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WORD COUNT 6574
TIME SUBMITTED 15-FEB-2022 08:32PM
PAPER ID 82597655

Roles of transient receptor potential channel 6 (TRPC6) in glucose-induced cardiomyocyte injury

Roles of TRPC6 in DCM

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Abstract:

Background: Diabetic cardiomyopathy is a serious complication of the end-stage diabetes, which has symptoms such as cardiac hypertrophy and heart failure. Transient receptor potential channel 6 (TRPC6) protein is a very important selective calcium channel, which is closely related to the development of various cardiomyopathy.

Aim: To explore whether TRPC6 affects cardiomyocyte apoptosis and proliferation inhibition in diabetic cardiomyopathy.

Methods: We compared the cardiac function and myocardial pathological changes of wild-type (WT) mice and mice injected with streptozotocin (STZ), and detected the expression of TRPC6 and P-calmodulin dependent protein kinase II (CaMKII) pathway proteins. At the same time, we treated H9C2 cardiomyocytes with high glucose, and added the SAR of TRPC6 inhibitor and the KN-93 of CaMKII inhibitor to H9C2 cells in a high glucose environment.

Results: We found that STZ-treated mice suffered from diabetic cardiomyopathy, decreased cardiac function, necrotic cardiomyocytes and limited proliferation. Western blot and immunofluorescence were used to detect the myocardial tissue and H9C2. It was found that compared with the control group, in the experimental group the expression of CC3 and Bax of apoptosis-related proteins increased significantly, and the expression of PCNA and CyclinD1 of proliferation-related proteins decreased significantly. In vivo and in vitro, the expression of TRPC6 and P-CaMKII of pathway proteins increased in a high glucose environment. However, after adding inhibitors to H9C2 cells in the HG environment, both apoptosis and proliferation inhibition were alleviated.

Conclusions: The apoptosis and proliferation inhibition of cardiomyocytes in a high glucose environment might be closely related to the activation of TRPC6 / P-CaMKII pathway.

Keywords: diabetic cardiomyopathy ; apoptosis ; proliferation; H9C2 cells ; TRPC6 ; P-CAMKII

The Core Tip:

Diabetic cardiomyopathy is a serious complication of the end-stage diabetes, which has symptoms such as cardiac hypertrophy and heart failure. Transient receptor potential channel 6 (TRPC6) protein is a very important selective calcium channel, which is closely related to the development of various cardiomyopathy. We found that the apoptosis and proliferation inhibition of cardiomyocytes in a high glucose environment might be closely related to the activation of TRPC6 / P-calmodulin dependent protein kinase II (P-CaMKII) pathway.

Introduction

Diabetes is a disorder of glucose metabolism characterized by elevated blood glucose, and one of its serious complications is diabetic cardiomyopathy (DCM),which can cause extensive necrosis of myocardial tissue, eventually resulting in heart failure and even cardiogenic shock [1, 2]. Pathological manifestations of myocardial necrosis caused by DCM is different from those by ischemia and hypoxia of coronary heart disease or myocardial hypertrophy caused by simple hypertension, and DCM is an independent and specific myocardial disease, which is characterized by myocardial collagen precipitation and interstitial fibrosis. Cardiomyocyte apoptosis and a large number of fibrous tissue proliferation are the important manifestations of DCM [3-5], its renin angiotensin is overactivated [6], and the main pathogenesis is the increase of oxygen free radicals [7].

The renin-angiotensin system (RAS) in the myocardium can regulate cardiovascular function and promote the growth of cardiomyocytes and vascular smooth muscle under normal conditions [8]. Under the high-glucose environment, an increase of Ang Ⅱ in the RAS system results in an increase of the secretion of the mineralocorticoid aldosterone, thereby causing an increase of the cardiac load [9]. Excessive secretion of cardiovascular endothelin can directly produce vasoconstrictor, make vascular smooth muscles proliferate, and accelerate the myocardial damage [10]. At the same time, endothelin stimulates the proliferation of myocardial fibroblasts and changes of collagen metabolism, causing myocardial interstitial remodeling and affecting cardiac systolic and diastolic functions [11]. The increase of oxygen free radicals (ROS) is another important cause of myocardial damage under the high-glucose environment. The level of lipid superoxide in the muscle tissue increases significantly in diabetes [12], while the expression of ROS scavenging enzymes such as catalase and superoxide dismutase (SOD) in the myocardium is relatively low. Cardiomyocytes are easy to become the

target group of oxidative radicals and oxidation reactions. Boyer and other reports show that the use of antioxidants has a protective effect on myocardial damage in diabetic patients, and the functions and morphological indexes of diabetic cardiomyopathy have been greatly improved in the experiment [13, 14].

Canonical transient receptor potential channel 6 (TRPC6) is a non-selective cationic calcium channel protein with a molecular weight of 106 kDa [15]. As an important member of the transient receptor potential (TRP) protein family, TRPC6 protein is expressed in various tissues such as lung, heart, kidney, skin and vascular [16-19], and plays an important role in the physiological and pathophysiological processes of various cells. Apoptosis is a mode of cell death, and intracellular calcium overload is an important factor triggering apoptosis [20]. The myocardial ischemia-reperfusion studies have found that selective knockout of TRPC6 can significantly reduce the degree of cardiomyocyte apoptosis and ischemia-reperfusion injury [21], suggesting that knockout of TRPC6 may inhibit ischemia-reperfusion-induced cardiomyocyte apoptosis by inhibiting calcium influx. Calmodulin dependent protein kinase II (CaMKII) is widely distributed in various tissues and cells, and is closely related to various life activities related to Ca^{2+}. In recent years, many studies have reported that CaMKII plays a very important role in various myocardial diseases such as myocardial hypertrophy, myocardial infarction and arrhythmia [22, 23]. However, there are few reports on the interaction between TRPC6 and CaMKII, and further researches are needed.

The pathogenesis of DCM is often related to the inflammatory process triggered by AngII, ROS and ERK [24], suggesting that TRPC6 may be involved in the pathogenesis of DCM. Therefore, in our present study, streptozotocin (STZ)-induced DCM mice models were constructed to detect the expression of apoptosis-related proteins including Bax, Cleaved Caspase 3 (CC3) and BCl2, and proliferation-related proteins including PCNA and

CyclinD1, and evaluate the level of cardiomyocyte injury under the high-glucose environment to further explore the mechanism of apoptosis and proliferation inhibition induced by the high-glucose environment through TRPC6 /P-CaMKII pathway in vitro. This study aimed to clarify the pathophysiological mechanism of DCM and provide a theoretical basis for finding new targets for the treatment of DCM.

Materials and methods

This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Hubei Provincial Center for Disease Control and Prevention. It's confirmed the study was reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

Reagents

DMSO (sigma, USA), high sugar DMEM medium (hyclone, USA), low sugar DMEM medium (hyclone, USA), Cell Counting Kit-8 (Beyotime ,China), fetal bovine serum (FBS) (Amresco, USA), Ripa lysate (Amresco, USA), protein concentration detection kit (Amresco, USA), PMSF (Amresco, USA), cocktail protease inhibitor (Amresco, USA), SDS (Amresco, USA), STZ(MCE, USA),KN-93 (MCE, USA), Tween-20 (Amresco, USA), SAR (sigma, USA), TRPC6 primary antibody (Abcam, USA), CC3 primary antibody (Abcam, USA), T-camkll primary antibody (Abcam, USA), P-camkll primary antibody (SAB, USA), BCl_2 primary antibody (Abcam, USA), GAPDH primary antibody (cell signaling, USA), Bax primary antibody (cell signaling, USA), PCNA primary antibody (Abcam, USA), CyclinD1 primary antibody (cell signaling, USA), Immunofluorescence Sheep anti mice secondary antibody (Abcam, USA).

Experimental animals and protocol

Animal feeding: C57 mice were purchased from Changzhou Cavens

Laboratory Animal Co, Ltd. Twenty male C57 mice aged 6-8 weeks with good vital signs were selected and raised in a standard animal room with the relatively stable temperature and humidity.

Modeling method: STZ solution with a concentration of 6.5 mg/ml was prepared with citric acid buffer (pH 4.5). After 5-hour fasting, mice in the experimental group were intraperitoneally injected with STZ solution (15 μL/g), and mice in the control group were injected with an equal amount of citric acid buffer for 3 days. After 7 days, the non-fasting blood glucose of mice in each group was measured. When the non-fasting blood glucose of STZ mice exceeded 17 mmol/L, the model was established successfully.

Blood glucose measurement: 1 week and 4 weeks after the last administration, the non-fasting blood glucose of mice was measured with peripheral blood. The tail tip was cut off with scissors, the peripheral blood was taken, and the blood glucose was measured by Abbott blood glucose meter, when the mice were in a stress-free state.

The mice were put under deep anesthesia, and euthanized by cervical dislocation, and then myocardial tissue was took for experiment.

Cell culture

H9C2 cells were donated by the laboratory of Anesthesiology Department of Union Hospital Affiliated to Huazhong University of science and technology. After passage, the cells were divided into 5 groups according to the different culture media and drugs: a low-glucose (LG) group: low-glucose medium with a concentration of 5.5 mmol/L; a control group:275ul mannitol solution of 500 mmol/L was added to 5ml low-glucose medium of 5.5 mmol/L to obtain the osmotic pressure of 33 mmol/L measured by OsmoPRO from Advanced Instruments lnc.; a high-glucose (HG) group: 275ul of glucose solution of 500 mmol/L was added to 5ml low-glucose medium of 5.5 mmol/L to obtain the osmotic pressure of 33 mmol/L measured by OsmoPRO from Advanced Instruments lnc.; a high

glucose plus TRPC6 inhibitor group (HG + SAR): the final concentration of 2 μmol/L sar7332 was added to the high-glucose medium with the concentration of 33.3 mmol/L; a high glucose plus CaMKII inhibitor group (HG + KN-93): KN-93 with a final concentration of 5 μmol/L was added to the high-glucose medium with a concentration of 33.3 mmol/L. After 72 hours, the next experiment was carried out.

Western blot

The proteins extracted from mice myocardium or H9C2 cells were added to 15% SDS-PAGE gel for separation, and transferred to PVDF membrane. The PVDF membrane with protein was put into skimmed milk powder for sealing. The 1:500 dilution of the primary antibody was evenly dropped on the PVDF membrane, and the PVDF membrane was incubated in a shake flask in a refrigerator at 4°C overnight. Then, we aspirated the primary antibody and rinsed the membrane. Goat anti-rabbit IgG or goat anti-mice IgG was diluted at a ratio of 1:1000, and was added into the PVDF membrane box, which was shaken on the shaking table for 1 hour. The chemiluminescent solution was mixed and configured, and then poured on the PVDF film with a pipette. After that, the strip drenched with luminescent solution was put into the developer for development. Finally, the strip was analyzed by image lab and image J.

Immunofluorescence

Cell specimens or tissue sections were fixed with 4% paraformaldehyde for 15 min at room temperature. Then, the samples were washed with phosphate buffered saline (PBS, pH 7.4, 0.1mol/L) solution three times for 8 min each time. With the help of PBS, triton was prepared into a membrane breaker with a concentration of 0.1%, and the membrane samples were broken at room temperature for 15 minutes. Then, the samples were washed with PBS solutionthree times for 8 min each time, and were sealed with Donkey Serum with a concentration of 3% at room temperature for 25 min. The slides were

washed again with warm PBS solution for 8 min. According to the instruction of the antibody, the primary antibody was incubated with PCNA dilution of 1:200, TRPC6 dilution of 1:400 and CC3 dilution of 1:400, and then the samples were placed in a refrigerator at 4°C overnight. The slides were rinsed with PBS solution three times for 8 min each time. The secondary antibody was anti-mice antibody or anti-rabbit antibody with a dilution ratio of 1:1000, and the samples were incubated in a 37°C incubator for 2 hours. The samples were washed three times with warm PBS for 8 min each time. Anti-fluorescence quenching sealing agent (ProLong® Gold Antifade Reagent, CST) was added to the samples, and pictures were took with confocal microscope.

Cell Counting Kit-8 (CCK-8) test

CCK-8, a highly sensitive kit, is widely used to detect cell proliferation and toxicity.

Cells were seeded at 1000 cells per well density in 96-well plates. Culture plates were pre-cultured in a 37C, 5% CO2 incubator, 10ml CCK-8 was added to each well. 96-well plates were placed in the incubator for 1-4h, then absorbance was determined at 450nm.

LDH detection

LDH is an enzyme in cells. When cells are stimulated or damaged, LDH is released from the inside to the outside. Therefore, the degree of apoptosis or necrosis can be evaluated by detecting LDH. The principle of this experiment is that lactic acid is converted into pyruvate under the catalysis of LDH, NAD+ is reduced to NADH, lipoamide dehydrogenase catalyzes NADH and INT to produce the Trichromate methyl, an absorption peak is generated at the wavelength of 490 nm, and the content of LDH is detected by colorimetry.

Cells with good conditions were selected according to 150 μL/well , seeded in 96-well plates, and detected when the cell density reached 80% - 85%. Then, the old culture medium was discarded, PBS was used to clean, and new

culture medium was added. After the pre-set time, the supernatant was aspirated and put into a centrifuge to centrifuge for about 5minutes at the speed of 300g. 120 μl of the supernatant was took, respectively, added to the next culture plate, and determined immediately.

ROS detection

Reactive oxygen species (ROS) include hydroxyl radical, hydrogen peroxide, superoxide and singlet oxygen, which can damage nucleic acid and biofilms. DCFH-DA itself has no fluorescence, and it can freely penetrate the cell membrane, and transform into DCFH in cells. Reactive oxygen species promote the oxidation of DCFH to produce fluorescent substances. Measurement of the green fluorescence intensity can evaluate the level of intracellular ROS.

The cells were inoculated into 12-well plates with small slides, different drugs were added according to different groups, and then the cells were cultured for 72 hours. When the cell density reached about 70%, DCFH-DA was directly added, and the cells were incubated in dark for 1 hour after reaching the initial concentration. We rinsed the cells with PBS solution to remove the residual DCFH-DA and reduce the background. The cells were observed and photographed under fluorescence microscope, and then were collected and detected by flow cytometry.

Apoptosis detected by flow cytometry

Annexin V, as a phospholipid-binding protein, has high affinity for phosphatidylserine. It binds to the cell membrane of early apoptotic cells through phosphatidylserine exposed outside the cells. Propidine iodide (PI) is a nucleic acid dye. However, at the middle and late stage of apoptosis and death, PI can penetrate the cell membrane and make the nucleus red. Therefore, by matching Annexin-V with PI, cells in the early and late stages of apoptosis can be isolated.

The cells of each group were collected, re-suspended in the culture medium,

and centrifuged at 4℃ for 5 min at the speed of 300g, and the supernatant was discarded. 1ml precooled PBS was added, and gently blew to mix the cells, and then the cells were centrifuged at 400g and 4℃ for 5min. We discarded the supernatant. The cells were re-suspended at 200uL PBS, 10uL annexin V-FITC and 10uL PI were added, and they were gently mixed, and incubated at 4 ℃ away from light for 30 min. 300uL PBS was added, and then flow detection was performed. NovoExpress analysis software was used for analysis.

Cell cycle detected by flow cytometry

The combination of PI and double-stranded DNA can produce fluorescence, and the fluorescence intensity is directly proportional to the content of double stranded DNA. After the DNA in the cell is stained with PI, the intracellular DNA content can be measured by a flow cytometry, thereby analyzing the cell cycle.

We took samples from each group, 1×10^7 cells were re-suspended in 1ml medium, centrifuged at 400g for 5min, and the supernatant was aspirated and discarded. Sedimentation in the 300μL PBS was re-suspended, and 700μL anhydrous ethanol was added, which was put in a refrigerator at - 20 ℃, and the cells were fixed for more than 24 hours. Then, the fixed sample was took out, centrifuged at 4 ℃ and 700g for 5min, and the supernatant was aspirated and discarded. We washed the sample twice with 1ml precooled PBS, and cell precipitation was re-suspended with 0.5ml PI/RNase holding buffer and incubated at room temperature for 15minutes. The DNA content of cells was measured by flow cytometry to determine the proportion of cells in each cell cycle. The results were analyzed using novoexpress software.

Histological analysis

HE staining is the abbreviation of hematoxylin-eosin staining. Hematoxylin dye is alkaline, which mainly makes the chromatin in the nucleus and ribosome in the cytoplasm purple blue. Eosin is an acidic dye, which mainly

makes the components in the cytoplasm and extracellular matrix red. Masson's trichrome is one of the main methods to show the dyeing of fibers in tissue. Masson stains muscle fibers in red and collagen fiber in blue, which is mainly used to distinguish collagen fiber from muscle fiber. In histology, periodic acid schiff (PAS) staining is mainly used to detect glycogen or other polysaccharide substances, which makes glycogen and the neutral mucilage material red and the nucleus blue.

The heart tissue was fixed with 10% formalin buffer, dehydrated with alcohol, and embedded in paraffin. We took 5 μm sections and stained them with HE, Masson and PAS. We took photos through an optical microscope, and Leica application suite image system was used to collect relevant parts of the sample.

Detection of myocardial injury markers

The main function of superoxide dismutase (SOD) is to catalyze the disproportionation of superoxide anion free radicals into hydrogen peroxide and oxygen. The superoxide anion free radicals are normal metabolites in organisms. Creatine kinase (CK) is a dimer composed of two subunits M and B. CK-MB in the myocardium accounts for about 10% - 32%, so it, as a marker of myocardial injury, has specificity. The destruction of cell membrane structure caused by apoptosis or necrosis will cause enzymes in the cytoplasm to be released into the culture medium, including lactate dehydrogenase (LDH) with stable enzyme activity. The three indicators were tested according to the steps described in the instruction.

Echocardiographic detection

Mice were intraperitoneally injected with 3% sodium pentobarbital at 40 Mg/kg. After reaching the state of mild anesthesia, they were fixed on the animal fixation plate in the supine position, and we touched them with fingers for apical pulsation. We removed the hair in the heart region on the body, and applied an appropriate amount of coupling agent to the heart. The

anterior zone was measured using Philips echocardiography. The 2-4 MHz ultrasound probe was placed on the left side of the sternum to show the short axis section of the left ventricle.

Statistical analysis

All data in this study were analyzed by graphpad prism 6.0. For the measurement data, the variance homogeneity test was carried out first, and then the unpaired two tailed t, t 'or rank sum test was performed accordingly. $P < 0.05$ was considered to have statistical difference.

Results

The flow chart of animal and cell experiments was showed in the supplementary figure 1

Establishment of mice DCM models

After STZ was injected in the experimental group, the blood glucose of mice in each group was measured regularly (Table 1). The fasting blood glucose of the control group had been in the normal range, while after 8 weeks of STZ injection, the fasting blood glucose of mice in the model group increased significantly compared with that before the injection ($p < 0.001$), and there was a significant difference in blood glucose compared with the control group ($p < 0.001$), which confirmed that the mice diabetes model was successfully established. Long axis ultrasound imaging and cardiac function measurement of the left ventricular showed that compared with the control group, LVEDD, LVEF and LVFS in the STZ group decreased significantly ($p < 0.05$), while LVESD in the STZ group increased significantly ($p < 0.05$), which suggested that cardiac function decreased in STZ group (Figure 1A and Table 2). In the detection of myocardial injury markers, compared with the control group, LDH and CK-MB in the STZ group significantly increased ($p < 0.0001$), while SOD significantly decreased ($P < 0.0001$), and a large amount of ROS were generated ($p < 0.0001$), indicating that there was serious oxidative stress injury

in myocardial tissue in the STZ group (Figure 1B and Table 3). The HE staining showed that compared with the control group, the cells in the STZ group were in disorder and hypertrophic, and the myocardial fibers were obviously broken and dissolved; the Masson staining showed that cardiomyocytes in the STZ group were hypertrophic and necrotic, and obvious fibrous tissue hyperplasia appeared in the myocardial stroma; the PAS staining showed that glycogen vacuoles, mucus and myocardial interstitial inflammatory cell infiltration increased in the STZ group (Figure 1C). The above results indicated that compared with the normal control group, the myocardial tissue in the STZ group had obvious pathological damage, which further confirmed that the DCM model was established successfully.

Apoptosis of cardiomyocytes in DCM mice

Cardiomyocyte apoptosis plays a very important role in the pathogenesis of DCM. In order to explore the apoptosis of cardiomyocytes under the high-glucose environment, we did the following experiments: ① the myocardial tissue of the two groups was made into the cell suspension, and then flow cytometry showed that the apoptotic rate of cardiomyocytes in the DCM model group significantly increased ($p < 0.0001$) (Figure 2A); ② western blot was used to detect apoptosis-related proteins in myocardium, and compared with the control group, there was no significant difference in the level of cardiomyocyte apoptosis related protein BCL2 in the DCM model group ($p > 0.05$), and the expression of Bax and CC3 increased significantly, and there was obvious apoptosis ($p < 0.01$) (Figure 2B); ③ immunofluorescence detection showed that the fluorescence intensity of CC3 in the DCM model group was significantly enhanced ($P < 0.01$) (Figure 2C).

Proliferation of cardiomyocytes in DCM mice

After the onset of DCM, calcium overload of cardiomyocytes and excessive energy consumption of mitochondria might lead to a decrease in the number of cells. In order to explore the proliferation of cardiomyocytes under the

high-glucose environment, we carried out the following experiments: ① the results of flow cytometry showed that the ratio of myocardial cells in the G1 phase in the DCM model group significantly increased ($P < 0.01$), indicating that the proliferation of many cells in the G1 phase was blocked (Figure 3A); the cell cycle related proteins in the two groups were detected by western blot, and compared with the control group, the expression of PCNA and CyclinD1 decreased significantly ($p < 0.01$) (Figure 3B); immunofluorescence detection also showed that the expression of PCNA in the myocardial tissue of model group decreased significantly ($p < 0.01$) (Figure 3C). These results suggested that the proliferation of cardiomyocyte was significantly inhibited.

Expression of TRPC6 and P-CAMKII protein in the myocardium of DCM mice

In order to clarify the role of TRPC6 in the high glucose-induced cardiomyocyte injury, we detected the expression of TRPC6 and its related signal pathway P-Camkll in the DCM mice. The myocardial tissues in the control group and DCM model group were detected by western blot. Compared with the control group, the expression level of calcium channel proteins such as TRPC6 and P-CAMKII in the DCM model group increased significantly ($p < 0.01$) (Figure 4A). The myocardial tissues of the two groups were detected by immunofluorescence, and the results showed that the expression of TRPC6 in the myocardium of the model group increased significantly ($p < 0.001$) (Figure 4B).

Pathological and biochemical changes of H9C2 cardiomyocytes in each group

In order to clarify the role and mechanism of TRPC6 and CaMKII in DCM-induced cardiomyocyte injury, H9C2 cardiomyocyte line was treated with high glucose to simulate DCM-induced cardiomyocyte injury in vitro. At the same time, sar7334 of TRPC6 inhibitor and KN-93 of CaMKII inhibitor were used to pretreat cells to evaluate their roles in HG-induced

cardiomyocyte injury. Compared with the LG group and the control group, the HG group had obvious cell necrosis and floating, and the number of cells decreased significantly ($P < 0.05$), and compared with the HG group, cell necrosis was improved and the number of cells increased significantly in the SAR7334 and KN-93 treatment groups ($P < 0.05$), which suggested that the inhibition of TRPC6 and CaMKII might alleviate the HG-induced cardiomyocyte injury. The absorbance of each group at 450 nm was detected by CCK-8 method, so as to evaluate the activity of H9C2 cells proliferation in each group, the results of which showed that compared with the LG group and control group, the viability of cells in the HG group (33 mmol/L) decreased significantly ($P < 0.01$), and the viability of cells in the HG + SAR group and HG + KN-93 group was significantly higher than that in the HG group ($P < 0.05$) (Figure 5A). Lactate dehydrogenase (LDH) is widely distributed in the myocardium and brain, and participates in the redox reaction in the cytoplasm. The amount of LDH release and the mortality of cardiomyocytes are commonly used to evaluate the degree of cardiomyocyte injury. The OD value is an index reflecting LDH activity. We examined LDH activity and cardiomyocyte mortality. Compared with the control group and LG group, the OD value of cells in the HG group increased significantly, while in the HG + SAR group and HG + KN-93 group the HG-induced LDH release was significantly inhibited; compared with that in the control group and LG group, the cell mortality in the HG group increased significantly, while compared with that in the HG group, the cell mortality in the HG + SAR group and HG + KN-93 group decreased significantly (Figure 5B). Excessive ROS can cause serious damage to proteins and nucleic acids in the cytoplasm. The level of ROS is closely related to the level of apoptosis. ROS fluorescence was detected by DCFH-DA, and our results showed that the fluorescence intensity of the HG group was significantly higher than that of the LG Group and control group ($p < 0.0001$), and compared with the HG

group, the level of ROS in cardiomyocytes of the HG + SAR group and HG + KN-93 group decreased significantly ($p < 0.0001$) (Figure 5C).

Effects of SAR7334 and KN-93 on HG-induced apoptosis of H9C2 cells

In order to further clarify whether the decline of cardiomyocyte viability induced by high glucose is related to mediating its apoptosis, we did the following research: the apoptosis of cells in each group was detected by flow cytometry, and HG (33 mmol/L)-induced was performed for 72 hours; compared with the LG group and control group, the apoptosis rate of the HG group was significantly higher ($p < 0.0001$); after using SAR (2 μmol/L) and KN-93 (5 μmol/L); compared with the HG group, the apoptosis rate of the HG + SAR group and HG + KN-93 group decreased significantly ($p < 0.0001$) (Figure 6A); WB was used to detect the changes of the apoptosis-related proteins including Bax, CC3 and BCl2 after the high-glucose processing, the expression of Bax and CC3 was significantly up-regulated ($p < 0.001$), H9C2 cells showed obvious apoptosis, and compared with the HG group, the level of Bax and CC3 proteins decreased significantly in the HG + SAR group and HG + KN-93 group ($p < 0.001$) (Figure 6B). The results of immunofluorescence showed that the fluorescence intensity of CC3 increased significantly in the HG group ($p < 0.001$), while the fluorescence intensity of CC3 in the HG + SAR group and HG + KN-93 group decreased significantly ($p < 0.01$) (Figure 6C).These results suggested that the effects of SAR7334 and KN-93 were similar, and they both inhibited HG-induced apoptosis and protected cardiomyocytes.

Effects of SAR7334 and KN-93 on HG-induced inhibition of H9C2 cell proliferation

In order to further explore whether calcium overload can inhibit the proliferation of cardiomyocyte, we carried out the following research: the cell cycle of each group was detected by flow cytometry; compared with the LG Group and control group, the ratio of cells in G1 phase in the HG group was

significantly higher ($p < 0.001$), and the ratio of cells in G1 phase in the HG + SAR7334 group and HG + KN-93 group was significantly lower than that in the HG group ($p < 0.01$) (Figure 7A); compared with the LG Group and control group, the cyclin of cells in each group was detected by western blot, the expression of PCNA and CyclinD1 in the HG group was significantly down-regulated ($p < 0.0001$), and compared with the HG group, the expression of PCNA and CyclinD1 was significantly up-regulated in the HG + SAR7334 group and HG + KN-93 group ($p < 0.001$) (Figure 7B); immunofluorescence also showed that the PCNA fluorescence intensity of the HG group decreased significantly ($p < 0.0001$), and after adding SAR7334 and KN-93 respectively, the PCNA fluorescence intensity increased significantly ($p < 0.01$) (Figure 7C). These results showed that after adding SAR7334 and KN-93, the cell proliferation was improved, and calcium overload inhibited the cell proliferation.

Changes of the signal pathway under the intervention of SAR7334 and KN-93

In order to explore the specific mechanism of cardiomyocyte injury caused by high glucose, the following steps were carried out in our present study: western blot was used to detect the expression of TRPC6 and P-Camkll in cells of each group, compared with the LG group and control group, the expression of TRPC6 and P-Camkll in the HG group increased significantly ($p < 0.001$), and compared with the HG group, the expression of TRPC6 and P-Camkll in the HG + SAR group and HG + KN-93 group decreased significantly ($p < 0.001$) (Figure 8A); immunofluorescence experiment also showed that the fluorescence intensity of TRPC6 in the HG group increased significantly ($p < 0.0001$), and the fluorescence intensity of TRPC6 decreased significantly after adding SAR7334 and KN-93 ($p < 0.0001$), suggesting that high glucose might activate TRPC6/P-Camkll pathway, cause intracellular calcium overload, and finally result in cardiomyocyte injury (Figure 8B).

Discussion

Normal metabolic function is very important for maintaining the homeostasis of the internal environment and the normal work of various organs. Metabolic dysfunction can cause the pathophysiological changes from tissue cells to the whole body through various ways, such as imbalance of internal environment homeostasis, activation of inflammatory pathways, activation of apoptosis-related pathways, and so on [25-27]. Diabetes is caused by congenital genetic factors or acquired pathogenic factors. It is manifested by a series of metabolic syndrome, which is characterized by abnormal blood glucose elevation [28, 29]. Diabetes usually leads to the activation of the molecules of signal pathways such as HIPPO/YAP [30], NF-b/NLRP3 [31], and Erk/nrf2/ho-1 [32], which leads to the pathological changes of peripheral blood vessels, smooth muscle and cardiomyocytes, diabetic foot, diabetic nephropathy, diabetic cardiomyopathy and other complications, and when the patient feels uncomfortable, the mortality rate of the patient is also greatly increased [33-36]. Among these complications, diabetic cardiomyopathy (DCM) is a serious complication of diabetes [37]. DCM refers to the irreversible damage of myocardial structure and function in diabetic patients due to hyperglycemia and metabolic disorders [38]. In terms of pathogenesis, studies have shown that DCM is an independent and specific myocardial injury, and the pathogenesis of DCM is different from that of hypertensive heart disease, coronary atherosclerotic heart disease and other heart diseases [39, 40]. Our present study suggested that high glucose might activate inflammatory pathways to induce apoptosis and fibrous tissue proliferation in diabetic patients, and play an important role [41, 42].

TRPC6 protein is a non-selective cation channel protein. It has been reported that TRPC6 can regulate Ca^{2+} influx under physiological conditions. In vitro, Sonnevedl *et al.* found that the high-glucose environment caused the

up-regulation of TRPC6 expression in an AngII expression dependent manner [43]. Zhang et al found that AngII could up-regulate the expression of TRPC6 in podocytes, increase Ca^{2+} influx, and promote podocyte apoptosis and autophagy [44]. Studies have shown that when the expression of TRPC6 increases, the intracellular Ca^{2+}concentration increases, resulting in the activation of calmodulin (CaM), and the nuclear translocation of NFAT2 in the cytoplasm into the nuclear membrane, which binds to the corresponding homeopathic elements in the target gene promoter to trigger the expression of downstream molecules [45]. Elucidating the exact mechanism of TRPC6 in the pathogenesis of DCM has important theoretical and practical significance for proposing relevant therapeutic targets. This study aimed to explore the role of TRPC6 in the pathogenesis of DCM by using STZ to construct DCM mode and simulate the high-glucose environment of cells in vitro.

In our present study, the DCM model was constructed by intraperitoneal injection of STZ. In order to clarify whether the STZ can induce DCM effectively, we used HE, Masson and PAS pathological staining to detect the changes of myocardial structure after modeling. Our results showed that compared with the control group, cardiomyocyte hypertrophy and necrosis, collagen fiber proliferation, and glycogen vacuoles in the STZ group increased. which suggested that STZ could effectively induce the construction of DCM model. In order to clarify the mechanism of STZ-induced DCM-related phenomena and to explore whether there was a process of cardiomyocyte apoptosis, we performed flow cytometry, western blot and immunofluorescence, and our results showed that the apoptosis rate of the STZ injection group increased significantly, and the expression of Bax and CC3 increased significantly, suggesting that the high-glucose environment might aggravate the degree of cardiomyocyte apoptosis, and the fluorescence intensity of CC3 also significantly increased. The same experimental method was also used to detect the cell cycle, and our results showed that in the

model group the ratio of cardiomyocytes in G1 phase significantly increased, the expression of PCNA and CyclinD1 decreased obviously, and the PCNA fluorescence intensity of cardiomyocytes decreased significantly, which suggested that high glucose might inhibit the proliferation of cardiomyocytes. TRPC6 and P-CaMKll of the signal pathway proteins were also detected, and it was found that the protein expression of the model significantly increased, suggesting that high glucose might promote cardiomyocyte apoptosis by activating the expression of TRPC6 and P-CaMKll.

For DCM, pathological oxidative stress and metabolic dysfunction can mediate the injury of cardiomyocytes through the activation of Ras and ROS systems [46]. Studies have shown that the activation of ROS signaling pathway is an important mechanism of cell injury [46, 47]. It has been reported that the production of intracellular ROS can directly damage the mitochondrial membrane, and cause the mutation of mitochondrial DNA, resulting in the imbalance of oxidative stress and Ca^{2+} regulation [47]. At the same time, ROS can also activate NF-kB in an indirect way, such as activating caspase-3 and its activator, thereby inducing the high expression of apoptosis-related genes [48]. As a non-selective calcium channel, TRPC6 can induce cell injury through calcium overload [49]. In order to further clarify the specific roles of TRPC6 in DCM, we observed the effect of TRPC6 on cardiomyocytes in the high-glucose environment by cell culture experiment in vitro.

Under the light microscope, we found that the apoptosis of H9C2 cells under the high glucose environment was severe. SAR7334 and KN-93 are specific inhibitors of TRPC6 and CaMKII. After adding SAR7334 and KN-93, the cell injury was alleviated. CCK-8 test showed that SAR7334 and KN-93 had a protective effect on cardiomyocytes. Lactate dehydrogenase (LDH) is widely distributed in the myocardium and brain, and participates in the redox reaction in cytoplasm. The amount of LDH release and the mortality of

cardiomyocytes are commonly used to evaluate the degree of cardiomyocyte injury. By detecting the LDH level in the supernatant of cultured cells, we confirmed that the inhibition of calcium calmodulin could alleviate the toxic effect of high glucose on cardiomyocytes. In addition, we used DCFH-DA for reactive oxygen species fluorescence detection. It was found that under the high-glucose stimulation, compared with that of the HG group, the content of ROS in cardiomyocytes of the HG + SAR group and the HG + KN-93 group decreased significantly, indicating that the production of ROS was very important in the process of HG-induced apoptosis, and this process was regulated by the concentration of Ca^{2+}. The activation of TRPC6 induced by high glucose further caused intracellular calcium overload and an increased expression of calmodulin, which was the initiating factor leading to apoptosis, and ROS as the downstream further resulted in the activation of apoptosis pathway.

In order to further clarify whether high glucose can induce the apoptosis and proliferation inhibition of cardiomyocytes, and clarify their related pathways, we used flow cytometry, western blot, and immunofluorescence to detect the rate of apoptosis, cell cycle, and the expression of apoptosis and cycle-related proteins, respectively. In the HG group, the apoptosis rate increased significantly, but the cells were more blocked in G1 phase. After the application of SAR and KN-93, the apoptosis rate decreased and the proportion of cells blocked in G1 phase decreased. The expression of apoptosis-related proteins including Bax and CC3 increased significantly in the HG group, and the expression of TRPC6 and P-CaMKll also showed the same trend. The expressions of the cell cycle-related proteins including PCNA and cyclinD1 decreased significantly in the HG group, and after adding calcium channel inhibitors, the situation was improved. The above results suggested that the change of Ca^{2+} concentration might play an important role.

Conclusion

In conclusion, the results of flow cytometry, immunofluorescence and western blot experiments suggested that the high-glucose environment might lead to the cardiomyocyte apoptosis and inhibition of cardiomyocyte proliferation by inducing TRPC6/P-CaMKll pathway, and the vitro experiments further validated that the inhibition of TRPC6 and CaMKII might protect cells under the high-glucose environment, improve cell activity, reduce cell mortality, and promote cell proliferation.

ARTICLE HIGHLIGHTS

[Research background]

Diabetic cardiomyopathy is a serious complication of the end-stage diabetes, which has symptoms such as cardiac hypertrophy and heart failure. Transient receptor potential channel 6 (TRPC6) protein is a very important selective calcium channel, which is closely related to the development of various cardiomyopathy.

[Research motivation]

In recent years, many studies have reported that CaMKII plays a very important role in various myocardial diseases such as myocardial hypertrophy, myocardial infarction and arrhythmia. However, there are few reports on the interaction between TRPC6 and CaMKII, and further researches are needed.

[Research objectives]

The purpose of this study is to explore whether TRPC6 affects cardiomyocyte apoptosis and proliferation inhibition in diabetic cardiomyopathy.

[Research methods]

We compared the cardiac function and myocardial pathological changes of wild-type (WT) mice and mice injected with streptozotocin (STZ), and detected the expression of TRPC6 and P-calmodulin dependent protein kinase

II (CaMKII) pathway proteins. At the same time, we treated H9C2 cardiomyocytes with high glucose, and added the SAR of TRPC6 inhibitor and the KN-93 of CaMKII inhibitor to H9C2 cells in a high glucose environment.

[Research results]

We found that STZ-treated mice suffered from diabetic cardiomyopathy, decreased cardiac function, necrotic cardiomyocytes and limited proliferation. Western blot and immunofluorescence were used to detect the myocardial tissue and H9C2. It was found that compared with the control group, in the experimental group the expression of CC3 and Bax of apoptosis-related proteins increased significantly, and the expression of PCNA and CyclinD1 of proliferation-related proteins decreased significantly. In vivo and in vitro, the expression of TRPC6 and P-CaMKII of pathway proteins increased in a high glucose environment. However, after adding inhibitors to H9C2 cells in the HG environment, both apoptosis and proliferation inhibition were alleviated.

[Research conclusions]

The apoptosis and proliferation inhibition of cardiomyocytes in a high glucose environment might be closely related to the activation of TRPC6 / P-CaMKII pathway.

[Research perspectives]

This might provide a new insight for the treatment of diabetic cardiomyopathy.

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