

Calcium inhibitor inhibits high glucose-induced hypertrophy of H9C2 cells

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Abstract. The aim of the present study was to explore whether the hypertrophy of H9C2 cardiomyocytes was induced by high glucose, to investigate whether the calcium channel inhibitor (Norvasc) could inhibit this process and to clarify the possible signaling pathways. The morphology of H9C2 cells was observed under an optical microscope, and the cell surface area was measured by Image Pro Plus 6.1 software. Furthermore, fluorescence spectrophotometry was used to detect intracellular calcium concentration ($[Ca^{2+}]_i$). ELISA was performed to detect calcineurin (CaN) activity; reverse transcription-quantitative PCR and western blotting were performed to detect the mRNA and protein expression levels of CaN A β subunit (CnA β), nuclear factor of activated T cells 3 (NFAT3) and β type myosin heavy chain (β -MHC). Cell size was increased with the increase in glucose concentration of culture medium at 48 and 72 h, respectively, and decreased with the addition of Norvasc compared with those without Norvasc ($P < 0.05$). There was no significant difference in cell size with the addition of Norvasc compared with cells cultured with 5 mM glucose ($P > 0.05$). The average $[Ca^{2+}]_i$ activity of single cells in the 48- and 72-h culture groups treated with 50 mM glucose was significantly higher than cells treated with 5 mM glucose ($P < 0.05$); and the fluorescent value of average $[Ca^{2+}]_i$ activity of single cells was lower, following the addition of Norvasc than that without Norvasc ($P < 0.05$). CaN activity in the 48- and 72-h culture group treated with 50 mM glucose was markedly higher than that treated with 5 mM glucose,

and the activity of CaN notably decreased with the addition of Norvasc compared with those without Norvasc. The mRNA and protein expression levels of CnA β , NFAT3 and β -MHC in the 48- and 72-h culture groups treated with 50 mM glucose were all significantly higher than those treated with 5 mM glucose ($P < 0.05$). The mRNA and protein expression of CnA β , NFAT3 and β -MHC cultured with 50 mM glucose were significantly decreased following the addition of Norvasc ($P < 0.05$). Thus, the calcium channel inhibitor Norvasc may inhibit high glucose-induced hypertrophy of H9C2 cardiomyocytes by inhibiting the Ca^{2+} -CaN-NFAT3 signaling pathway.

Introduction

China has a high incidence of diabetes mellitus, with the number of diabetic patients exceeding 90 million (1). At present, ~300 million individuals have been diagnosed with diabetes mellitus worldwide (2). Large-scale clinical studies have reported that patients with diabetes have a significantly higher risk of heart injury and cardiac insufficiency compared with those without diabetes (3-7). Diabetic cardiomyopathy is defined as a heart injury, which is independent of other diseases and is caused by diabetes mellitus itself (8), and manifests as left ventricular diastolic dysfunction at the early stage of onset and systolic dysfunction at the later stage. If not treated properly, diabetic cardiomyopathy can develop into heart failure, arrhythmia, cardiogenic shock and even sudden death in severe cases (9).

There are multiple clinical strategies to prevent and treat diabetic cardiomyopathy. However, the effect of reducing the incidence and mortality of cardiovascular complications requires improvement in patients with diabetes. Therefore, the study of diabetic cardiomyopathy requires extensive investigation. It is currently hypothesized that several mechanisms are involved in the pathogenesis of diabetic cardiomyopathy, including changes in myocardial energy metabolism and calcium signaling (10-13).

As an important secondary messenger, calcium plays an important role in the excitation-contraction coupling of the heart and regulates the expression of cardiac-related genes.

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The role of Ca^{2+} signaling and its dependent signal transduction pathway in cardiac hypertrophy and myocardial apoptosis has been widely recognized by researchers. Ca^{2+} is ubiquitous in cells and plays an important role not only in the electrical activity of the heart but also as a direct activator of myofilament contraction. Calcium enters the cardiomyocytes through voltage-gated calcium channels which selectively permeate calcium ions into the cell when there is a change from a high voltage to a low voltage inside of the cell. Voltage-gated calcium ion channels are subdivided into six fundamental types according to their electrophysiology and sensitivity to certain drugs and toxins. These voltage-gated calcium ion channels are named L-, T-, N-, P-, Q- and R-channels: L-channels have a long activation and high conductance, and are mainly located in skeletal, cardiac and vascular muscle and are involved in its contraction; T-channels have a transient opening and are involved in calcium ion entry when the membrane is depolarized; N-channels are involved in neurotransmitter release; P/Q-channels are mainly localized in the nerve terminal of cells of the cerebellum and are involved in neurotransmitter release; and R-channels are mainly localized in cell bodies and are involved in Ca^{2+} -dependent action potentials (14).

In the myocardium, the L-type calcium channel is the main pathway for calcium to enter the cell (15).

The role of the Ca^{2+} -calcineurin (CaN)-nuclear factor of activated T cells 3 (NFAT3) signaling pathway in cardiac development has gained interest in recent years, and several classes of antihypertensive drugs, including angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers and calcium channel blockers (CCBs), have been demonstrated to reverse hypertrophy in humans (16,17). CCBs were introduced for the treatment of hypertension in the 1980s. Their use was subsequently expanded to disorders such as angina pectoris, paroxysmal supraventricular tachycardia, hypertrophic cardiomyopathy, coronary spasm and cerebral vasospasm (18). Evidence from experimental studies indicate that CCBs nifedipine, nisoldipine and amlodipine additionally lead to regression of interstitial and perivascular myocardial fibrosis, which may contribute to the improvement of diastolic function and coronary reserve (19).

Norvasc is the brand name of amlodipine besylate. Amlodipine does not increase cardiovascular morbidity or mortality in patients with severe heart failure (20) and multiple studies have reported that amlodipine can reduce cardiac remodeling in spontaneously hypertensive rats and myocardial infarction in rats (21-23). Therefore, Norvasc was selected for the current study to determine whether it could inhibit hypertrophy of H9C2 cardiomyocytes induced by high glucose.

The aim of the present study was to investigate high glucose-induced hypertrophy of H9C2 cells and its possible pathogenesis, from the perspective of the calcium signaling pathway, to provide a theoretical basis for the identification of potential targets for clinical prevention and treatment of this disease.

Materials and methods

Materials. Cell culture reagents: FBS (cat. no. SH30087.01; Hyclone; GE Healthcare Life Sciences); DMEM-high glucose culture medium (50 mM; cat. no. 11965-092; Gibco; Thermo

Fisher Scientific, Inc.); DMEM-low glucose culture medium (5 mM; cat. no. 10567-014; Gibco; Thermo Fisher Scientific, Inc.). H9C2 cells were rat embryonic cardiomyocytes purchased from the Cell Resource Center of Shanghai Academy of Life Sciences, Chinese Academy of Sciences; Norvasc was obtained from Sigma-Aldrich (Merck KGaA); ELISA assay kit of CaN (cat. no. E-EL-R0134c) was purchased from Elabscience Biotechnology Co., Ltd.; Fluo-3 AM (calcium ion fluorescent probe; cat. no. S1056) was from Beyotime Institute of Biotechnology; DNase I (RNase-free) was purchased from Beijing Transgen Biotech Co., Ltd.; reverse transcription kit and real-time PCR kit were obtained from Vazyme; CnA β , nuclear factor of activated T cells 3 (NFAT3), β type myosin heavy chain (β -MHC) and b-actin primary antibodies (cat. nos. ab3673, ab66781, ab207926 and ab8227, respectively) were all purchased from Abcam.

Cell culture. Following thawing, cells (1×10^4 - 1×10^5 /ml) were cultured in DMEM containing 10% FBS in a 5% CO_2 incubator, with saturated humidity at 37°C.

Experimental grouping. The experimental groups were as follows: i) A1, 48-h culture group treated with 5 mM glucose; ii) B1, 48-h culture group treated with 50 mM glucose; iii) C1, 48-h culture group treated with 50 mM glucose + 25 nmol/l Norvasc; iv) A2, 72-h culture group treated with 5 mM glucose; v) B2, 72-h culture group treated with 50 mM glucose; vi) C2, 72-h culture group treated with 50 mM glucose + 25 nmol/l Norvasc. All cell treatments used DMEM containing 10% FBS.

Determination of cell surface area. Cell morphology were observed using an optical microscope, and the cell surface area was measured by Image-Pro Plus 6.1 software (Media Cybernetics, Inc.). A total of 3 fields of view were observed at magnification $\times 20$.

Determination of intracellular calcium ($[\text{Ca}^{2+}]_i$) activity. Cell culture media was removed and cells (1×10^4 - 1×10^5 /ml) were washed with PBS. Fluo-3 (2 μM), diluted in serum-free media was added for 1.5 h at 37°C, 5% CO_2 . Fluo-3 was removed, followed by two PBS washes and the addition of cell culture media. Fluorescence microscopy (magnification, $\times 20$) was performed to detect $[\text{Ca}^{2+}]_i$.

Determination of cellular CaN enzyme activity. Enzyme activity of CaN was measured using a rat CaN ELISA assay kit, according to the manufacturer's instructions.

Detection of CaN, NFAT3 and β -MHC mRNA expression in myocardial cells by reverse transcription-quantitative (RT-q) PCR. The extraction of RNA was achieved using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). Next, the reaction mixture was prepared with RNase-free DNase I, followed by digestion for 30 min at 37°C and inactivation for 10 min at 65°C to remove DNA. Reverse transcription was conducted with the addition of template RNA and primer mixtures at 42°C for 1 h, then resting on ice for 2 min. This was followed by the addition of cDNA (10X) to the primer reaction mixture. The RNA concentration was measured with NanoDrop™ 1000

(Thermo Fisher Scientific, Inc.). The cycling conditions for qPCR were as follows: 95°C for 30 sec, 40 cycles at 95°C for 10 sec followed by 60°C for 34 sec. The melting curve was drawn within the temperature range of 60-95°C. The primer sequences were: Rat-GAPDH forward (F), 5'-CATCAACGA CCCCTTCATTG-3' and reverse (R), 5'-GAAGATGGTGAT GGGTTTCC-3'; rat-CaN F, 5'-ATGTTGCCTAGTGGAGTG TT-3' and R, 5'-GGAGAGTATCCTCGTATTGCTT-3'; NFAT3 F, 5'-CCACAAGGCATTGAGACACAT-3' and R, 5'-TCACCA GCAGCAGCAGCAG-3'; and rat-β-MHC F, 5'-AATGAACAC CGGAGCAAGG-3'; and R, 5'-CGGGTCAGCTGAGAGATA AGAGC-3'. mRNA levels were quantified by relative quantification and analyzed using the $2^{-\Delta\Delta C_q}$ method (24).

Detection of CaN, NFAT3 and β-MHC protein expression in myocardial cells by western blotting. Culture plates of H9C2 cells (1×10^4 - 1×10^5 /ml) were washed with pre-cooled PBS and treated with pre-cooled lysis buffer (CWBio) on ice for 30 min. The lysis products were centrifuged for 20 min at $8,300 \times g$ at 4°C to collect the supernatant. Furthermore, 2 μl supernatant was used to determine the protein concentration by using a BCA protein quantitative kit. Protein samples (50 μg/well) were separated by SDS-PAGE on a 10% gel. Electrophoresis was performed for 20 min at a constant voltage of 100 V, and for 40 min at a constant voltage of 140 V. Subsequently, the proteins were transferred to a PVDF membrane, followed by three TBS washes. Following the removal of TBS, the membranes were blocked using TBS with Tween-20 (TBST) buffer (20 mM Tris-HCl, 150 mM NaCl and 0.1% Tween-20) containing 5% non-fat milk for 2 h at room temperature. The membrane was then incubated with TBST buffer (20 mM Tris-HCl, 150 mM NaCl and 0.1% Tween-20) containing 5% non-fat milk with the following primary antibodies, polyclonal rabbit anti-human CaN (1:1,000), polyclonal rabbit anti-human NFAT3 (1:1,000), polyclonal rabbit anti-human β-MHC (1:1,000) and polyclonal rabbit anti-human β-actin, overnight at 4°C. Following three washes with 1X PBS, the PVDF membrane was incubated with a horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:10,000; cat. no. 111-035-003; Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 2 h. The PVDF membrane was washed five times with PBS (15 min each), and developed using an ECL kit (Beijing ComWin Biotech Co., Ltd.).

Statistical analysis. GraphPad Prism 7 (GraphPad Software, Inc.) was used for statistical analysis. Data were analyzed with two-way ANOVA adjusted for multiple comparisons. Multiple comparisons between the groups was performed using Bonferroni tests. The bilateral inspection level for α was 0.05, and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cell swelling analysis. The cell size in B1 and B2 groups was increased compared with cells in A1 and A2 groups after 48 and 72 h of culture under different conditions. Cell size was decreased following the addition of Norvasc compared with those without the addition of Norvasc under the same conditions. There was no significant difference between the C1 and

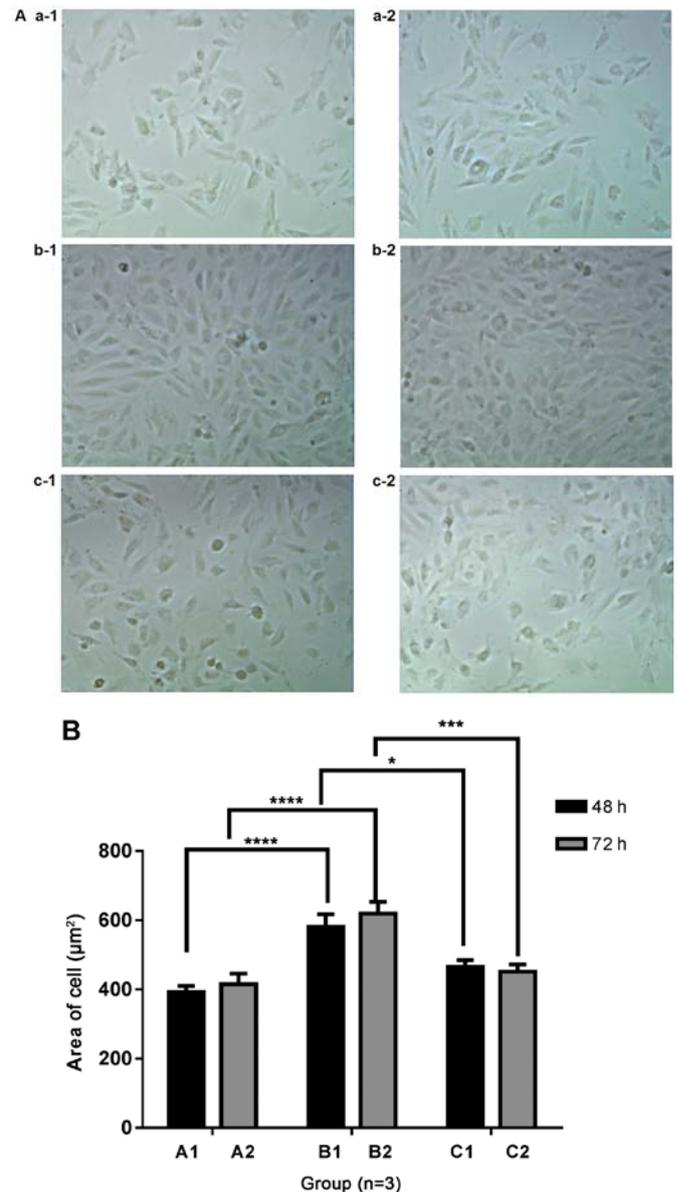


Figure 1. Measurement of cell size by Image Pro Plus 6.1 software in each group (n=3). (A) Representative images of H9C2 cells (magnification, x20) and (B) quantification of cell area. A1: 48-h culture group treated with 5 mM glucose; B1: 48-h culture group treated with 50 mM glucose; C1: 48-h culture group treated with 50 mM glucose + Norvasc; A2: 72-h culture group treated with 5 mM glucose; B2: 72-h culture group treated with 50 mM glucose. C2: 72-h culture group treated with 50 mM glucose + Norvasc. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

C2 and A1 and A2 groups, respectively. Furthermore, there was no significant difference in cell size between the A1 and A2 groups, B1 and B2 groups, and C1 and C2 groups, respectively. The statistical analysis chart is presented in Fig. 1.

Determination of $[Ca^{2+}]_i$ activity in each group. Following 48 and 72 h of culture, the fluorescent values of $[Ca^{2+}]_i$ activity in B1, B2, C1 and C2 groups were increased compared with the A1 and A2 groups, respectively. Following the addition of Norvasc, the fluorescent value of $[Ca^{2+}]_i$ activity in each group was notably lower than that without Norvasc under the same conditions. In addition, no obvious difference was found in the fluorescent values of intracellular $[Ca^{2+}]_i$ activity

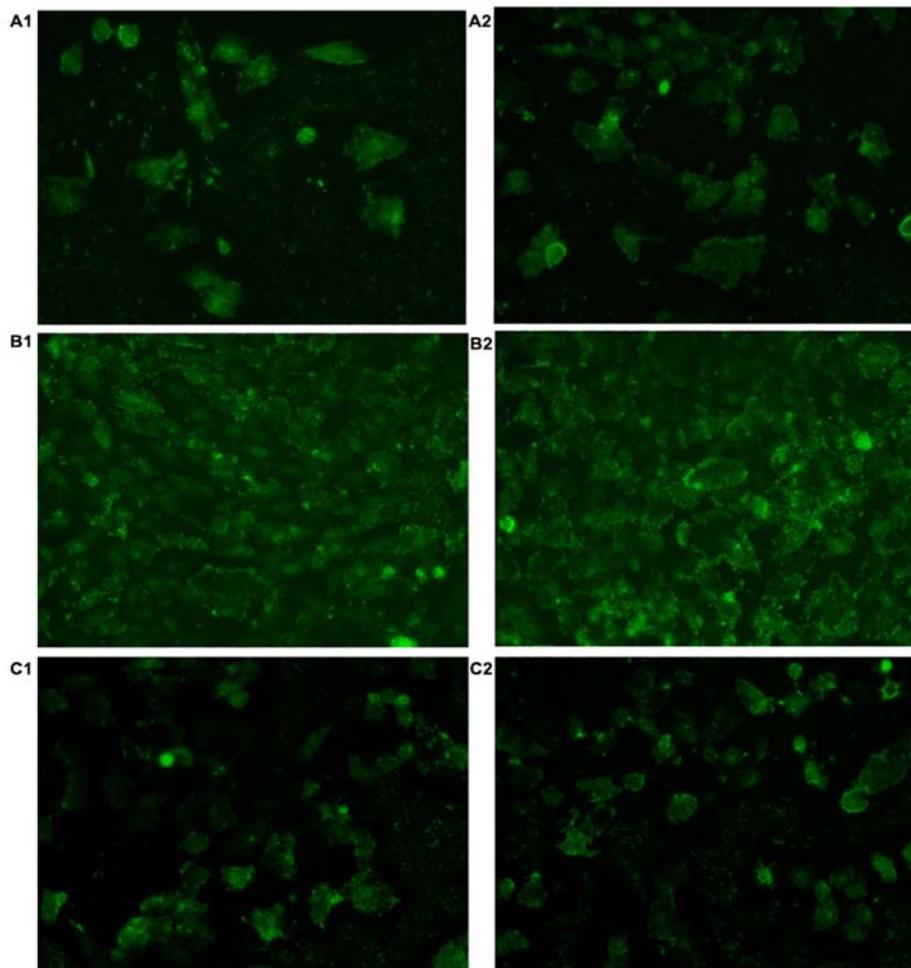


Figure 2. Images of fluorescent staining of intracellular calcium concentration activity in H9C2 cells (magnification, x20). (n=3) A1: 48-h culture group treated with 5 mM glucose; B1: 48-h culture group treated with 50 mM glucose; C1: 48-h culture group treated with 50 mM glucose + Norvasc; A2: 72-h culture group treated with 5 mM glucose; B2: 72-h culture group treated with 50 mM glucose; C2: 72-h culture group treated with 50 mM glucose + Norvasc.

between the A1 and A2 groups, B1 and B2 groups, and C1 and C2 groups, respectively. Images of fluorescent staining are presented in Fig. 2. The statistical analysis chart is presented in Fig. 3.

Cellular CaN enzyme activity. The CaN concentration was increased in the B1 and B2 groups compared with the A1 and A2 groups, following 48 and 72 h of culture, respectively. With the addition of Norvasc, the concentration of CaN in each group was significantly lower than that without Norvasc. The concentration standard curve is presented in Fig. 4A and the statistical analysis chart is presented in Fig. 4B.

CaN, NFAT3 and β -MHC mRNA expression levels of H9C2 cells determined by RT-qPCR

Changes in CaN mRNA expression in H9C2 cells. The mRNA expression of CaN in B1 and B2 groups was increased compared with that in the A1 and A2 groups, following 48 and 72 h of culture, respectively. Furthermore, the mRNA expression level of CaN was lower with the addition of Norvasc than that without Norvasc. The mRNA expression of CaN in the C1 and C2 groups had no significant difference between the A1 and A2 groups respectively. The statistical analysis chart is presented in Fig. 5.

Changes in NFAT3 mRNA expression in H9C2 cells. Following 48 and 72 h of culture, the mRNA expression of NFAT3 in the B1 and B2 groups was increased compared with the A1 and A2 groups, and expression in the C1 group was increased compared with the A1 group. The addition of Norvasc resulted in decreased NFAT3 mRNA expression than that without Norvasc. Additionally, no significant difference was observed in the mRNA expression level of NFAT3 in the C2 groups compared with that in the A2 groups. The statistical analysis chart is presented in Fig. 6.

Changes in β -MHC mRNA expression in H9C2 cells. A trend towards increased β -MHC mRNA expression was observed in B1 and B2 groups compared with the A1 and A2 groups following 48 and 72 h of culture, under different culture conditions. Following the addition of Norvasc, the mRNA expression of β -MHC was lower than that without Norvasc under the same conditions. The mRNA expression level of β -MHC in groups C1 and C2 were without any significant difference between groups A1 and A2, respectively. Furthermore, there was no statistical difference between the A1 and A2 groups or C1 and C2 groups. With the prolongation of culture time, a trend towards decreased β -MHC mRNA expression was observed in the B2 group compared with the B1 group. The statistical analysis chart is shown in Fig. 7.

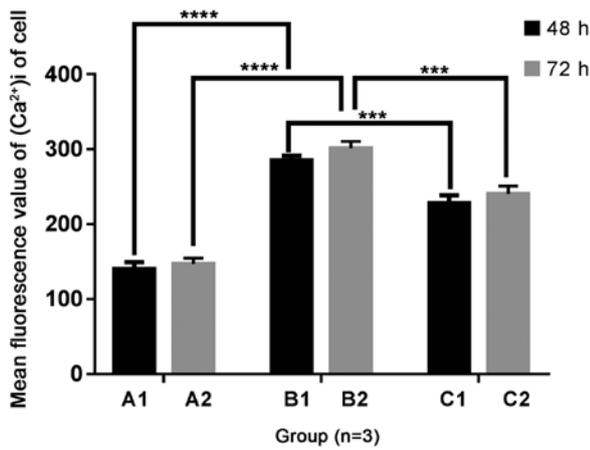


Figure 3. Fluorescence measurement of intracellular calcium concentration activity in H9C2 cells (n=3). A1: 48-h culture group treated with 5 mM glucose; B1: 48-h culture group treated with 50 mM glucose; C1: 48-h culture group treated with 50 mM glucose + Norvasc; A2: 72-h culture group treated with 5 mM glucose; B2: 72-h culture group treated with 50 mM glucose; C2: 72-h culture group treated with 50 mM glucose + Norvasc. ****P<0.001, ****P<0.0001. [Ca²⁺]_i, intracellular calcium concentration.

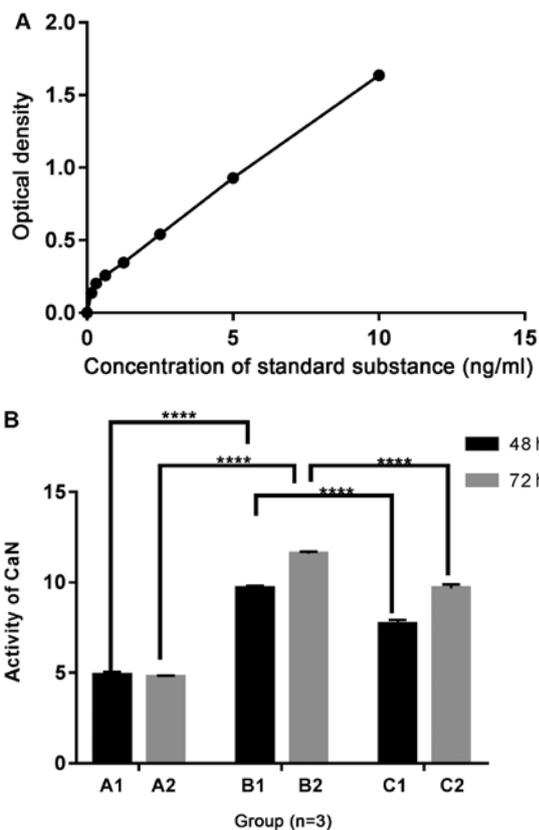


Figure 4. Measurement of CaN in H9C2 cells. (A) Standard curve of CaN determination. (B) Determination of CaN concentration in each group (n=3). A1: 48-h culture group treated with 5 mM glucose; B1: 48-h culture group treated with 50 mM glucose; C1: 48-h culture group treated with 50 mM glucose + Norvasc; A2: 72-h culture group treated with 5 mM glucose; B2: 72-h culture group treated with 50 mM glucose; C2: 72-h culture group treated with 50 mM glucose + Norvasc. ****P<0.0001. CaN, calcineurin.

Detection of CaN, NFAT3 and β -MHC protein expression in H9C2 cells by western blotting. Representative western blotting images are presented in Fig. 8.

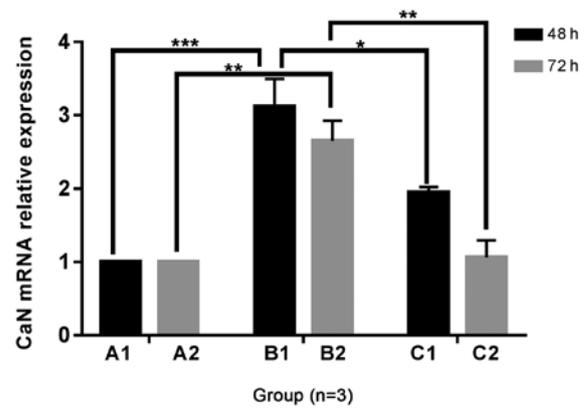


Figure 5. Detection of CaN mRNA expression in H9C2 cells by reverse transcription-quantitative PCR (n=3). A1: 48-h culture group treated with 5 mM glucose; B1: 48-h culture group treated with 50 mM glucose; C1: 48-h culture group treated with 50 mM glucose + Norvasc; A2: 72-h culture group treated with 5 mM glucose; B2: 72-h culture group treated with 50 mM glucose; C2: 72-h culture group treated with 50 mM glucose + Norvasc. *P<0.05; **P<0.01; ***P<0.001. CaN, calcineurin.

Detection of CaN protein expression in H9C2 cells by western blotting. The protein expression of CaN in B1 and B2 groups was increased compared with the A1 and A2 groups, respectively, following 48 and 72 h of culture under different culture conditions. Protein expression of CaN was significantly decreased in groups C1 and C2 compared with the B1 and B2 groups, respectively, following the addition of Norvasc. There was no significant difference between the C1 and C2 groups and A1 and A2 groups, respectively. The statistical analysis chart is presented in Fig. 9.

Detection of NFAT3 protein expression in H9C2 cells by western blotting. The protein expression of NFAT3 was increased in the B1 and B2 groups compared with that of the A1 and A2 groups, respectively, following 48 and 72 h of culture under different culture conditions. However, the protein expression of NFAT3 was decreased with the addition of Norvasc compared to that without Norvasc, under the same conditions. The NFAT3 protein expression was also increased in the C1 and C2 groups compared with that in the A1 and A2 groups. The statistical analysis chart is presented in Fig. 10.

Detection of β -MHC protein expression in H9C2 cells by western blotting. The trend of increased protein expression of β -MHC was observed in the B1 and B2 groups compared with that in the A1 and A2 groups, respectively, following 48 and 72 h of culture under different culture conditions. The addition of Norvasc resulted in decreased β -MHC protein expression compared with that without the addition of Norvasc, under the same conditions. Additionally, the expression of β -MHC protein in the C1 and C2 groups was not significantly different with that in the A1 and A2 groups. There was no significant difference in the protein expression of β -MHC in group A and B and C with the prolongation of culture time. The statistical analysis chart is presented in Fig. 11.

Discussion

The World Health Organization predicts that 300 million individuals worldwide will be diagnosed with diabetes mellitus

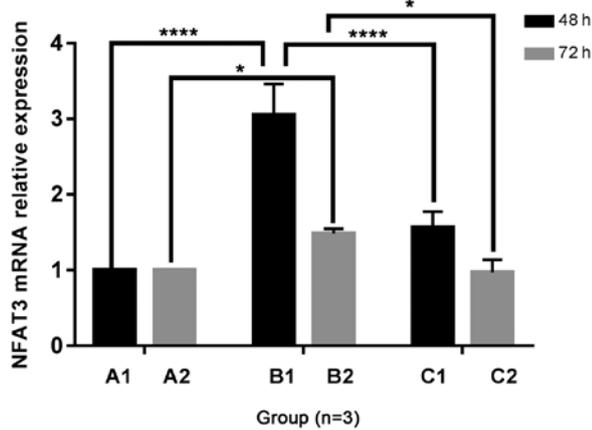


Figure 6. Detection of NFAT3 mRNA expression levels in H9C2 cells by reverse transcription-quantitative PCR (n=3). A1: 48-h culture group treated with 5 mM glucose; B1: 48-h culture group treated with 50 mM glucose; C1: 48-h culture group treated with 50 mM glucose + Norvasc; A2: 72-h culture group treated with 5 mM glucose; B2: 72-h culture group treated with 50 mM glucose; C2: 72-h culture group treated with 50 mM glucose + Norvasc. *P<0.05; ****P<0.0001. NFAT3, nuclear factor of activated T cells 3.

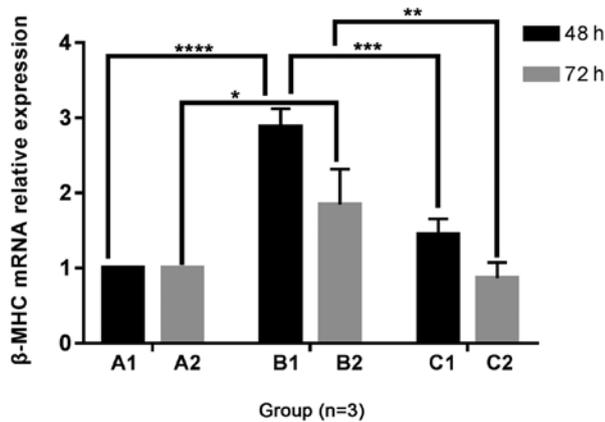


Figure 7. Detection of β -MHC mRNA expression levels in H9C2 cells by reverse transcription-quantitative PCR (n=3). A1: 48-h culture group treated with 5 mM glucose; B1: 48-h culture group treated with 50 mM glucose; C1: 48-h culture group treated with 50 mM glucose + Norvasc; A2: 72-h culture group treated with 5 mM glucose; B2: 72-h culture group treated with 50 mM glucose; C2: 72-h culture group treated with 50 mM glucose + Norvasc. *P<0.05; **P<0.01; ****P<0.0001. β -MHC, β type myosin heavy chain.

by 2025 (25). Patients with diabetes are prone to cardiovascular diseases, and the probability of myocardial infarction is 10 times higher than that of non-diabetic patients (26,27). First proposed by Rubler *et al* (28) in 1972, diabetic cardiomyopathy is a type of heart disease that is independent of coronary heart disease, valve disease and hypertensive heart disease, and is mainly characterized by diastolic dysfunction in the early stage and systolic dysfunction at the late stage. The pathogenesis is complex, with the main pathological changes including myocardial inflammation, metabolic disorders, myocardial cell apoptosis and fibrosis (29,30). The duration of hyperglycemia serves as an important indicator in determining the severity of heart failure caused by diabetic cardiomyopathy (31). Therefore, hyperglycemia is studied as an independent risk factor of myocardial injury caused by diabetic cardiomyopathy (32,33).

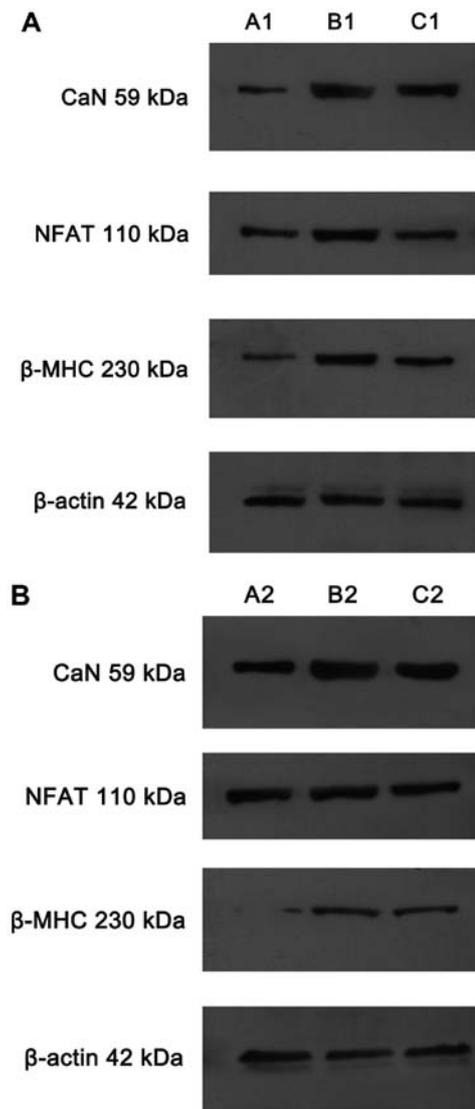


Figure 8. Representative western blot images extracted from the original western blot using Photoshop (version 8.0.1). (A) A1: 48-h culture group treated with 5 mM glucose; B1: 48-h culture group treated with 50 mM glucose; C1: 48-h culture group treated with 50 mM glucose + Norvasc. Data are representative of three independent experiments performed in triplicate. Initial study groups including those treated with 25 mM glucose and 25 mM glucose + Norvasc for 48 h (data not shown). (B) A2: 72-h culture group treated with 5 mM glucose; B2: 72-h culture group treated with 50 mM glucose; C2: 72-h culture group treated with 50 mM glucose + Norvasc. Data are representative of three independent experiments performed in triplicate. Initial study groups including those treated with 25 mM glucose and 25 mM glucose + Norvasc for 48 h (data not shown). CaN, calcineurin; NFAT3, nuclear factor of activated T cells 3; β -MHC, β type myosin heavy chain.

High glucose will stimulate cardiac hypertrophy in a variety of ways (34-37). In the present study, it was found that high glucose could lead to an increase in the average individual volume of H9C2 cells. Research investigating the aberrant molecular processes that occur during cardiac hypertrophy has used primary cardiomyocytes from neonatal rat hearts as the standard experimental *in vitro* system. In addition, some studies have made use of the H9C2 rat cardiomyoblast cell line (38,39), as it was found by Watkins *et al* (40) that the H9C2 cell line and primary neonatal cardiomyocyte cells exhibit similar hypertrophic responses *in vitro*. Therefore, the H9C2 cell line was selected for the present study, which also has

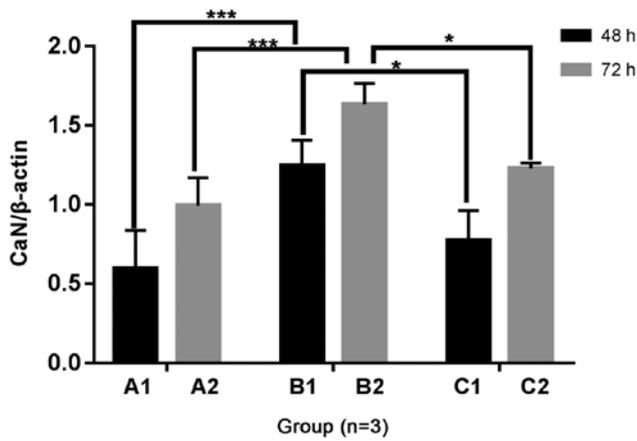


Figure 9. CaN protein expression in H9C2 cells detected by western blotting (n=3). A1: 48-h culture group treated with 5 mM glucose; B1: 48-h culture group treated with 50 mM glucose; C1: 48-h culture group treated with 50 mM glucose + Norvasc; A2: 72-h culture group treated with 5 mM glucose; B2: 72-h culture group treated with 50 mM glucose; C2: 72-h culture group treated with 50 mM glucose + Norvasc. *P<0.05; ***P<0.001. CaN, calcineurin.

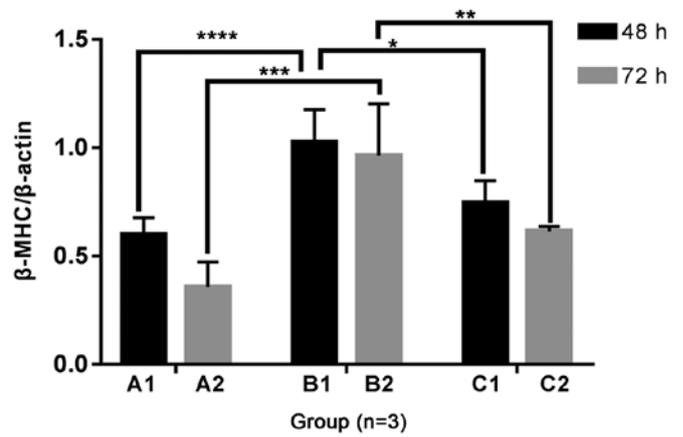


Figure 11. β-MHC protein expression in H9C2 cells detected by western blotting (n=3). A1: 48-h culture group treated with 5 mM glucose; B1: 48-h culture group treated with 50 mM glucose; C1: 48-h culture group treated with 50 mM glucose + Norvasc; A2: 72-h culture group treated with 5 mM glucose; B2: 72-h culture group treated with 50 mM glucose; C2: 72-h culture group treated with 50 mM glucose + Norvasc. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. β-MHC, β type myosin heavy chain.

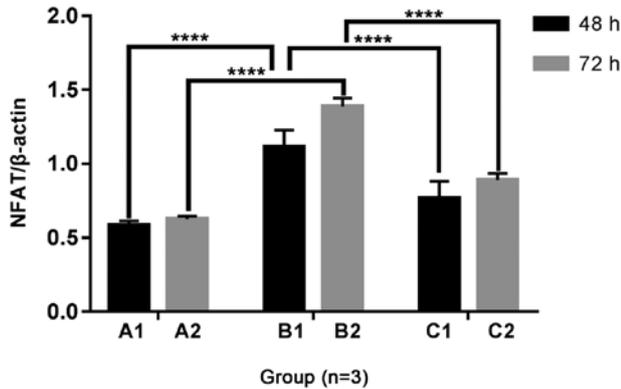


Figure 10. NFAT3 protein expression in H9C2 cells detected by western blotting (n=3). A1: 48-h culture group treated with 5 mM glucose; B1: 48-h culture group treated with 50 mM glucose; C1: 48-h culture group treated with 50 mM glucose + Norvasc; A2: 72-h culture group treated with 5 mM glucose; B2: 72-h culture group treated with 50 mM glucose; C2: 72-h culture group treated with 50 mM glucose + Norvasc. ****P<0.0001. NFAT3, nuclear factor of activated T cells 3.

the advantage of being an animal-free alternative. However, this is also a limitation of the present study because primary neonatal cardiomyocyte cells were not used. The role of the Ca²⁺-CaN-NFAT3 signaling pathway in cardiac development has become a research area of interest in recent years. The activation of Ca²⁺-dependent CaN A subunit (CnA) has been frequently observed in human heart hypertrophy and heart failure (41,42). In mice, increased intracellular calcium is known to activate CnA, which can bind and dephosphorylate the NFAT transcription factor family of activated T-nuclear factors. Subsequently, NFAT is transferred from the cytoplasm to the nucleus, activating the specific expression of various genes associated with cardiac hypertrophy, such as atrial natriuretic factor, brain natriuretic peptide (BNP), and β-MHC in the heart. This leads to increased nucleic acid synthesis of myocardial cell protein and increased cardiac cell volume, and finally cardiac hypertrophy (43,44). Transgenic mice

overexpressing a consistently active form of CnA-specific cardiomyocytes, MHC-CnA, have been found to suffer from cardiac hypertrophy at 18 days following birth, with varying degrees of progression to heart failure and sudden death (45).

Several different studies have found that the Ca²⁺-CaN-NFAT3 signaling pathway plays a role in cardiac hypertrophy. Daskoulidou *et al* (46) found that increased Orai 1 expression could mediate an increase in the calcium current of the store-operated calcium entry channel, in order to regulate calcium in cardiac cells, which may be induced by hyperglycemia through the activation of the CaN/NFATc3 signaling pathway. Somvanshi *et al* (47) revealed that the activation of the somatostatin receptor 2 (SSTR2) not only inhibited the expression and activity of CaN phosphatase, but also hindered the dephosphorylation of NFAT and nuclear translocation, which provided evidence that SSTR2 could protect the heart by regulating the Ca²⁺-associated signaling pathway, leading to cardiac hypertrophy. The T-type calcium channel, Cav3.2 could be induced by Egr1 (early growth response 1), which is released at the early stage of myocardial hypertrophy due to the early pressure overload, to regulate cardiac hypertrophy through the CaN phosphatase-NFAT signaling pathway (48). A previous study also revealed that the activity of the CaN signaling pathway may be activated by the bacteria *Porphyromonas gingivalis*, and further lead to cardiomyocyte hypertrophy and cell death of cultured H9C2 cardiomyocytes (49). The Ca²⁺-CaN-NFAT3 pathway is considered to be the crucial mechanism in mediating the development of cardiac hypertrophy and therefore, the role of the Ca²⁺-CaN-NFAT3 signaling pathway in hyperglycemia-induced myocardial hypertrophy was investigated in the present study. Following the determination of [Ca²⁺]_i and mRNA and protein expression levels of CaN, NFAT and β-MHC, it was revealed that high glucose could induce an increase in [Ca²⁺]_i and CaN concentration. Furthermore, high glucose increased the mRNA and protein levels of CaN, NFAT and β-MHC, and in combination with the cell morphology data, suggest the involvement of the Ca²⁺-CaN-NFAT signaling pathway in hyperglycemia-induced H9C2 cells.

The activation of CaN mainly depends on the increase in intracellular Ca^{2+} , and its activity is regulated by the change in intracellular Ca^{2+} concentration (50). The continuous maintenance of NFAT in the nucleus requires a continuous and oscillating increase in calcium (51) in order to maintain the activated form of CaN phosphatase. This CaN-dependent signal is sensitive to the inhibition of calcium channel blockers, such as verapamil, or CaN inhibitors, such as cyclosporine A (CSA) and tacrolimus (FK-506) (52-54). L-type Ca^{2+} channels are widely distributed in the heart, smooth and skeletal muscles (55,56). In the heart and smooth muscle cells, the L-type Ca^{2+} channels are responsible for the inward movement of Ca^{2+} , thereby causing contraction. In skeletal muscles, these channels act as voltage sensors in excitation-contraction coupling. L-type calcium channels consist of several subunits, namely, α_1 , α_2 , β , γ and δ (14). The α_1 subunit consists of four homologous structural domains, and contains transmembrane pores, through which calcium ions can be obtained, as well as calcium antagonist binding sites adjacent to the pores, which are connected to each other and to the calcium channel via allosteric junctions (57,58).

The L-type calcium channel is considered to be the main source of calcium that can activate CaN-NFAT3 signal transduction (59). Thus, L-type calcium channel inhibitors have become a research area of interest and an important therapeutic target for cardiac hypertrophy. Cardiac remodeling, which involves structural and functional changes at the molecular, cellular, tissue and whole-organ levels, can be used to determine the clinical course of heart failure (60). A number of studies have reported that amlodipine can reduce cardiac remodeling in spontaneously hypertensive rats and myocardial infarction in rats (61,62). Valsartan monotherapy as well as combination therapy with valsartan and either amlodipine or cilnidipine similarly attenuated hypertension and left ventricular hypertrophy (LVH) in hypertensive rats (22). Compared with amlodipine alone, amlodipine combined with atorvastatin had a greater beneficial effect on the myocardial hypertrophy. These benefits may be associated with the cumulative effect of the drug on inhibiting NADPH oxidase-mediated reactive oxygen species (63). Amlodipine has also been found to improve cardiomyocyte hypertrophy by inhibiting the phosphorylation of the epithelial growth factor receptor (64). Losartan, amlodipine, and particularly fosinopril can inhibit myocardial cell apoptosis, prevent myocardial fibrosis, and reverse cardiac hypertrophy; the inhibition of the cardiac renin-angiotensin-aldosterone system may be the mechanism of the cardioprotective effects of these three drugs (65). Meo *et al* (66) reported a decrease in L-type calcium current in cardiomyocytes in a glycosuria mouse model, induced by streptozotocin. In addition to the traditional L-type calcium channel, it is generally considered that Orai 1-mediated calcium store-operated calcium channel also participates in the calcium regulation of cardiomyocytes. In a diabetic model, Orai 1 expression was increased in both cardiomyocytes and smooth muscle cells (46,67). Amlodipine sulfonate is an L-type calcium channel blocker, which can selectively inhibit the transmembrane domain to inhibit calcium ions from entering cardiomyocytes. Several studies have demonstrated that amlodipine sulfonate could inhibit CaN-NFAT3 and thus inhibit cardiac hypertrophy (68,69). Therefore, the L-type calcium channel amlodipine besulfonate, Norvasc, was

selected to investigate whether intracellular influx of Ca^{2+} was observed in H9C2 cells cultured with high glucose, in order to induce changes in intracellular calcium concentration and the activation of CaN, thereby activating the signaling pathway, and ultimately causing cardiomyocyte hypertrophy.

The main purpose of the present study was to investigate whether Norvasc could induce the recovery of cardiomyocyte hypertrophy and inhibit Ca^{2+} -CaN-NFAT3 signaling. Following the addition of Norvasc, the activity of $[\text{Ca}^{2+}]_i$, CaN concentration, CaN mRNA and protein expression in the corresponding groups were all significantly decreased, which indicated that Norvasc reduced CaN expression. Thus, intracellular calcium could activate CaN and promote its expression. The present study demonstrated that calcium channel inhibitors could alleviate cell hypertrophy and are more effective in inhibiting cell hypertrophy following prolonged action. Bugyei-Twum *et al* (70) also found that high glucose *in vitro* induced the activation of Smad in H9C2 cells and promoted cardiac fibrosis and hypertrophy through transcriptional coregulator p300, and Chen *et al* (71) revealed that lercanidipine may ameliorate cardiomyocyte hypertrophy, partially by blocking Cn-NFAT3 and CaMKII-HDAC4 signaling. These findings are consistent with the present study.

The signal transduction mechanism of myocardial hypertrophy is complex. In addition to Ca^{2+} -CaN-NFAT3 signaling pathway, there may be other signaling pathways, which may have interactions with CaN-NFAT3. Thus, further studies are required, including the use of specific inhibitors, such as CSA, to identify other pathways. Liu *et al* (72) demonstrated that lipopolysaccharide (LPS) treatment leads to myocardial hypertrophy via the calcineurin/NFAT-3 signaling pathway in H9C2 cells. They further provided a link between the LPS-induced inflammatory response and the calcineurin/NFAT-3 signaling pathway that mediates the development of cardiac hypertrophy. LPS treatment was found to significantly promote the activation and nuclear translocation of NFAT3 and mediated the development of cardiac hypertrophy as a transcription factor. The cell size, actin fiber, atrial natriuretic peptide and BNP levels were assessed following the use of ERK1/2 inhibitors, p38 MAPK inhibitors, JNK 1/2 inhibitors, CaN inhibitors and NF-KB inhibitors, which demonstrated that only the CaN inhibitor could significantly inhibit NFAT3 nuclear localization. The present study provides findings that can be used to determine subsequent future studies.

In summary, the present experimental design and results suggest that, the calcium channel inhibitor Norvasc may inhibit hyperglycemia in H9C2 cells, which may result from the activation of the Ca^{2+} -CaN-NFAT3 signaling pathway by high glucose levels.

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Availability of data and materials

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

Authors' contributions

XX, LR, XT, FP and GL made substantial contributions to conception and design of the study. XX, LR, XT, FP and CY provided resources, contributed to research and validation of the results, interpreted data and wrote the original draft preparation. XX, LR, FP and GL reviewed and edited the manuscript. XX, LR and GL supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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