Calhex$_{231}$ alleviates high glucose-induced myocardial fibrosis via inhibiting Itch-ubiquitin proteasome pathway in Vitro

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Abstract

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. Calhex231, the CaSR inhibitor, is not clear whether it regulates myocardial fibrosis in DCM. In the present study, type 1 diabetic (T1D) rats and primary neonatal rat cardiac fibroblasts were used to observe the role of Calhex231. In vivo experiments showed that in the T1D 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-SMA, TGF-β1, collagen I/III, MMP2, MMP9, along with cardiac fibroblast migration and proliferation. We further demonstrated that CaSR activation increased intracellular Ca²⁺ 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 Calhex231 clearly inhibited the above-mentioned changes. Collectively these results suggest that Calhex231 could inhibit Itch-ubiquitin proteasome and TGF-β1/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 Calhex231 would be a new therapeutic agent for the treatment of DCM.

Keywords: Diabetic cardiomyopathy; Myocardial fibrosis; Calcium sensitive receptor; Calhex231; TGF-β1/Smads; Itch
Introduction

In recent years, the incidence and mortality of diabetes have shown a rapid growth trend worldwide\(^1\). 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\(^4,5\).

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 Ang II via inhibiting CaSR expression\(^8\). Recently, we found that the expression of CaSR in the myocardium tissues of diabetic rats\(^9\), 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-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) T1D group: intraperitoneal injection of streptozotocin \(^{(10)}\) (STZ: Sigma-Aldrich, Munich, Germany; 60 mg/kg); (3) T1D+Calhex\(_{231}\) group: the rats were intraperitoneally injected with Calhex\(_{231}\) until 12 weeks (10umol/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

Rat cardiac fibroblasts (CFs) were isolated from the hearts of 1-3 day old Wistar rats. In brief, the heart was quickly removed, immediately placed in D-hanks solution, cut into pieces (0.5mm\(^3\)), digested with trypsin for 8 min; the digestion was then terminated by adding 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% CO\(_2\) 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 Calhex\(_{231}\), Itch-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 ELISA kit (Beyotime, Nantong, China). Serum levels of triacylglycerol (TG), total cholesterol (TC) were then analyzed using a standard biochemistry panel (Beyotime,
Nantong, China). Blood glucose in blood samples from the tail vein was measured using a blood glucose analyzer (ACCU-CHEK, Roche, Germany). Culture media of the cardiac fibroblasts were collected to determine collagen I/III, TGF-β1 levels by enzyme-linked immunosorbent assays (ELISAs, Boster, Wuhan, China), following the supplier's protocols.

**Echocardiographic analysis of cardiac structure and function**

A Vivid 7 Dimension echocardiography machine was used to assess cardiac function and dimensions (GE Healthcare, Waukesha, WI, USA). The rats were anesthetized by intraperitoneal injection of 10% pentobarbital sodium (0.3 ml/100 g). We then measured ejection fraction (EF). The heart weight to body weight ratio (HW/BW) was also calculated.

**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 10mM 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-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 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, China). 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 millimeters and used for Sirius red staining. Images were acquired using optical microscopy.

**Western blot analysis**

The rat hearts and cardiac fibroblasts cells were homogenized in 0.5 ml of RIPA buffer before being transferred into small tubes and rotated for 1h 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, Shanghai, China). Protein lysates from cells of each group were separated by electrophoresis with SDS-PAGE and electro-transferred onto a 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, Texas, USA); α-SMA, Smad2/3, p-Smad2/3, Smad7(Cell Signaling Technology, Danvers, MA); MMP2/9 (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, USA).

**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, Dallas, Texas, USA) overnight at 4 °C before coupled to Protein A/G Magnetic Beads (Selleckchem, Houston, TX, USA) for 2h according to the instructions of...
Protein A/G Magnetic Beads for IP (Biotool). After that, SDS-PAGE and other protocol were performed using the above methods of Western-blot.

**EdU and the scratch wound repair assay**

A 5-ethyl-2'-deoxyuridine (EdU) kit (Ribobio, Guangzhou, China) was used to detect cell proliferation. CFs, at a density of $5 \times 10^3$/well, were seeded in 96-well plates. The cells were then treated with high glucose (40mM) and Calhex$_{231}$ (3μM). Then, EdU (10 μM) was added and the cells were incubated for 24 h at 37˚C. Thereafter, the cells were washed with PBS, fixed in 4% formaldehyde for 10 min, stained with Apollo staining solution (100 μl/well) for 30 min and then stained with 0.5 μg/ml Hoechst for 10 min at room temperature. Images were acquired using a fluorescence microscope (BX61; Olympus, Tokyo, Japan). Three fields were randomly selected from each dish and at least three dishes from each group were counted to determine the number of EdU-positive cells.

Rat cardiac fibroblasts treated with HG(40mM) and Calhex$_{231}$(3μM), were then subjected to scratch assays as described previously.$^{11}$ Images were captured at 0 and 24 hours after treatment using phase-contrast microscopy.

**Measurements of intracellular Ca$^{2+}$ using Fluo-3/AM probes**

CFs treated with HG(40mM), Calhex$_{231}$(3μM) and were stained using 5 mM fluo-3 AM (ab145254) for 30 min at 37˚C in the dark. Then, cells were washed with Ca$^{2+}$-free Tyrode’s solution to remove residual dye. The fluorescence of Ca$^{2+}$ was then measured by fluorescence microscopy (Olympus, 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$^5$ 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 with PBS. CFs were transfected with Control siRNA (Con-siRNA) and Itch-siRNA (Santa, Dallas, TX, USA) using Lipofectamine™ 3000 transfection reagent from Invitrogen™ (Thermo Fisher Scientific, Scotland, UK). siRNA and the transfection reagent complex were added to the reduced serum media (Gibco™ Opti-MEM™, Thermo Fisher Scientific, USA).
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Scientific, UK) for 8h, the transfection continued for another 24h 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 mean (SEM). 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, USA). *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 T1D group and T1D+Calhex231 group (Fig. 1A-B). This indicated that the type 1 diabetic model had been successfully created.

**Calhex231 ameliorated diabetic myocardial fibrosis in T1D 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 T1D rats, the ratio of HW/BW was significantly increased in T1D 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 T1D group. However, the opposite results were observed in the T1D+Calhex231 group (Fig. 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/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 (Fig. 3 A-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 Ca$^{2+}$ 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-β1 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-β1, 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).

**Calhex231 alleviates 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 Itch expression. The results showed that Itch expression increased significantly in the HG group and R568 group, while Calhex231 obviously inhibited the action of HG. These results suggested that downregulated expression of CaSR can decrease Itch expression (Fig.5A). Cardiac fibroblasts were transfected with Itch-siRNA according to the experiment protocol, the results showed that the 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).
expressions of collagen I and collagen III were significantly decreased in cardiac fibroblasts transfected with Itch-siRNA, consistent with Calhex$_{231}$ treatment (Fig. 5D).

**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 trans-differentiation and can activate the transcription and secretion of extracellular matrix proteins via the activation of Ang II, TGF-β, Erk signaling pathway, and ROS *in vitro* $^{13-15\)}$. Our team has demonstrated that Calhex$_{231}$ ameliorates myocardial hypertrophy via inhibiting CaSR expression. However, these previous studies did not elucidate the role of Calhex$_{231}$ in high glucose-induced myocardial fibrosis.

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 Calhex$_{231}$ 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\(^{16}\) and TGF-β\(_1\)/Smads signaling pathway participate in the regulation of myocardial fibrosis\(^{17}\). TGF-β\(_1\) 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 protease degradation system\(^{18}\). The present study shows that high glucose levels and CaSR agonists can significantly increase TGF-β\(_1\) and p-Smad2/3, and degrade Smad7, while CalHex231 exert the opposite effects, suggesting that Calhex231 could downregulate TGF-β\(_1\)/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\(^{2+}\)]\(_i\)\(^{19,20}\). Follow-up experiments confirmed that CaSR activation increased intracellular Ca\(^{2+}\) concentration via the G protein-PLC-IP3 pathway\(^{21}\). However, Why CalHex231 can reduce effects of HG on myocardial fibrosis? We speculated that increase of intracellular calcium could induce Itch expression, subsequently, we demonstrated that high glucose and R568 can significantly increase Itch protein.
expression, while Calhex$_{231}$ abolished these effects, which indicated that the expression of Itch was regulated by intracellular Ca$^{2+}$\textsuperscript{22, 23}.

As mentioned earlier, Itch (an E3 ligase) can degrade Smad7 protein by ubiquitin proteasome pathway\textsuperscript{24, 25}. Thereafter, we used siRNA to disrupt the synthesis of Itch 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 Itch-siRNA significantly reduced the above effects. This class of E3 ligases consists of Nedd4-1, Nedd4-2, Itch, Smurf1, Smurf2, WWp1 and WWp2,2,3 and promotes ubiquitination and subsequent proteasomal or lysosomal degradation of target proteins(Smad-7). We hypothesize that Calhex231 may also decrease one of E3 activity abovementioned and Itch is the main factor\textsuperscript{24, 25}. Therefore, Itch activation can degrade Smad7 protein by ubiquitin proteasome pathway\textsuperscript{24, 25} in HG/R568 condition and upregulate Smad2/3 expression\textsuperscript{18}.

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 Itch-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\textsuperscript{26} and inhibit Itch-ubiquitin proteasome to downregulate TGF-β\textsubscript{1}/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.
Acknowledgments

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Conflict of interest
The authors declare no conflict of interest.
Reference


Figure 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. T1D rats (n=8).
Figure 2. Calhex$_{231}$ ameliorated diabetic myocardial fibrosis in T1D rats

(A) EF, Sirius red staining (collagen fibers shown in red) and HW/B W. (B-C) Representative images of CaSR, α-SMA, Itch, Col-I, Col-III immunohistochemistry. (D) Western blot results of α-SMA, TGF-β$_1$, 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.)
Figure 3. Calhex$_{231}$ reduced the deposition of collagens and depressed the migration and proliferation of cardiac fibroblasts

(A) Cultured cardiac fibroblasts in the control group (5.5mM), HG group (40mM) and Calhex$_{231}$(3μM). The protein expression of CaSR, α-SMA, Col-I, Col-III, MMP2 and MMP9 were evaluated by western blot analysis. The intensity of each band was quantified by densitometry, and the data were normalized to β-actin. (B) Contents of Col-I and Col-III in the cell supernatants were determined using ELISA. (C) Cardiac fibroblast migration and proliferation were detected by scratch wound repair and EdU assays *P<0.05 vs. Control; **P<0.05 vs. HG (n ≥ 3).
Figure 4. Measurement of intracellular Ca²⁺ and signaling pathways associated with myocardial fibrosis in cardiac fibroblasts

Cardiac fibroblasts undergoing HG (40mM), R568 (5μM) and Calhex231 (3μM) were cultured for 24 h at 37 °C in control group, HG group, HG+R568, and HG+Calhex231 group. (A) Cytosolic Ca²⁺ was stained with Fluo-3/AM and assessed by fluorescence intensity. (B) Contents of TGF-β1 in the cell supernatants were determined using ELISA. (C) Representative western blot of TGF-β₁, Smad7 in comparison with β-actin expression and p-Smad2 and p-Smad3 in comparison with t-Smad2 and t-Smad3 expression in cardiac fibroblasts. *P<0.05 vs. Control, #P<0.05 vs. HG (n≥3).

(Color figure can be accessed in the online version.)
Figure 5. Calhex<sub>231</sub> alleviates high glucose-induced myocardial fibrosis via inhibiting Itch-ubiquitin proteasome pathway

(A) Representative western blot of Itch in comparison with β-actin expression in CFs exposed to HG in the presence of 5 μM R568 or 3 μM Calhex<sub>231</sub>. (B) Representative western blot of Itch in CFs which were transfected with Itch-siRNA or Con-siRNA. (C) The ubiquitination level of Smad7 in CFs which were transfected with Itch-siRNA or Con-siRNA and treated by 40mM HG or 5μM R568, respectively by immunoprecipitation. (D) Cardiac fibroblasts were transfected with Itch-siRNA or Con-siRNA and treated with HG (40mM), R568(5μM), Calhex<sub>231</sub>(3μM) for 48h. Protein expressions of Col-I, Col-III were detected by western blot analysis. (E) Schematic diagram showing Calhex<sub>231</sub> alleviates high glucose-induced myocardial fibrosis via inhibiting Itch-ubiquitin proteasome pathway in cardiac fibroblasts. *P<0.05 vs. Control/Con-siRNA, #P<0.05 vs. HG (n≥3), &P<0.05 vs. Con-siRNA+HG, @P<0.05 vs. Con-siRNA+HG+R568 (n≥3).
(Color figure can be accessed in the online version.)