Activation of calcium-sensing receptor-mediated autophagy in high glucose-induced cardiac fibrosis in vitro

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Abbreviations: MF, myocardial fibrosis; CaSR, calcium sensing receptor; DCM, diabetic cardiomyopathy; MMP2/9, matrix metalloproteinase 2/9; α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1; CFs, cardiac fibroblasts; SnoN, ski-related novel gene; Smurf2, smad ubiquitin regulatory factor 2

Key words: diabetic cardiomyopathy, myocardial fibrosis, calcium sensitive receptor, fibroblasts, smad ubiquitin regulatory factor 2, autophagy

Abstract. Myocardial fibrosis is a major complication of diabetic cardiomyopathy (DCM) that is primarily caused by cardiac fibroblasts that are highly activated by persistent hyperglycemic stimulation, resulting in excessive collagen deposition. Calcium sensing receptor (CaSR) is a member of the G protein-coupled receptor superfamily and regulates intracellular calcium concentrations, which are associated with numerous diseases, including myocardial infarction, tumors and pulmonary hypertension. However, whether CaSR participates in the pathological process of myocardial fibrosis in DCM remains unknown. The present study aimed to investigate the mechanism via which CaSR regulates high glucose (HG)-induced cardiac fibrosis in vitro. HG treated-cardiac fibroblast (CFs) were used and western blotting, immunoprecipitation, Cell Counting Kit-8 assay, ELISA and transfection technology were performed to examine the role of CaSR. In the HG group, treatment with HG increased CaSR, α-smooth muscle actin, collagen I/III and matrix metalloproteinase 2/9 expression and enhanced autophagosome generation and CF proliferation. Furthermore, CaSR activation upregulated the expression of Smad ubiquitin regulatory factor 2 (Smurf2), which led to increased intracellular Ca2+ concentrations, increased ubiquitination levels of SKI like proto-oncogene and Smad7 and autophagy activation. Furthermore, the CaSR agonist (R568) or the CaSR inhibitor (Calhex231) and Smurf2-small interfering RNA promoted or inhibited HG-induced alterations, including the enhanced and weakened effects, respectively. Taken together, the results from the present study suggested that increased CaSR expression in CFs activated the Smurf2-ubiquitin proteasome and autophagy, causing excessive CF proliferation and extensive collagen deposition, which resulted in HG-induced myocardial fibrosis. These findings indicated a novel pathogenesis of DCM and may provide a novel strategy for the diagnosis and treatment of DCM.

Introduction

Diabetes is a chronic metabolic disease and has become a major global health issue, which is attributed to diet, smoking, lifestyle and environmental changes (1). According to statistics, there are an increasing number of cases of diabetes worldwide, and it is predicted that the global diabetes population will increase to 591.9 million individuals by 2035 (2). Diabetes can lead to the damage of multiple organs including eyes, heart and kidney, and diabetic cardiomyopathy (DCM) is a primary complication of the disease (3). Currently, drug treatment is still the main therapeutic strategies, however, diet control, regular exercise, hyperbaric oxygen therapy and stem cell therapy can also reduce the diabetic complications (1).

Myocardial fibrosis is a major pathological process of DCM and the characteristic is an imbalance of extracellular matrix leading to excessive deposition of collagens, which alters the cardiac structure and impairs the systolic and diastolic function of the heart, eventually leading to heart failure (4). A recent study demonstrated that continuous hyperglycemia leads to high activation of cardiac fibroblasts (CFs) and induces CF differentiation into myofibroblasts, resulting in cardiac extracellular matrix unbalance and myocardial fibrosis.
in the heart tissue (5-7). However, the underlying mechanisms of myocardial fibrosis in DCM remain unclear.

Calcium sensitive receptor (CaSR) is a member of the C family of the G protein-coupled receptor superfamily, which is composed of 1,078 amino acids and consists of four major regions (8-10). CaSR is widely expressed in prokaryotic and eukaryotic cells, where it regulates the release of parathyroid hormone and maintains the homeostasis of calcium and other metal ions (11,12).

The ubiquitin-proteasome signaling pathway is a predominant protein degradation pathway (13), and its activation is regulated by numerous proteins, such as the Smad ubiquitin regulatory factor 2 (Smurf2) that regulates the transforming growth factor (TGF)-β1/Smads signaling pathway (14). Autophagy is the primary metabolic process of eukaryotic organisms (15). During autophagy, substances in the cytoplasm are phagocytized by autophagosomes, which are spherical structures with bilayer membranes, and are transported to lysosomes for degradation. After binding to endosomes or lysosomes, autophagosomes and their contents are degraded (16) and can therefore provide energy for the synthesis of macromolecules. Numerous studies have indicated that autophagy can improve heart diseases. For example, TGF-β1 treatment causes fibrogenesis and increases autophagy in human atrial fibroblasts (17). Furthermore, exogenous H2S treatment promotes the clearance of ubiquitin aggregates via autophagy in a type 2 diabetes model mice (18).

Previous studies have indicated that CaSR is associated with a variety of heart and lung diseases (19,20). The preliminary results indicated that high glucose (HG)-treated CFs displayed significantly upregulated CaSR expression and an increased number of autophagosomes. To explore the functional role and underlying mechanisms of CaSR in HG-induced myocardial fibrosis, a CaSR agonist and inhibitor were used in the present study.

Materials and methods

Isolation and culture of neonatal rat CFs. Primary CFs were isolated from neonatal Wistar rat (3 days of age). All experiments were approved by the Mudanjiang Medical University Medical Science Ethics Committee. As previously described (8,21,22), neonatal rats were sacrificed via 2% isoﬂurane inhalation and subsequent cervical dislocation, the heart was excised (8), and digested using trypsin for 8 min under sterile conditions at 37.5˚C. Dulbecco’s modiﬁed Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientiﬁc, Inc.) was added to terminate the digestion process. The aforementioned step was repeated 8 times. Cells were obtained by centrifugation for 10 min at 800 x g at 4˚C. Following incubation for 2 h, the unattached cells were discarded, the attached cells (CFs) were plated in a petri dish with DMEM containing 5.5 mM glucose composed the control group.

Western blotting. Total proteins were extracted from CFs by RIPA buffer (Beyotime Institute of Biotechnology) at 4˚C and quantified using the BCA assay kit (Beyotime Institute of Biotechnology). Proteins (20 µg) were separated by SDS-PAGE (10 or 14%) and transferred onto PVDF membranes. After blocking with 5% non-fat dried milk for 2 h at room temperature, membranes were incubated overnight at 4˚C with primary antibodies (1:1,000) against CaSR, collagen I/III (Col-I/III), p62, Beclin1, microtubule associated protein 1 light chain 3 α-I/II (LC3-I/II), SKI like proto-oncogene (SnoN), α-smooth muscle actin (α-SMA), β-actin (cat. nos. sc-47741, 59772/271249, 28359, 48341, 398822, 136958, 130617 and 47778, respectively; Santa Cruz Biotechnology, Inc.), Smurf2, smad2, phosphorylated (p)-smad2, smad3, p-smad3, transforming growth factor (TGF-β), MMP9 (cat. nos. 12024, 5339, 18338, 9513, 9520, 3709 and 3852, respectively; Cell Signaling Technology, Inc.), MMP2 and smad7 (cat. nos. 10373-2-AP and 25840-1-AP, respectively; ProteinTech Group, Inc.). Membranes were then incubated with the secondary antibodies against rabbit or anti-rabbit immunoglobulin G (1:10,000; cat. nos. bs-0295M-HRP or bs-0296HR-HRP; BIOSS) for 1 h at room temperature. Bands were detected using ECL Western Blotting Substrate kit (Beyotime Institute of Biotechnology). Relative expression levels were normalized to endogenous control β-actin using Gel-Pro-Analyzer Software Version 6.3 (Media Cybernetics, Inc.).

Immunoprecipitation. CFs were seeded into 35-mm culture dishes (1x104/dish) and cultured at 37˚C for 48 h. After treatments, CFs were harvested and treated with lysed in lysis buffer plus 1% PMSF (Roche Diagnostics) for 30 min at 4˚C. Cells were centrifuged at 4˚C at 3,000 x g for 25 min. Subsequently, cells were incubated with ubiquitin specific antibody (1:1,000; cat. no. sc-8017; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. Protein A/G Magnetic Beads (Selleck Chemicals) were used for binding according to the manufacturer’s protocol. Following incubation for 2 h, the beads were eluted, centrifuged and the supernatant was collected. Subsequently, western blotting was performed as aforementioned to detect the level of protein ubiquitination.

Cell proliferation detection by EdU and cell cycle analysis. Cell proliferation was detected using an EdU kit (Guangzhou RiboBio Co., Ltd.). Briefly, CFs were seeded into 96-well plates (5x104/well) and treated with HG (40 mM), R568 (5 µM) or Calhex231 (3 µM) for 24 h. Subsequently, cells were stained using the EdU kit according to the manufacturers’ instructions and the nucleus was stained with Hoechst at 37˚C for 10 min. Stained cells were observed using an EVOS M50000 fluorescence microscope (magnification, x200; Thermo Fisher Scientific, Inc.).

For cell cycle analysis, CFs were seeded into 6-well plates (1x105 cells per well) and treated with HG (40 mM), R568 (5 µM) or Calhex231 (3 µM) for 48 h. Subsequently, CFs were washed three times with cold PBS, fixed with 70%
ethanol at 4°C overnight and centrifuged at 800 x g at room temperature for 3 min. Cells were then treated with staining buffer, suspended and incubated with RNaseA (cat. no. C1052; Beyotime Institute of Biotechnology). CFs were stained with PI (50 µg/ml; Beyotime Institute of Biotechnology) for 30 min at 37°C. Cell cycle phase analysis was detected by flow cytometry using a BD C6 flow cytometer (BD Biosciences) and data analysis of the cytometric files was performed using BD ModFit LT version 2.0 (Verity Software House).

Detection of cell viability by the cell counting kit (CCK)-8 assay. CFs were seeded (2x10⁵ cells/well) into 96-well plates and treated with HG (40 mM), R568 (5 µM) or Calhex231 (3 µM). Cell viability was detected at 0, 12, 24, 36, 48, 60 or 72 h using a CCK-8 assay kit (cat. no. AR1199; Wuhan Boster Biological Technology, Ltd.) according to the manufacturer's protocol. The absorbance was read at 450 nm on a microplate reader.

Transmission electron microscopy (TEM). To observe autophagosomes of CFs, ultrastructural analysis was performed as previously described (23,24). Briefly, CFs were fixed with 2.5% glutaraldehyde at 4°C overnight, and with 1% osmium tetroxide for 2 h at room temperature. Subsequently, CFs were dehydrated using a graded ethanol series (50, 70, 90 and 100%), embedded in epoxy resin, stained with 3% uranyl acetate and 3% lead citrate for 30 min at 37°C. CFs were washed 3 times with PBS, transfection was performed using Lipofectamine® 3000 transfection reagent (Thermo Fisher Scientific, Inc.) in Opti-MEM medium and control siRNA (5'-CCCTTAAGTTTATCTGGCG-3') or Smurf2-siRNA (5'-GGTGTGTATGGCGAGGACATA-3') at a final concentration of 300 nM (Santa Cruz Biotechnology, Inc.) for 12 h. After transfection, CFs were cultured in regular medium for 24 h and then the cells were subjected to further analysis.

Measurement of intracellular calcium. CFs were treated with HG (40 mM), HG + R568 (40 mM + 5 µM) or HG + Calhex231 (40 mM + 3 µM). Subsequently, Fluo-3 AM (5 mM; cat. no. ab145254) was added to each group in the dark for 30 min at 37°C. CFs were washed with Ca²⁺-free Tyrode's solution and subsequently observed using a BX61 fluorescence microscope (magnification, x200; Olympus Corporation).

ELISA. CF culture media was collected to detect Col-I/III and TGF-β₁, levels using ELISA detection kits (cat. nos. EK0411, EKO424 and EK0513, respectively; Boster Biological Technology) according to the manufacturer's protocols.

Statistical analysis. All experiments were performed at least 3 times independently. Data were presented as the means ± standard error of the mean. Statistical analysis was performed by a two-tailed Student's t-test or one-way ANOVA followed by the Bonferroni multiple comparisons test using SPSS 18.0 software (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of CaSR on Col-I/III and MMPs expression in CFs and on CF proliferation. Following 48 h culture, the expression of CaSR, α-SMA, Col-I, Col-III, MMP2 and MMP9 was significantly increased in the HG group compared with control group. Furthermore, treatment with RG568 and Calhex231 enhanced or attenuated the effects of HG, respectively (Fig. 1A and B). In CF supernatant, the concentration of Col-I/III was significantly higher in the HG and HG + R568 groups, but significantly lower in the HG + Calhex231 group (Fig. 1C).

To detect cell proliferation, EdU assay was performed at the 24 h time point. Cell proliferation was significantly higher in the HG and HG + R568 groups compared with the control group. However, cell proliferation was significantly lower in the HG + Calhex231 group compared with the HG group (Fig. 2A).

Cell proliferation is closely related to the cell cycle, including the transition from the G1 phase to the S and G2 phases (27). Compared with the control group, the results from cell cycle analysis demonstrated that the S and G2 phase distribution of CFs was significantly increased in the HG and HG + R568 groups, and that the G1 phase distribution was significantly decreased in the HG group (Fig. 2B). In addition, CF viability at different time points (0, 12, 24, 36, 48, 60 and 72 h) was detected with the CCK-8 assay. CF viability in the HG and HG + R568 groups was significantly increased compared with the control group. However, compared with the HG group, cell viability was significantly lower in the HG + Calhex231 group at each time point (Fig. 2C).

CaSR activation enhances HG-induced autophagy. CFs were treated with HG, R568 or Calhex231 for 48 h. A significant increase in the number of autophagosomes was observed in the HG and HG + R568 groups via TEM and the CYTO-ID autophagy detection kit; however, Calhex231 significantly decreased the number of autophagosomes (Fig. 3A). The results from western blotting demonstrated that p62 protein expression was significantly downregulated, whereas Beclin1 and LC3-II expression was significantly upregulated in the HG and HG + R568 groups. Compared with the HG group, opposite results were observed in the Calhex231 group (Fig. 3B).
CaSR regulates intracellular Ca²⁺ and the TGF-β/Smads signaling pathway. To further investigate the underlying mechanism of myocardial fibrosis, Fluo-3/AM fluorescent dyes were used to detect the fluorescence intensity of cytosolic Ca²⁺. Compared with the control group, the results indicated that fluorescence intensity was higher in the HG and HG + R568 groups. Compared with the HG group, fluorescence intensity was lower in the HG + Calhex231 group (Fig. 4A). To detect CF TGF-β₁ secretion, the ELisa assay was performed. The concentration of TGF-β₁ was significantly higher in the HG and HG + R568 groups compared with the control group. Conversely, the concentration of TGF-β₁ was significantly lower in the HG + Calhex231 group compared with the HG group (Fig. 4B).

CaSR, calcium sensing receptor; CFs, cardiac fibroblasts; HG, high glucose; α-SMA, α-smooth muscle actin; Col-I, collagen I; Col-III, collagen III; MMP2, matrix metalloproteinase 2; MMP9, matrix metalloproteinase 9.

Activation of the TGF-β₁/Smads signaling pathway can lead to myocardial fibrosis (28); therefore, western blotting was performed to detect the expression of primary proteins. The expression of TGF-β₁ and p-Smad2/3 proteins was significantly increased, whereas Smad7 expression was significantly decreased in the HG and HG + R568 groups compared with the control group. As expected, the HG + Calhex231 group displayed the opposite results compared with the HG group (Fig. 4C).

Smurf2-ubiquitin proteasome pathway and autophagy are involved in HG-induced myocardial fibrosis. The results from western blotting demonstrated that Smurf2 expression was
significantly increased in the HG and R568 groups compared with the control group, but Calhex231 displayed the opposite effect compared with the HG group, suggesting CaSR activation increased Smurf2 expression (Fig. 5A). Furthermore, Smurf2 expression was significantly inhibited following Smurf2-sirna transfection compared with transfection with control siRNA (Fig. 5B). Although CFs were treated with HG and R658, the detection of ubiquitination levels demonstrated that the expression levels of SnoN and Smad7 were significantly suppressed by Smurf2‑siRNA. The results also suggested that Smurf2‑siRNA decreased the enhancing effect of HG and R658 (Fig. 5C). Furthermore, compared with the Con‑siRNA + HG and Con‑siRNA + HG + R568 groups, Smurf2‑siRNA significantly decreased the expression levels of Col‑I/III, Beclin1 and LC3‑II, while p62 expression was significantly increased in transfected CFs of Smurf2‑siRNA + HG and Smurf2‑siRNA + HG + R568 groups (Fig. 6A).

Discussion

Diabetes mellitus is a metabolic syndrome characterized by hyperglycemia that is caused by insufficient insulin secretion or resistance, which can eventually lead to DCM (29). Myocardial fibrosis is a process of cardiac remodeling and inflammatory gradual infiltration, which causes numerous conditions, including DCM, myocardial infarct (30). According to previous data, CFs serve a key role in the pathological process of myocardial fibrosis (31) by maintaining homeostasis and remodelling the extracellular matrix (32). Previous studies have confirmed that durative hyperglycemia
can induce CF proliferation, boost myofibroblast trans-differentiation and increase the secretion of extracellular matrix proteins; however, the underlying mechanisms remain unclear (33-35).

Collagen secretion (Col-I/III) and increased extracellular matrix accumulation that occurs during myocardial fibrosis can lead to the proliferation and activation of CFs (36). A previous study indicated that CaSR is expressed in CFs (37);
however, no study has investigated the relationship between CaSR expression and myocardial fibrosis in DCM. R568 is a positive allosteric modulator (calcimimetics) and can activate CaSR expression to increase intracellular Ca\(^{2+}\) concentration. Calhex231 is an inhibitor of the CaSR via negative allosteric modulation and can reduce intracellular calcium (38,39).

In the present study, when CFs were treated with HG, the protein expression of CaSR, \(\alpha\)-SMa, col-I, col-III, MMP2 and MMP9 was significantly increased. EdU staining, CCK-8 assays and the cell cycle analysis indicated that HG enhanced CF proliferation and promoted the secretion of Col-I/III into the supernatant. In addition, the CaSR agonist (R568) and the CaSR inhibitor (Calhex231) promoted and inhibited HG-induced alterations, respectively. The results indicated that CaSR was closely associated with HG-induced myocardial fibrosis in DCM.

Autophagy is a highly conservative catabolic process that is associated with several cardiac pathologies (40). Autophagy can also stabilize and maintain the metabolic process of intracellular environmental balance. In injured cells, damaged substances and organelles are phagocytosed by autophagosomes of spherical double layer membranes, and then transported to lysosomes via specific mechanisms to degradation and reuse (41). Previous studies have confirmed that autophagy participates in the process of fibrosis (42). The present study investigated therefore the role of CaSR in CF autophagy. In CFs treated with HG and R568, a large number of autophagosomes were observed, and the expression of Beclin1 and LC3-II was increased, whereas p62 expression was decreased. These results suggested that CaSR activation induced fibroblast proliferation and phenotype conversion, which may be associated with the autophagy pathway.

The TGF-\(\beta_1\)/Smads signaling pathway is the primary route of tissue fibrosis, which occurs during autophagy. p62, LC3-II and Beclin1 are the key proteins associated with autophagy (28). Smad7 can degrade Smad2/3, preventing nuclear translocation and restraining activation of the TGF-\(\beta_1\)/Smads signaling pathway (43). SnoN is a member of the SKI proto-oncogene family, which can inhibit Smad2/3 and Smad4 to form a combined complex, resulting in fibrosis inhibition (44). The present study further indicated that HG and CaSR agonists remarkably enhanced the expression of TGF-\(\beta_1\) and p-Smad2/3, and degraded Smad7. However, CaSR antagonists had the opposite effect.

As a primary secondary messenger, intracellular Ca\(^{2+}\) concentrations regulate numerous physiological functions such as electrophysiology of cardiac myocytes, the contraction of smooth muscle cells and immune cell response (45-47). Previous studies have indicated that CaSR could increase intracellular calcium via the G protein-phospholipase C-inositol triphosphate signaling pathway (20,48). However, why the activation of CaSR can enhance the effects of HG on myocardial fibrosis has not been reported. It was speculated that increased intracellular calcium could induce Smurf2 expression, which...
could degrade SnoN and Smad7 proteins via the ubiquitin proteasome signaling pathway. This hypothesis was investigated in the present study by detecting the ubiquitination level of SnoN and Smad7. Smurf2-siRNA significantly reduced the ubiquitination level of SnoN and Smad7, which prevented the stimulating effects of HG and R568. Col-I/III are essential components of the extracellular matrix; therefore, Col-I/III content increases can account for myocardial fibrosis. The present study detected the relationship between autophagy and alterations to Col-I/III expression in CFs. Smurf2-siRNA
significantly decreased the expression of Col-I/III, Beclin1 and LC3-II in CFs. However, the expression of p62 was significantly increased. Taken together, these results indicated that downregulation of autophagy could inhibit collagen secretion by CFs.
In summary, it has been hypothesized that the continuous stimulation of hyperglycemia could upregulate CaSR expression in CFs to increase intracellular Ca\(^{2+}\) (51) and activate Smurf2-ubiquitin proteasome and autophagy. The present study demonstrated that proliferative and activated CFs promoted collagen deposition, which may be a cause of myocardial fibrosis (Fig. 6B), but this requires further investigation. However, the present study only investigated the functional role of CaSR in HG-treated CFs. To identify the underlying mechanisms of CaSR-mediated autophagy in HG-induced cardiac fibrosis, further investigation is required in vivo, which may help the discovery of novel strategies for the prevention and treatment of DCM.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HY and ZW conceived and supervised the study. HY, JX, YZ and LL designed experiments. XX and HY performed experiments. XX and HY analyzed data. HY wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Ethics approval and consent to participate

The animal raising and handling procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Mudanjiang Medical University Medical Science Ethics Committee (Mudanjiang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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