. Image-Pro Plus 6.0 software was used to quantify brown-stained areas in each image.

**Sirius Red Staining** After anesthesia, the heart was quickly removed and washed with phosphate buffer. The cardiac tissue was fixed in 10% buffered formaldehyde, embedded in paraffin, sliced at 4 mm and used for Sirius red staining. Images were acquired using optical microscopy.

**Western Blot Analysis** The rat hearts and cardiac fibroblasts were homogenized in 0.5 mL of RIPA buffer before being transferred into small tubes and rotated for 1 h at 4°C. Solubilized proteins were collected after centrifugation at 3000 × g for 30 min. The supernatant was then collected and stored at −80°C. The protein concentration of each sample was quantified using the BCA Protein Assay kit (Beyotime). Protein lysates from cells of each group were separated by electrophoresis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). Non-specific proteins on membranes were blocked with 5% non-fat dried milk for 2 h at room temperature, the membranes were incubated overnight with the following primary antibodies (at a 1:1000 dilution, 4°C): CaSR, TGF-β1, Itch, Col-I/III (Santa Cruz Biotechnology, Dallas, TX, U.S.A.); α-SMA, Smad2/3, p-Smad2/3, Smad7 (Cell Signaling Technology, Danvers, MA, U.S.A.); matrix metalloproteinase-2/9 (MMP-2/9) (Cell Signaling Technology), β-actin, β-tubulin (Santa Cruz Biotechnology). And then the membranes were incubated with anti-rat/anti-rabbit IgG antibody (Bioss, Beijing, China) at a 1:5000 dilution for 1 h at room temperature. The specific complex was visualized using the ECL plus Western blot detection system. The relative intensities of protein bands were finally quantified using a Bio-Rad Chemi EQ densitometer and Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

**Immunoprecipitation** CFs were seeded in 35 mm petri dish. After treatments, cells were collected and lysed in lysis buffer plus PMSF (Roche) for 30 min at 4°C. After 13500 × g centrifugation for 25 min, the lysates were immunoprecipitated with 2 μg specific antibody of anti-ubiquitin (Santa Cruz Biotechnology) overnight at 4°C before coupled to Protein A/G Magnetic Beads (Selleckchem, Houston, TX, U.S.A.) for 2 h according to the instructions of Protein A/G Magnetic Beads for IP (Biotool). After that, SDS-PAGE and other protocols were performed using the above methods of Western-blot.

**EdU and the Scratch Wound Repair Assay** A Vivid 7 Dimension echocardiography machine was used to assess cardiac function and dimensions (GE Healthcare, Waukesha, WI, U.S.A.). The rats were anesthetized by intraperitoneal injection of 10% pentobarbital sodium (0.3 mL/100 g). We then measured ejection fraction (EF). The rats were anesthetized by intraperitoneal injection of 10% pentobarbital sodium (0.3 mL/100 g). We then measured ejection fraction (EF). The rats were anesthetized by intraperitoneal injection of 10% pentobarbital sodium (0.3 mL/100 g). We then measured ejection fraction (EF). The rats were anesthetized by intraperitoneal injection of 10% pentobarbital sodium (0.3 mL/100 g). We then measured ejection fraction (EF).

**Measurements of Intracellular Ca²⁺ Using Fluo-3/AM Probes** CFs treated with HG (40 mM) and Calhex231 (3 μM) were then subjected to scratch assays as described previously. Images were captured at 0 and 24 h after treatment using phase-contrast microscopy.

**EdU and the Scratch Wound Repair Assay** A Vivid 7 Dimension echocardiography machine was used to assess cardiac function and dimensions (GE Healthcare, Waukesha, WI, U.S.A.). The rats were anesthetized by intraperitoneal injection of 10% pentobarbital sodium (0.3 mL/100 g). We then measured ejection fraction (EF). The rats were anesthetized by intraperitoneal injection of 10% pentobarbital sodium (0.3 mL/100 g). We then measured ejection fraction (EF). The rats were anesthetized by intraperitoneal injection of 10% pentobarbital sodium (0.3 mL/100 g). We then measured ejection fraction (EF). The rats were anesthetized by intraperitoneal injection of 10% pentobarbital sodium (0.3 mL/100 g). We then measured ejection fraction (EF). The rats were anesthetized by intraperitoneal injection of 10% pentobarbital sodium (0.3 mL/100 g). We then measured ejection fraction (EF). The rats were anesthetized by intraperitoneal injection of 10% pentobarbital sodium (0.3 mL/100 g). We then measured ejection fraction (EF). The rats were anesthetized by intraperitoneal injection of 10% pentobarbital sodium (0.3 mL/100 g). We then measured ejection fraction (EF). The rats were anesthetized by intraperitoneal injection of 10% pentobarbital sodium (0.3 mL/100 g). We then measured ejection fraction (EF). The rats were anesthetized by intraperitoneal injection of 10% pentobarbital sodium (0.3 mL/100 g). We then measured ejection fraction (EF).
pus, BX61). The excitation wavelength was 488 nm, and the emission wavelength was 530 nm.

**Transfection of Itch-siRNA in Vitro** CFs were seeded at equal number of cells (2 × 10^4 per dish) in 35 mm petri dish and maintained in the absence of antibiotic culture medium for 24 h before transfection, then washed three times

**Fig. 1. Successful Replication of Type 1 Diabetic Cardiomyopathy**
Random blood glucose was measured in weeks 2, 4, 8 and 12. Other indicators were analyzed in week 12 after successful modeling. (A, B) Blood glucose, insulin levels, triacylglycerol (TG) and total cholesterol (TC) in the serum. *p < 0.05 vs. Control rats; †p < 0.05 vs. TID rats (n = 8).

**Fig. 2. Calhex_{231} Ameliorated Diabetic Myocardial Fibrosis in T1D Rats**
(A) EF, Sirius red staining (collagen fibers shown in red) and HW/BW. (B, C) Representative images of CaSR, α-SMA, Itch, Col-I, Col-III immunohistochemistry. (D) Western blot results of α-SMA, TGF-β1, Col-I, Col-III in comparison with β-actin expression in rats. *p < 0.05 vs. Control rats; †p < 0.05 vs. T1D rats (n = 8). (Color figure can be accessed in the online version.)
with PBS. CFs were transfected with Control siRNA (Con-siRNA) and Itch-siRNA (Santa Cruz Biotechnology) using Lipofectamineä 3000 transfection reagent from Invitrogenä (Thermo Fisher Scientific, Scotland, U.K.). siRNA and the transfection reagent complex were added to the reduced serum media (Gibcoä Opti-MEM, Thermo Fisher Scientific) for 8 h, the transfection continued for another 24 h in serum-containing regular medium. After that, the cells were subjected to research.

**Statistical Analyses** All experiments were replicated at least three times independently. All data were expressed as mean value ± standard error of the mean (S.E.M.). Statistical analysis was performed by two-tailed Student t-test or one-way ANOVA, followed by the Bonferroni multiple comparison test using SPSS 18.0 software (SPSS Inc., IL, U.S.A.). p < 0.05 was considered statistically significant.

**RESULTS**

**Successful Replication of Type 1 Diabetic Cardiomyopathy** We determined blood glucose on weeks 2, 4, 8, and 12, along with insulin levels, TGs, and TCs in serum on week 12. The results showed that compared with the control group, the blood glucose levels at each time point were higher, the insulin level was significantly decreased, TGs, TCs were obviously increased in the TID group and TID + Calhex231 group (Figs. 1A, B). This indicated that the type 1 diabetic model had been successfully created.

**Calhex231 Ameliorated Diabetic Myocardial Fibrosis in TID Rats** In week 12, cardiac morphology detections showed that EF was significantly decreased and Sirius red staining showed large amounts of collagen deposition in the interstitial and perivascular areas in TID rats, the ratio of HW/BW was significantly increased in TID group (Fig. 2A). Immunohistochemical analysis and Western blot results of cardiac tissue showed that compared with the control group, the expression of α-SMA, Itch, TGF-β1, collagen I/III were increased in the TID group. However, the opposite results were observed in the TID + Calhex231 group (Figs. 2C, D).

**Calhex231 Alleviated High Glucose-Induced Myocardial Fibrosis in Cardiac Fibroblasts** After the 48-hour culture of cardiac fibroblasts in the high glucose group, the expression of CaSR, α-SMA, Col-I, Col-III, MMP2/9 were significantly upregulated, along with the higher contents of Col-I, Col-III in supernatant. Treatment with Calhex231 obviously attenuated the effects of HG (Figs. 3A, B).

Results from the scratch wound repair assay showed that the rate of cardiac fibroblast migration was higher in the HG group than in the control group, while the migration was significantly slower in the HG + Calhex231 group. The proliferation of cardiac fibroblasts at 24 h was detected using EdU assays. Compared with the control group, the cell proliferation was greater in the HG group, but was significantly decreased in the HG + Calhex231 group (Fig. 3C).

**CaSR Activation Increased Intracellular Calcium Concentration and Upregulated TGF-β1/Smads Pathway** To further study the mechanism of high glucose-induced myocardial fibrosis, cytosolic Ca2+ was determined with Fluo-3/AM and assessed by fluorescence intensity. The results indicated that the fluorescence intensity was higher in the HG group,
and was lower in the HG + Calhex231 group (Fig. 4A). The content of TGF-\(\beta_1\) was higher in HG and HG + R568 groups but was significantly lower in the HG + Calhex231 group (Fig. 4B).

Western blot detection results showed that the expression of TGF-\(\beta_1\), p-Smad2, and p-smad3 proteins were significantly upregulated and that Smad7 was markedly downregulated in HG and HG + R568 groups. However, the opposite results were observed in the Calhex231 group (Fig. 4C).

**DISCUSSION**

Diabetes is a metabolic disease characterized by hyperglycemia due to impaired insulin secretion or insulin resistance. Persistent hyperglycemia and metabolic disorders can lead to the impairment of tissues and organs, particularly the cardiovascular system, nervous system and kidneys. DCM is a heart disease independent of congenital heart disease, coronary heart disease, and valvar heart disease, and is also a significant cause of the increased mortality in patients with diabetes.12)

According to the existing literature, high glucose levels can stimulate the proliferation of fibroblasts, promote myofibroblast transdifferentiation and can activate the transcription and secretion of extracellular matrix proteins via the activation of Ang II, TGF-\(\beta\), Erk signaling pathway, and reactive oxygen species (ROS) in vitro.13–15) Our team has demonstrated that Calhex231 ameliorates myocardial hypertrophy via inhibiting CaSR expression. However, these previous studies did not elucidate the role of Calhex231 in high glucose-induced myocardial fibrosis via inhibiting Itch-ubiquitin proteasome pathway. To determine the effects of Itch-ubiquitin proteasome pathway on high glucose-induced myocardial fibrosis, we firstly detected the expression of Itch in cardiac fibroblasts (Fig. 5A). Cardiac fibroblasts transfected with Itch-siRNA significantly inhibited the expression of Itch, but con-siRNA had little effects on control group (Fig. 5B). Compared with the HG and HG + R568 group, the ubiquitination levels of Smad7 were markedly decreased in Itch-siRNA + HG group and Itch-siRNA + HG + R568 group, suggesting that Itch-siRNA can weaken the influence of HG and R658 (Fig. 5C). The expressions of collagen I and collagen III were significantly decreased in cardiac fibroblasts transfected with Itch-siRNA, consistent with Calhex231 treatment (Fig. 5D).
In present study, we replicated a rat model of type 1 diabetes. Polydipsia, polyuria, obvious emaciation, increased blood glucose, TC, TG, and decreased insulin activity were observed in rats treated with streptozotocin, thus indicating that a T1D rat model had been successfully created.

Twelve weeks after modeling, HW/BW was significantly increased and EF was decreased in the T1D group, which may have been related to weight loss and an increase in the myocardial extracellular matrix. This speculation is supported by cardiac morphology and related protein analysis. Sirius red staining showed large amounts of collagen deposition in the interstitial and perivascular areas and Calhex231 could inhibit these changes obviously. The expression of collagen I and collagen III proteins in the myocardial tissue was increased significantly in the T1D group, but was decreased significantly in the T1D + Calhex231 group. These results demonstrated that myocardial remodeling and myocardial fibrosis had clearly occurred in the T1D rats and Calhex231 could alleviate high glucose-induced myocardial fibrosis.

In cell experiments, we observed that the protein expression of CaSR, α-SMA, Col-I, Col-III, MMP2 and MMP9 significantly increased in CFs treated with high glucose, the scratch wound repair assay and EdU staining showed that high glucose treatment increased the migration and proliferation of cardiac fibroblasts, along with the increased contents of Col-I, Col-III in supernatant. Furthermore, treatment with Calhex231 could inhibit these changes, which were consistent with the experimental results of animals.

Recent studies have shown that increase intracellular Ca^{2+} can upregulate the expression of TGF-beta via activation of p-PKC/p-38 protein and TGF-β1/Smads signaling pathway participate in the regulation of myocardial fibrosis. TGF-β1 is associated with its type 2 receptor (TβRII), which activates TβRI kinase, causing Smad2,3 phosphorylation, which then combines with Smad4 and forms a complex, this complex is then translocated to the nucleus and regulates the transcription of target genes. Smad7 is an inhibitory Smad, which can degrade Smad2/3 via the ubiquitin proteasome degradation system. The present study shows that high glucose levels and CaSR agonists can significantly increase TGF-β1 and p-Smad2/3, and degrade Smad7, while Calhex231 exert the opposite effects, suggesting that Calhex231 could downregulate TGF-β1/Smads signaling pathway.
Intracellular calcium is an important second messenger and Ca$^{2+}$ concentration can be increased through Na$^{+}$/Ca$^{2+}$ exchanger and L-type calcium channel pathways. In our previous study, Cardiomyocytes/cardiac fibroblasts treated with Na$^{+}$/Ca$^{2+}$ exchanger inhibitor (NiCl$_2$) and L-type calcium channel blocker (CdCl$_2$), CaSR agonist (GdCl$_3$) could lead to intracellular calcium release and increase of [Ca$^{2+}$]$\text{_{i}}$. Follow-up experiments confirmed that CaSR activation increased intracellular Ca$^{2+}$ concentration via the G protein-PLC-IP3 pathway. However, why Calhex$_{231}$ can reduce the secretion and treatment with Calhex$_{231}$ could also reduce the secretion of collagen.

As mentioned earlier, Itoh (an E3 ligase) can degrade Smad7 protein by ubiquitin proteasome pathway. Thereafter, we used siRNA to disrupt the synthesis of Itoh protein and detected the ubiquitination level of Smad7 by immunoprecipitation. The results showed that the ubiquitination level of Smad7 were significantly increased in cardiac fibroblasts treated with HG and R568, however, the Itoh-siRNA significantly reduced the above effects. This class of E3 ligases consists of Nedd4-1, Nedd4-2, Itoh, Smurf1, Smurf2, WWp1 and WWp2,2,3 and promotes ubiquitination and subsequent proteasomal or lysosomal degradation of target proteins (Smad-7). We hypothesize that Calhex$_{231}$ may also decrease one of E3 activity abovementioned and Itoh is the main factor. Therefore, Itoh activation can degrade Smad7 protein by ubiquitin proteasome pathway in HG/R568 condition and upregulate Smad2/3 expression.

Since excessive extracellular matrix (ECM) is the main cause of high glucose-induced myocardial fibrosis, we detected the effects of Calhex$_{231}$ on collagen I/III expression changes in CFs, the expressions of collagen I/III were significantly decreased in cardiac fibroblasts transfected with Itoh-siRNA and treatment with Calhex$_{231}$ could also reduce the secretion of collagens.

CONCLUSION

Taken together, based on the above experimental results and literature, we demonstrated that Calhex$_{231}$ could decrease intracellular calcium and inhibit Itoh-ubiquitin proteasome to downregulate TGF-$\beta$/Smads pathway, and then depress the migration and proliferation of cardiac fibroblasts, along with the reduction deposition of collagens, thus alleviated glucose-induced myocardial fibrosis. Our findings indicate an important new mechanism for myocardial fibrosis, and suggest Calhex$_{231}$ would be a new therapeutic agent for the treatment of DCM.

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Conflict of Interest The authors declare no conflict of interest.

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