

# Protective effect of angiotensin-(1-7) against hyperglycaemia-induced injury in H9c2 cardiomyoblast cells via the PI3K/Akt signaling pathway

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**Abstract.** Angiotensin-(1-7) [Ang-(1-7)], a heptapeptide mainly generated from cleavage of AngI and AngII, possesses physiological and pharmacological properties, including anti-inflammatory and antidiabetic properties. Activation of the phosphoinositide 3-kinase and protein kinase B (PI3K/Akt) signaling pathway has been confirmed to participate in cardioprotection against hyperglycaemia-induced injury. The aim of the present study was to test the hypothesis that Ang-(1-7) protects H9c2 cardiomyoblast cells against high glucose (HG)-induced injury by activating the PI3K/Akt pathway. To examine this hypothesis, H9c2 cells were treated with 35 mmol/l (mM) glucose (HG) for 24 h to establish a HG-induced cardiomyocyte injury model. The cells were co-treated with 1  $\mu$ mol/l ( $\mu$ M) Ang-(1-7) and 35 mM glucose. The findings of the present study demonstrated that exposure of H9c2 cells to HG for 24 h markedly induced injury, as evidenced by an increase in the percentage of apoptotic cells, generation of reactive oxygen species and level of inflammatory cytokines, as well as a decline in cell viability and mitochondrial luminosity. These injuries were significantly attenuated by co-treatment of the cells with Ang-(1-7) and HG. In addition, PI3K/Akt phosphorylation was suppressed by HG treatment, but this effect was abolished when the H9c2 cells were co-treated with Ang-(1-7) and HG. Furthermore, the cardioprotection of Ang-(1-7) against HG-induced injury in

H9c2 cardiomyoblasts was highly attenuated in the presence of either D-Ala7-Ang-(1-7) (A-779, an antagonist of the Mas receptor) or LY294002 (an inhibitor of PI3K/Akt). In conclusion, the present study provided new evidence that Ang-(1-7) protects H9c2 cardiomyoblasts against HG-induced injury by activating the PI3K/Akt signaling pathway.

## Introduction

Diabetes severely affects human health, and epidemiological studies have reported that the number of diabetic patients is expected to reach 592 million worldwide by 2035 (1). Diabetes is tightly associated with both microvascular (including neuropathy, nephropathy and retinopathy) and macrovascular (including cardiovascular diseases) complications (2-6). As a common complication of diabetes, diabetic cardiomyopathy (DCM) represents the main cause of morbidity and mortality among diabetic patients (7). DCM is generally considered to be manifested by a series of structural and functional anomalies in the myocardium of diabetic patients, including myocardial fibrosis, impaired diastolic and systolic contractility, cardiomyocyte hypertrophy, cardiac autonomic neuropathy and apoptosis (8-11). Hyperglycaemia is the key element of diabetes, and plays a crucial role in the evolution of DCM (11,12). Accumulating reports have revealed that multifarious factors may contribute to hyperglycaemia-induced myocardial damage, including reactive oxygen species (ROS) generation (13-18), insufficiency of antioxidant systems (16-21) and mitochondrial dysfunction (13,21,22). Cardiac inflammatory reactions, characterized by increased levels of pro-inflammatory cytokines, may also play an important part in the manifestation of DCM (23-25). However, the pathogenesis of hyperglycaemia-induced cardiomyocyte injury has not been fully elucidated.

The phosphoinositide 3-kinase and protein kinase B (PI3K/Akt) signaling pathway plays a key role in the conditioning of cell proliferation and survival (26). It has been reported that the evolution of DCM is interlinked with Akt pathway deactivation (27,28). In the myocardium of diabetic

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rats, Akt phosphorylation may be inhibited by increased circulating free fatty acids and inflammatory cytokines (29). However, in diabetic mice, cardiac systolic function and cardiomyocyte proliferation may be improved via benfotiamine-induced activation of the Akt pathway (27). Activation of the PI3K/Akt pathway may protect cardiomyocytes against hyperglycaemia-triggered oxidative stress as well as inflammation, along with an increase in cell viability (29,30). Jadhav *et al* (31) also reported that increased expression of the PI3K/Akt signaling pathway may lead to reduction of pro-inflammatory cytokines and account for enhanced glucose metabolism and amelioration of cardiac injury in DCM. Accordingly, it is reasonable to hypothesize that the molecules that activate PI3K/Akt signaling may exert cardioprotective effects against hyperglycaemia-induced cardiomyocyte injury.

Angiotensin-(1-7) [Ang-(1-7)] is a heptapeptide, mainly generated by cleavage of AngI and AngII by the angiotensin-converting enzyme (ACE) 2 (32-34), that possesses cardioprotective properties against myocardial hypertrophy, pathological cardiac remodeling, fibrosis and inflammation (35-40). Ang-(1-7) has been found to activate the PI3K/Akt pathway in cardiomyocytes (41-43); thus, it has been hypothesized that Ang-(1-7) exerts protective effects on the myocardium against diabetes, due to its range of therapeutic properties. The aim of the present study was to investigate the cytoprotective effect of Ang-(1-7) on H9c2 cardiomyoblasts against hyperglycaemia and its effects on the PI3K/Akt signaling pathway, which is involved in anti-inflammation and cell survival.

## Materials and methods

**Materials.** Ang-(1-7) and D-Ala<sup>7</sup>-Ang-(1-7) (A-779) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA), and stored at -20°C. 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA), fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM)-F12 were purchased from Gibco-BRL (Thermo Fisher Scientific; Grand Island, NY, USA). Hoechst 33258, rhodamine 123 (Rh123) and LY294002 (an inhibitor of PI3K/Akt) were obtained from Sigma-Aldrich (Merck KGaA; St. Louis, MO, USA). The Cell Counting Kit-8 (CCK-8) was supplied by Dojindo Laboratories (Kumamoto, Japan). Anti-phospho-PI3K rabbit mAb (cat. no. 4228), anti-total-PI3K rabbit mAb (cat. no. 4292), anti-phospho-Akt rabbit mAb (cat. no. 12178), anti-total-Akt rabbit mAb (cat. no. 14702), anti-cleaved caspase-1 rabbit mAb (cat. no. 2225), anti-cleaved caspase-3 rabbit mAb (cat. no. 9662) and anti-cleaved caspase-12 rabbit mAb (cat. no. 2202) were supplied by Cell Signaling Technology, Inc. (Boston, MA, USA), horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. KC5G5) and bicinchoninic acid (BCA) protein assay kit were obtained from Kangchen Biotech, Inc. (Shanghai, China). Enhanced chemiluminescence (ECL) solution was purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). Interleukin (IL)-1 $\beta$ , -6 and tumor necrosis factor (TNF)- $\alpha$  enzyme-linked immunosorbent assay (ELISA) kits were provided by Abcam (Cambridge, UK).

**Cell culture and treatments.** H9c2 cells, a rat cardiac myoblast cell line, were supplied by Sun Yat-Sen University Experimental Animal Center (Guangzhou, China). H9c2 cardiomyoblasts were

cultured in DMEM-F12 supplemented with 10% FBS under an atmosphere of 5% CO<sub>2</sub> and at 37°C with 95% air. H9c2 cardiomyoblasts were treated with 35 mmol/l (mM) glucose (high glucose, HG) in the presence or absence of 1  $\mu$ mol/l ( $\mu$ M) Ang-(1-7) for 24 h. To further ascertain whether the protective effect of Ang-(1-7) and the activation of the PI3K/Akt pathway were induced by Ang-(1-7), H9c2 cardiomyoblasts were co-treated with 1  $\mu$ M Ang-(1-7) and 35 mM glucose in the presence of 1  $\mu$ M A-779 or 10  $\mu$ M LY294002 for 24 h.

**Western blot analysis.** After the indicated treatments, H9c2 cardiomyoblasts were harvested and lysed with cell lysis solution at 4°C for 30 min and total protein was quantified using the BCA protein assay kit. Loading buffer was added to cytosolic extracts, followed by boiling for 5 min; the same amount of supernatant from each sample was fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the total proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% fat-free milk for 60 min in fresh blocking buffer [0.1% Tween-20 in Tris-buffered saline (TBS-T)] at room temperature, and incubated with either anti-phospho-PI3K (1:1,000 dilution), anti-total-PI3K (1:1,000 dilution), anti-phospho-Akt (1:1,000 dilution), anti-total-Akt (1:1,000 dilution), anti-cleaved caspase-1 (1:1,000 dilution), anti-cleaved caspase-3 (1:1,000 dilution), or anti-cleaved caspase-12 (1:1,000 dilution) in freshly prepared TBS-T with 3% free-fat milk overnight with gentle agitation at 4°C. The membranes were washed for 15 min with TBS-T and incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:3,000 dilution; Kangchen Biotech, Inc., Shanghai, China) in TBS-T with 3% fat-free milk for 1.5 h at room temperature. The membranes were then washed 3 times with TBS-T for 15 min. The immunoreactive signals were visualized using ECL detection. In order to quantify protein expression, the X-ray films were scanned and analyzed with ImageJ 1.47i software. The experiment was performed 3 times.

**Measurement of cell viability.** H9c2 cardiomyoblasts were seeded in 96-well plates at a density of 1x10<sup>4</sup>/ml, incubated at 37°C, and the CCK-8 assay was employed to assess cell viability. After the indicated treatments, 10  $\mu$ l CCK-8 solution (1/10 dilution) was added to each well, and the plate was then incubated for 2 h in the incubator. Absorbance at 450 nm was assayed using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) as previously described (44). The means of the optical density (OD) of 3 wells in the indicated groups were used to calculate the percentage of cell viability as follows: Cell viability (%) = (OD<sub>treatment group</sub>/OD<sub>control group</sub>) x 100%. The experiment was performed 3 times.

**Hoechst 33258 nuclear staining for apoptosis assessment.** Apoptotic cell death was tested using Hoechst 33258 staining followed by photofluorography. First, H9c2 cells were plated in 35-mm dishes at a density of 1x10<sup>6</sup> cells/well. After the above-mentioned indicated treatments, the H9c2 cells were fixed with 4% paraformaldehyde in 0.1 mol/l phosphate-buffered saline (PBS; pH 7.4) for 10 min at 4°C, and the slides were then washed 5 times with PBS, followed by 5 mg/ml Hoechst 33258 for 10 min and washing 5 times with PBS. Finally, the cells were visualized under a fluorescence microscope (BX50-FLA; Olympus, Tokyo,

Japan). Viable H9c2 cells displayed a uniform blue fluorescence throughout the nucleus and normal nuclear size, whereas apoptotic H9c2 cells exhibited condensed, distorted or fractured nuclei. The experiment was performed 3 times.

**Measurement of mitochondrial membrane potential (MMP).** The MMP ( $\Delta\Psi_m$ ) was tested using a fluorescent dye, Rh123, a cell-permeable cationic dye that preferentially enters mitochondria due to the highly negative MMP. Depolarization of the membrane results in loss of MMP from the mitochondria and a decrease in green and red fluorescence. H9c2 cells were cultured in a slide with EMEM-F12. After the abovementioned treatments, the slides were washed 3 times with PBS. The cells were incubated with 1 mg/l Rh123 at 37°C for 45 min in the incubator, washed briefly with PBS 3 times and air-dried. Fluorescence was measured over the entire field of vision using a fluorescence microscope connected to an imaging system (BX50-FLA, Olympus). The mean fluorescence intensity (MFI) of Rh123 from 5 random fields was analyzed using the ImageJ 1.47i software; MFI was considered as an index of the levels of MMP. The experiment was performed 3 times.

**Examination of intracellular ROS generation.** Intracellular ROS generation was determined based on the oxidative conversion of cell-permeable oxidation of DCFH-DA to fluorescent DCF. H9c2 cardiomyoblasts were cultured in a slide with EMEM-F12 medium. After the abovementioned treatments, the slides were washed twice with PBS. DCFH-DA (10  $\mu\text{mol/l}$ ) solution in serum-free medium was added to the slides, and the cells were then incubated at 37°C for a further 30 min in the incubator. The slides were washed 5 times with PBS, and DCF fluorescence was measured over the entire field of vision using a fluorescence microscope connected to an imaging system (BX50-FLA, Olympus). The MFI from 5 random fields was measured using ImageJ 1.47i software and the MFI was used as an index of the amount of ROS. The experiment was performed 3 times.

**ELISA.** H9c2 cells were cultured in 96-well plates. After the indicated treatments, the medium was collected and used for ELISA. IL-1 $\beta$ , -6 and TNF- $\alpha$  assays were performed according to the manufacturer's instructions with the respective ELISA kits. The experiment was performed 3 times.

**Statistical analysis.** All data are presented as the mean  $\pm$  standard error of the mean. Differences between groups were analyzed with one-way analysis of variance using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA), followed by the LSD post-hoc comparison test. Statistical significance was set at  $P < 0.05$ .

## Results

**Ang-(1-7) attenuates HG-induced decrease in cell viability in H9c2 cardiomyoblast cells.** To evaluate whether Ang-(1-7) protects H9c2 cardiomyoblasts against HG (35 mM), a dose-response study with varying doses of Ang-(1-7) (0.1, 1, 5, 10, 20 and 40  $\mu\text{M}$ ) was performed to calculate the effective cytoprotective dose of Ang-(1-7). The data shown in Fig. 1A indicate that exposure of H9c2 cells to 35 mM

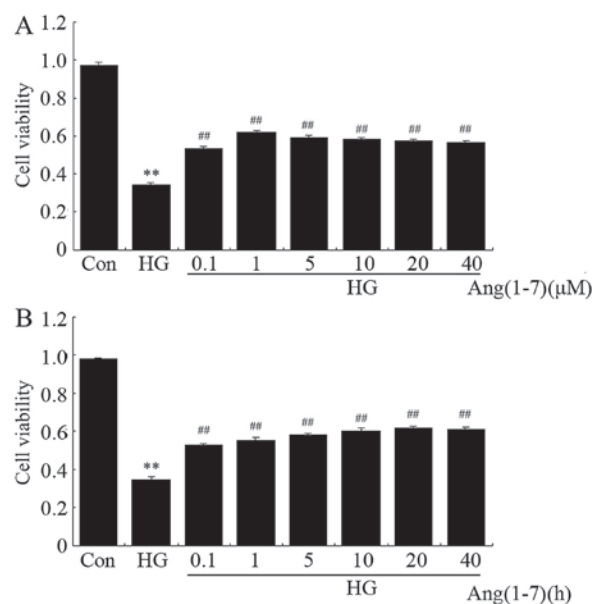


Figure 1. Ang-(1-7) protects against HG-triggered decrease in viability of H9c2 cells. Cell viability was detected using the Cell Counting Kit-8. (A) H9c2 cells were treated with 35 mM HG for 24 h in the absence or presence of the indicated concentrations of Ang-(1-7). (B) H9c2 cells were treated with HG for the indicated times in the absence or presence of 1  $\mu\text{M}$  Ang-(1-7). Data are presented as mean  $\pm$  standard error of the mean ( $n=3$ ). \*\* $P < 0.01$  compared with the control group; ## $P < 0.01$  vs. the HG-treated group. Ang-(1-7), angiotensin-(1-7); HG, high glucose (35 mM); LY, LY294002.

glucose for 24 h was markedly cytotoxic, decreasing cell viability to 34.7% ( $P < 0.01$ ) compared with the non-treated group. However, the cytotoxic effect of HG on H9c2 cells was notably inhibited by treatment with Ang-(1-7) at the indicated concentrations for 24 h. The maximum inhibitory effect was observed with 1  $\mu\text{M}$  Ang-(1-7). Ang-(1-7) (1  $\mu\text{M}$ ) alone did not obviously alter the viability of H9c2 cells. Therefore, 1  $\mu\text{M}$  Ang-(1-7) was used in the subsequent time-response study with different pretreatment times (1, 3, 6, 12, 24 and 48 h). As shown in Fig. 1B, co-treatment of H9c2 cells with 1  $\mu\text{M}$  Ang-(1-7) and 35 mM glucose for the indicated times all markedly reduced HG-induced cytotoxicity, achieving the maximal inhibitory ability at 24 h. Based on the abovementioned results, H9c2 cardiomyoblasts were co-treated with 1  $\mu\text{M}$  Ang-(1-7) and 35 mM glucose for 24 h in all the subsequent experiments.

**Ang-(1-7) alleviates HG-induced dephosphorylation of PI3K/Akt in H9c2 cardiomyoblasts.** To investigate the potential mechanism underlying the cytoprotective effect of Ang-(1-7) on H9c2 cells, PI3K/Akt activation was subsequently examined. As shown in Fig. 2, PI3K/Akt phosphorylation was suppressed by HG treatment compared with the control group, but this effect was abolished when the H9c2 cells were co-treated with Ang-(1-7) and HG. Moreover, the function of Ang-(1-7) in restoring PI3K/Akt phosphorylation may be abolished by the presence of 1  $\mu\text{M}$  A-779 (an antagonist of the Mas receptor). Treatment with either Ang-(1-7) or A-779 alone did not affect PI3K/Akt phosphorylation.

**Activation of the PI3K/Akt pathway contributes to the cytoprotective effect of Ang-(1-7) against the HG-induced decline in H9c2 cell viability.** To determine whether the increase in PI3K/Akt

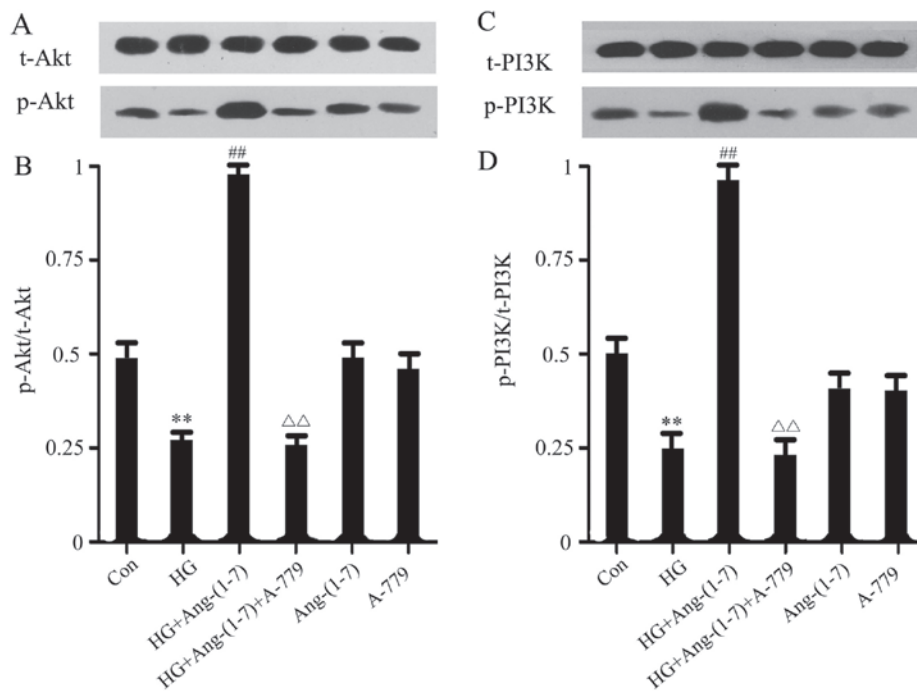


Figure 2. Ang-(1-7) mitigates HG-induced dephosphorylation of phosphoinositide 3-kinase and protein kinase B (PI3K/Akt) in H9c2 cells. H9c2 cells were treated for 24 h with 35 mM glucose alone, or co-treated with 1  $\mu$ M Ang-(1-7) and 35 mM glucose, or co-treated with 35 mM glucose, 1  $\mu$ M Ang-(1-7) and 1  $\mu$ M A-779, or treated with 1  $\mu$ M Ang-(1-7) alone, or 1  $\mu$ M A-779 alone. The expression of PI3K and Akt in the indicated groups. (B and D) Densitometric analysis for the results in (A) and (C), respectively. Data are presented as mean  $\pm$  standard error of the mean (n=3). \*\*P<0.01 compared with the control group; ##P<0.01 compared with the HG-treated group;  $\Delta\Delta$ P<0.01 vs. the group co-treated with Ang-(1-7) and HG. Ang-(1-7), angiotensin-(1-7); HG, high glucose (35 mM); LY, LY294002.

phosphorylation by Ang-(1-7) contributes to the cardioprotective effect of Ang-(1-7) against HG-induced cytotoxicity, H9c2 cardiomyoblasts were co-conditioned with 1  $\mu$ M Ang-(1-7) and 35 mM glucose in the presence of 10  $\mu$ M LY294002 (a selective inhibitor of PI3K/Akt). As shown in Fig. 3, co-treatment with HG and Ang-(1-7) blunted the cytotoxic effect and increased cell viability, but the presence of A-779 eliminated the cytoprotective effect of Ang-(1-7). Of note, treatment with LY294002 eliminated the protective effect of Ang-(1-7) in H9c2 cardiomyoblasts against HG-induced decreased cell viability. However, treatment with Ang-(1-7) or A-779 or LY294002 alone did not decrease H9c2 cell viability. These findings indicate that Ang-(1-7) protects H9c2 cardiomyoblasts against HG-induced cytotoxicity, at least partially via PI3K/Akt pathway activation.

*Activation of the PI3K/Akt pathway promotes the cytoprotective effect of Ang-(1-7) against HG-induced apoptosis in H9c2 cardiomyoblasts.* An increasing number of studies have proposed that HG leads to increased apoptosis in myocardial injury. Thus, the effect of Ang-(1-7) on HG-induced cell apoptosis was observed in H9c2 cardiomyoblasts. It was demonstrated that treatment of H9c2 cells with 35 mM glucose for 24 h significantly increased apoptosis (Fig. 4B). However, the abovementioned phenomenon may be clearly reversed by co-treatment with Ang-(1-7) and HG for 24 h (Fig. 4C). It was observed that treating the H9c2 cardiomyoblasts with 35 mM glucose and 1  $\mu$ M Ang-(1-7) in the presence of A-779 for 24 h did not significantly reduce apoptosis (Fig. 4D). Of note, apoptosis was increased in H9c2 cardiomyoblasts by co-treatment with 1  $\mu$ M Ang-(1-7) and 35 mM glucose in the

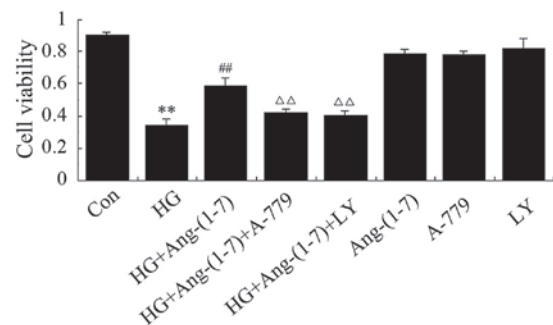


Figure 3. Activation of phosphoinositide 3-kinase and protein kinase B (PI3K/Akt) participates in the protective effect of Ang-(1-7) against HG-induced decreased viability of H9c2 cells. H9c2 cells were treated for 24 h with 35 mM glucose alone, or co-treated with 1  $\mu$ M Ang-(1-7) and 35 mM glucose, or co-treated with 35 mM glucose, 1  $\mu$ M Ang-(1-7) and 1  $\mu$ M A-779, or co-treated with 35 mM glucose, 1  $\mu$ M Ang-(1-7) and 10  $\mu$ M LY294002, or treated with 1  $\mu$ M Ang-(1-7) alone, or 1  $\mu$ M A-779 alone, or 10  $\mu$ M LY294002 alone. Cell viability was detected using the Cell Counting Kit-8. Data are presented as mean  $\pm$  standard error of the mean (n=3). \*\*P<0.01 compared with the control group; ##P<0.01 compared with HG-treated group;  $\Delta\Delta$ P<0.01 vs. the group co-treated with Ang-(1-7) and HG. Ang-(1-7), angiotensin-(1-7); HG, high glucose (35 mM); LY, LY294002.

presence of 10  $\mu$ M LY294002 (Fig. 4E). Ang-(1-7), A-779 or LY294002 alone did not exert any effect on myocardial apoptosis (Fig. 4F-H). These findings indicated that the PI3K/Akt pathway may participate in the anti-apoptotic function of Ang-(1-7) in HG-exposed H9c2 cardiomyoblast cells.

*Activation of the PI3K/Akt pathway is associated with the cytoprotection of Ang-(1-7) against HG-induced ROS*

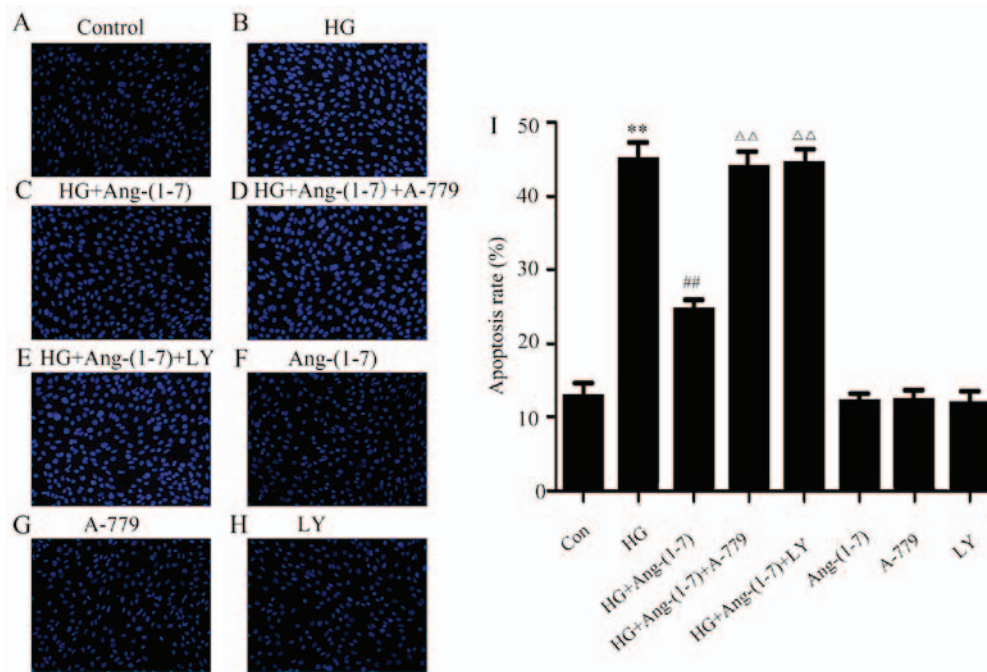


Figure 4. Activation of phosphoinositide 3-kinase and protein kinase B (PI3K/Akt) is involved in the protective effect of Ang-(1-7) against HG-induced apoptosis in H9c2 cells. (A-H) After the indicated treatments, cellular apoptosis was assessed by Hoechst 33258 staining followed by photofluorography. (A) Control group. (B) H9c2 cells were exposed to 35 mM glucose for 24 h. (C) H9c2 cells were co-treated with 1  $\mu$ M Ang-(1-7) and 35 mM glucose for 24 h. (D) H9c2 cells were co-treated with 1  $\mu$ M Ang-(1-7) and 35 mM glucose in the presence of 1  $\mu$ M A-779 for 24 h. (E) H9c2 cells were co-treated with 1  $\mu$ M Ang-(1-7) and 35 mM glucose in the presence of 10  $\mu$ M LY294002 for 24 h. (F) H9c2 cells were treated with 1  $\mu$ M Ang-(1-7) for 24 h followed by 24-h culture. (G) H9c2 cells were treated with 1  $\mu$ M A-779 for 24 h followed by 24-h culture. (H) H9c2 cells were treated with 10  $\mu$ M LY294002 for 24 h followed by 24-h culture. (I) The apoptosis rate was analysed with a Cell Counting Kit-8 and ImageJ 1.41o software. Data are presented as mean  $\pm$  standard error of the mean (n=3). \*\*P<0.01 compared with the control group; ##P<0.01 compared with the HG-treated group;  $\Delta\Delta$ P<0.01 vs. the group co-treated with Ang-(1-7) and HG. Ang-(1-7), angiotensin-(1-7); HG, high glucose (35 mM); LY, LY294002.

production in H9c2 cardiomyoblast cells. As shown in Fig. 5B, exposure to 35 mM glucose for 24 h induced an increase in the generation of ROS in H9c2 cardiomyoblast cells, and the increased ROS production was suppressed by the presence of Ang-(1-7) (Fig. 5C). Furthermore, co-treatment with A-779, Ang-(1-7) and HG diminished the aforementioned effect of Ang-(1-7), further indicating the cardioprotective function of Ang-(1-7) against HG-induced ROS production (Fig. 5D). However, ROS production increased when H9c2 cardiomyoblasts were co-treated with 1  $\mu$ M Ang-(1-7) and 35 mM glucose in the presence of 10  $\mu$ M LY294002 (Fig. 5E). Our study demonstrated that Ang-(1-7), A-779 or LY294002 alone did not affect ROS production in H9c2 cells (Fig. 5F-H). These results revealed that PI3K/Akt pathway activation is involved in the cytoprotective function of Ang-(1-7) against HG-triggered ROS overproduction in H9c2 cardiomyoblasts.

**Activation of the PI3K/Akt pathway facilitates the cytoprotective function of Ang-(1-7) against HG-induced loss of MMP in H9c2 cardiomyoblasts.** The cardioprotective effect of Ang-(1-7) on HG-induced loss of MMP was further examined in H9c2 cardiomyoblast cells. As shown in Fig. 6B, treatment of H9c2 cells with 35 mM glucose for 24 h diminished MMP, while MMP was elevated by co-treatment with 1  $\mu$ M Ang-(1-7) and 35 mM glucose (Fig. 6C). Of note, exposure to 35 mM glucose in the presence of Ang-(1-7) and A-779 still resulted in loss of MMP (Fig. 6D). Importantly, co-treatment of H9c2 cells with 10  $\mu$ M LY294002, 1  $\mu$ M Ang-(1-7) and 35 mM glucose did not attenuate the loss of MMP caused by HG (Fig. 6E). These find-

ings demonstrated that the cytoprotective effect of Ang-(1-7) on the HG-induced loss of MMP in H9c2 cardiomyoblasts was mediated in part by PI3K/Akt pathway activation. Ang-(1-7), A-779 or LY294002 alone did not have any effect on MMP in H9c2 cardiomyoblasts (Fig. 6F-H).

**Ang-(1-7) decreases HG-induced inflammation in H9c2 cells, while inhibitors of PI3K/Akt reverse the effect of Ang-(1-7).** In the present study, it was examined by ELISA whether exposure to HG in H9c2 cardiomyoblasts triggers inflammatory responses and the role of Ang-(1-7) in this process. It was demonstrated that cardiac expression of IL-1 $\beta$ , -6 and TNF- $\alpha$  increased following exposure to 35 mM glucose for 24 h, while co-treatment with 1  $\mu$ M Ang-(1-7) and 35 mM glucose significantly lowered the level of these inflammatory cytokines. By contrast, exposure of H9c2 cardiomyoblasts to 35 mM glucose in the presence of both Ang-(1-7) and A-779 increased the expression of these inflammatory mediators. Of note, inflammatory reactions were suppressed by co-treatment of H9c2 cells with 10  $\mu$ M LY294002, 1  $\mu$ M Ang-(1-7) and 35 mM glucose, whereas treatment with Ang-(1-7), A-779 or LY294002 alone did not affect the inflammatory responses in H9c2 cardiomyoblasts (Fig. 7).

**Ang-(1-7) diminishes the HG-induced increased expression of cleaved caspase-1, -3 and -12 in H9c2 cells, while PI3K/Akt inhibitors block the action of Ang-(1-7).** In order to further verify the protective effect of Ang-(1-7) against HG-induced cardiomyoblast apoptosis and inflammation, the expression

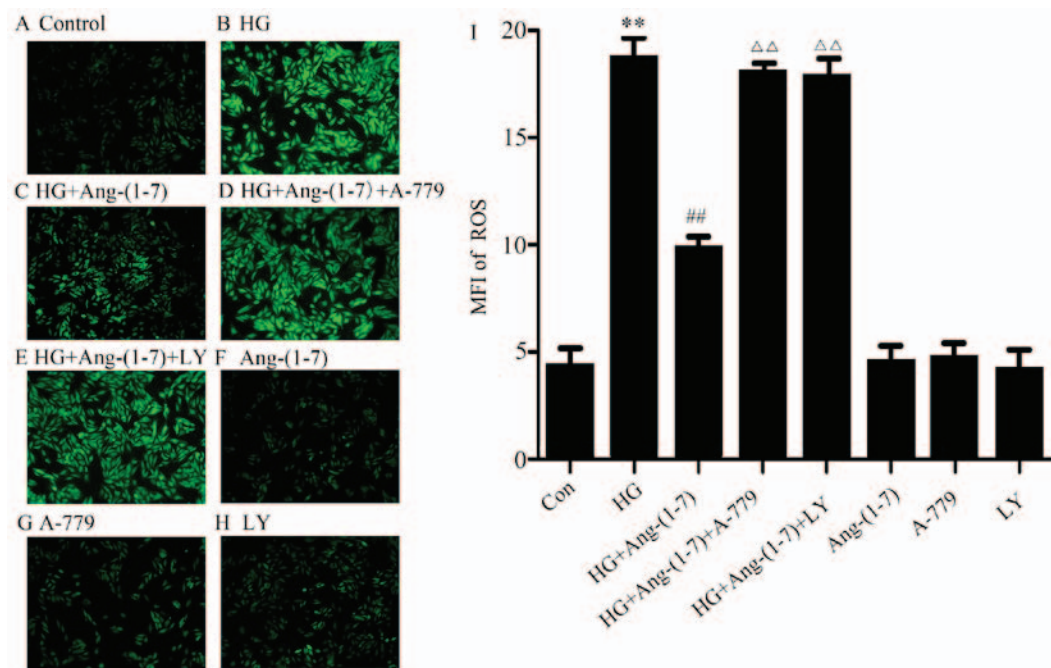


Figure 5. Activation of phosphoinositide 3-kinase and protein kinase B (PI3K/Akt) is implicated in the protective effect of Ang-(1-7) against HG-induced reactive oxygen species (ROS) production in H9c2 cells. (A-H) Intracellular ROS level was observed with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining followed by photofluorography. (A) Control group. (B) H9c2 cells were exposed to 35 mM glucose for 24 h. (C) H9c2 cells were co-treated with 1  $\mu$ M Ang-(1-7) and 35 mM glucose for 24 h. (D) H9c2 cells were co-treated with 1  $\mu$ M Ang-(1-7) and 35 mM glucose in the presence of 1  $\mu$ M A-779 for 24 h. (E) H9c2 cells were co-treated with 1  $\mu$ M Ang-(1-7) and 35 mM glucose in the presence of 10  $\mu$ M LY294002 for 24 h. (F) H9c2 cells were treated with 1  $\mu$ M Ang-(1-7) for 24 h followed by 24-h culture. (G) H9c2 cells were treated with 1  $\mu$ M A-779 for 24 h followed by 24-h culture. (H) H9c2 cells were treated with 10  $\mu$ M LY294002 for 24 h followed by 24-h culture. (I) Quantitative analysis of the mean fluorescence intensity (MFI) of DCFH-DA in (A-H) with ImageJ 1.41o software. Data are presented as means  $\pm$  standard error of the mean (n=3). \*\*P<0.01 compared with the control group; ##P<0.01 compared with the HG-treated group;  $\Delta\Delta$ P<0.01 vs. the group co-treated with Ang-(1-7) and HG. Ang-(1-7), angiotensin-(1-7); HG, high glucose (35 mM); LY, LY294002.

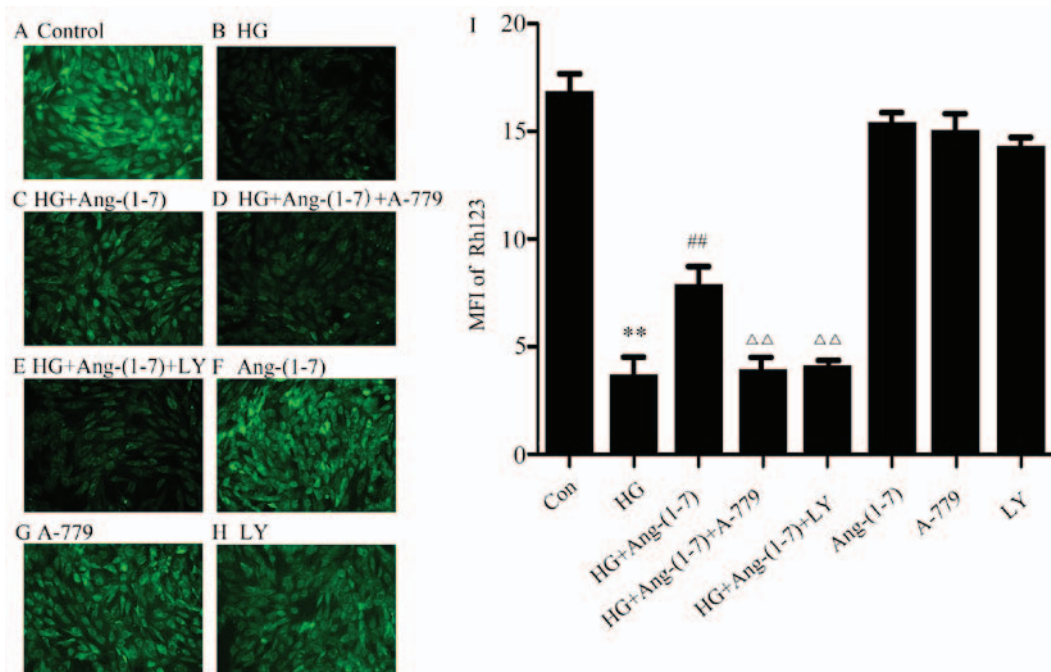


Figure 6. Activation of phosphoinositide 3-kinase and protein kinase B (PI3K/Akt) contributes to the protective effect of Ang-(1-7) against HG-induced reduction of mitochondrial membrane potential (MMP). (A-H) MMP was detected with rhodamine 123 (Rh123) staining followed by photofluorography. (A) Control group. (B) H9c2 cells were exposed to 35 mM glucose for 24 h. (C) H9c2 cells were co-treated with 1  $\mu$ M Ang-(1-7) and 35 mM glucose for 24 h. (D) H9c2 cells were co-treated with 1  $\mu$ M Ang-(1-7) and 35 mM glucose in the presence of 1  $\mu$ M A-779 for 24 h. (E) H9c2 cells were co-treated with 1  $\mu$ M Ang-(1-7) and 35 mM glucose in the presence of 10  $\mu$ M LY294002 for 24 h. (F) H9c2 cells were treated with 1  $\mu$ M Ang-(1-7) for 24 h followed by 24-h culture. (G) H9c2 cells were treated with 1  $\mu$ M A-779 for 24 h followed by 24-h culture. (H) H9c2 cells were treated with 10  $\mu$ M LY294002 for 24 h followed by 24-h culture. (I) Quantitative analysis of the mean fluorescence intensity (MFI) of Rh123 in (A-H) using ImageJ 1.41o software. Data are shown as means  $\pm$  standard error of the mean (n=3). \*\*P<0.01 compared with the control group; ##P<0.01 compared with the HG-treated group;  $\Delta\Delta$ P<0.01 vs. the group co-treated with Ang-(1-7) and HG. Ang-(1-7), angiotensin-(1-7); HG, high glucose (35 mM); LY, LY294002.

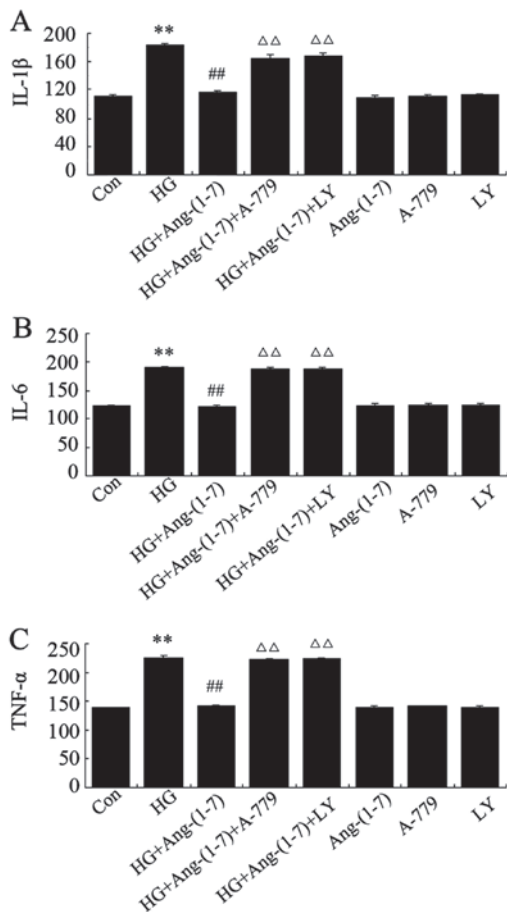


Figure 7. Ang-(1-7) protects against HG-triggered inflammation in H9c2 cells. H9c2 cells were treated for 24 h with 35 mM glucose alone, or co-treated with 1  $\mu$ M Ang-(1-7) and 35 mM glucose, or co-treated with 35 mM glucose, 1  $\mu$ M Ang-(1-7) and 1  $\mu$ M A-779, or co-treated with 35 mM glucose, 1  $\mu$ M Ang-(1-7) and 10  $\mu$ M LY294002, or treated with 1  $\mu$ M Ang-(1-7) alone, 1  $\mu$ M A-779 alone, or 10  $\mu$ M LY294002 alone. After the indicated treatments, the release of (A) IL-1 $\beta$ , (B) IL-6 and (C) TNF- $\alpha$  was assessed via ELISA. Data are presented as mean  $\pm$  standard error of the mean (n=3). \*\*P<0.01 compared with the control group; ##P<0.01 compared with the HG-treated group;  $\Delta\Delta$ P<0.01 vs. the group co-treated with Ang-(1-7) and HG. Ang-(1-7), angiotensin-(1-7); HG, high glucose (35 mM); LY, LY294002; IL, interleukin; TNF, tumor necrosis factor.

level of cleaved caspase-1, -3 and -12 in H9c2 cardiomyoblasts was evaluated by western blot analysis. As shown in Fig. 8, exposure to 35 mM glucose for 24 h induced a significant increase of cleaved caspase-1, -3 and -12 expression level. As we hypothesized, co-treatment with 1  $\mu$ M Ang-(1-7) and 35 mM glucose markedly reduced the HG-induced increased expression level of these proteins. Incubating H9c2 cardiomyoblasts with 35 mM glucose and 1  $\mu$ M Ang (1-7) in the presence of 1  $\mu$ M A-779 enhanced the expression of these proteins; similarly, co-treatment of H9c2 cells with 10  $\mu$ M LY294002, 1  $\mu$ M Ang-(1-7) and 35 mM glucose increased the expression level of these proteins. Finally, treatment with Ang-(1-7), A-779 or LY294002 alone did not affect the expression level of these proteins.

## Discussion

Growing evidence indicates that hyperglycaemia plays a pivotal role in the development of DCM, but the pathophysio-

logical and molecular mechanisms of hyperglycaemia-induced cardiomyocyte injury remain unclear. In the present study, the HG (35 mM glucose)-induced H9c2 cardiomyoblast injury model was used to investigate the cardioprotective effects of Ang-(1-7) against HG-induced cardiomyocyte injury and the underlying mechanisms.

Consistent with previous studies (13-22), our findings verified several detrimental events induced by HG on H9c2 cardiomyoblasts, such as cytotoxicity, apoptosis, oxidative damage, mitochondrial dysfunction and inflammation, as demonstrated by an increase in the apoptotic cell percentage, ROS generation and inflammatory cytokine level, as well as a decline in cell viability and mitochondrial luminosity. In addition, caspase-1, -3 and -12 are known to be involved in cell apoptosis and inflammatory response (29,30,45-47); thus we investigated the expression of these proteins and found that HG treatment significantly increased their levels, further confirming that HG treatment may trigger apoptosis and inflammation in H9c2 cardiomyoblasts.

An important finding in the present study was the protective effects of Ang-(1-7) against HG-induced injury of H9c2 cardiomyoblasts. Ang-(1-7), which is formed from AngI and AngII by the action of ACE2 (32-34), exhibits physiological functions that are different from those of AngII, including prevention of myocardial hypertrophy, mitigation of cardiac remodeling, antifibrotic effect and vasodilatory function (35-39,48-50). It has been reported that Ang-(1-7) may enable glucose uptake in neonatal cardiomyocytes (51). Additionally, in streptozotocin-induced diabetic rats, Ang-(1-7) treatment may suppress right ventricular (RV) fibrosis and ameliorate RV oxidative stress (52). Taking into consideration these reports, we further investigated the protective role of Ang-(1-7) against hyperglycaemia in H9c2 cardiomyoblasts. First, it was observed that Ang-(1-7) clearly restrained HG-induced cytotoxicity, since co-treatment with Ang-(1-7) and HG increased cell survival rate compared with the HG treatment group. These results are consistent with those of previous studies (36,38,39,52). Second, we investigated the anti-apoptotic function of Ang-(1-7) in HG-treated H9c2 cells, which is supported by recent reports that ischemia/reperfusion-induced cardiomyocyte apoptosis may be significantly inhibited by Ang-(1-7) (53). Third, in line with previous reports (52,53), we observed that Ang-(1-7) suppresses HG-induced oxidative stress in H9c2 cardiomyoblasts, as shown by a marked decrease in the generation of ROS. Fourth, the results of the present study demonstrated that Ang-(1-7) protected mitochondria against HG-triggered loss of MMP, which was consistent with the findings of a previous study (54) demonstrating that the Ang-(1-7) peptidomimetic AVE 0991 exerted protective effects in the kidneys in ApoE-knockout mice by partially reversing atherosclerosis-related changes in the mitochondrial proteome. Fifth, the HG-induced cardiac inflammatory reaction may be blocked by Ang-(1-7), with lower levels of IL-1 $\beta$ , -6 and TNF- $\alpha$  compared with the HG group. Similarly, Papinska *et al* (55) observed that Ang-(1-7) treatment reduced inflammatory cell infiltration of the heart tissue in a mouse model of type 2 diabetes. Finally, Ang-(1-7) treatment in H9c2 cardiomyoblasts decreased cleaved caspase-1, -3 and -12 expression under HG conditions, further verifying the protective effect of Ang-(1-7) against HG-induced apoptosis and inflammation in H9c2

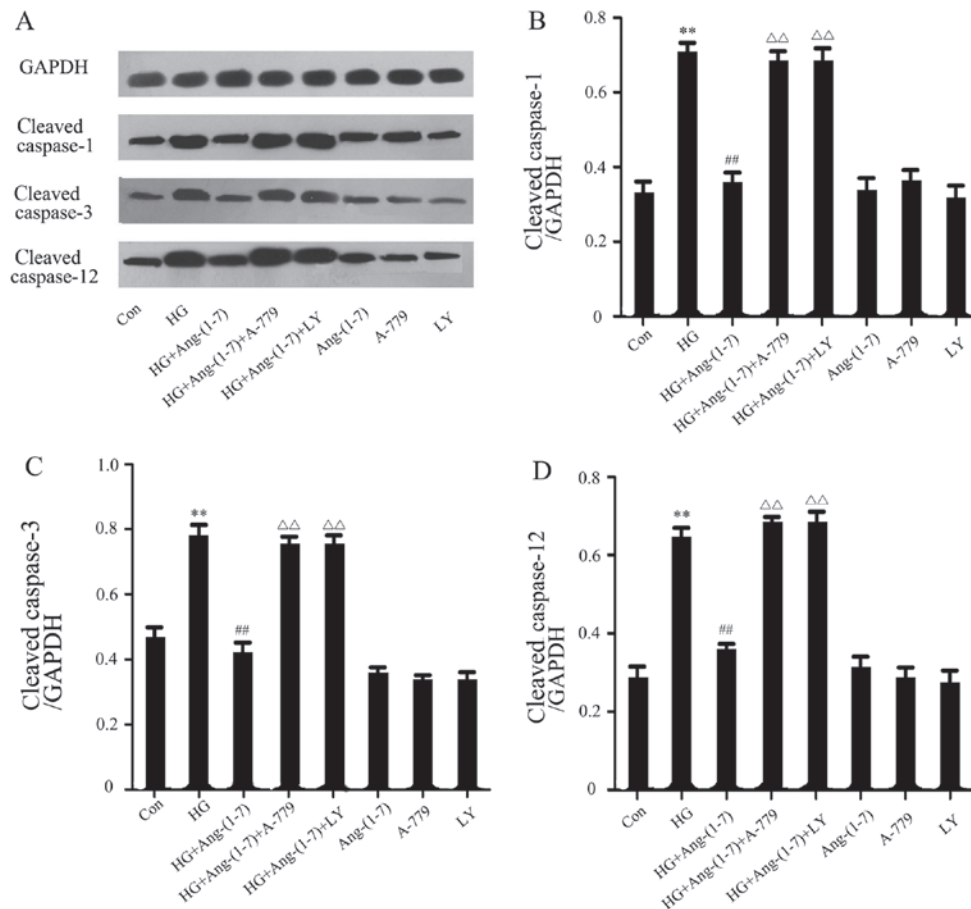


Figure 8. Ang-(1-7) reduced HG-induced increased expression levels of cleaved caspase-1, -3 and -12 in H9c2 cells. H9c2 cells were treated for 24 h with 35 mM glucose alone, or co-treated with 1  $\mu$ M Ang-(1-7) and 35 mM glucose, or co-treated with 35 mM glucose, 1  $\mu$ M Ang-(1-7) and 1  $\mu$ M A-779, or co-treated with 35 mM glucose, 1  $\mu$ M Ang-(1-7) and 10  $\mu$ M LY294002, or treated with 1  $\mu$ M Ang-(1-7) alone, or 1  $\mu$ M A-779 alone, or 10  $\mu$ M LY294002 alone. The expression of cleaved caspase-1, -3 and -12 was detected by western blot analysis. (A) Changes in the expression levels of cleaved caspase-1, -3 and -12 in the indicated groups. (B-D) Densitometric analysis of the results in (A). Data are presented as mean  $\pm$  standard error of the mean (n=3). \*\*P<0.01 compared with the control group; ##P<0.01 compared with the HG-treated group;  $\Delta\Delta$ P<0.01 vs. the group co-treated with Ang-(1-7) and HG. Ang-(1-7), angiotensin-(1-7); HG, high glucose (35 mM); LY, LY294002.

cardiomyoblasts. Of note, co-administration of Ang-(1-7) and A-779 reversed the abovementioned protective effects of Ang-(1-7), suggesting that HG-related injuries may reappear with inhibition of Ang-(1-7). The findings of the present study offer convincing evidence regarding the cardioprotective effects of Ang-(1-7) against HG-induced injury.

The potential mechanism underlying the cardioprotective effect of Ang-(1-7) against HG-induced injury was then investigated. As is known, a group of survival protein kinases, including PI3K/Akt, constitute a target for cardioprotection against ischemia/reperfusion injury (56,57). Therefore, in this study the function of the PI3K/Akt signaling pathway was examined under HG conditions. Another novel finding of our study was that the activation of the PI3K/Akt signaling pathway is involved in the cardioprotective effect of Ang-(1-7) against HG. First, we observed that HG treatment triggered the dephosphorylation of the PI3K and Akt proteins in H9c2 cardiomyoblasts, in accordance with previous findings (27-29). Furthermore, co-treatment with Ang-(1-7) and HG not only protects H9c2 cells against HG, but also considerably reverses the HG-induced dephosphorylation of PI3K/Akt in these cells. Of note, the presence of LY294002, an inhibitor of PI3K/Akt, markedly inhibited the cardioprotective effect of Ang-(1-7) against HG-triggered

cytotoxicity, cell apoptosis, oxidative stress, mitochondrial damage and inflammatory reaction. Therefore, activation of PI3K/Akt signaling by Ang-(1-7) may, at least in part, be involved in its cardioprotective effect against HG. Several recent studies reported that PI3K/Akt pathway activation participates in cardiac cell resistance to apoptosis, oxidative stress and inflammation, and improves myocardial systolic function (27-32); those findings were supported by our results.

Interestingly, the mechanisms of the cardioprotection of Ang-(1-7) against HG may be multifarious. Endoplasmic reticulum stress (ERS) is known to play a key role in the progression of DCM. HG-activated ERS may reduce the myocardial protein expression of p-PI3K and p-Akt (58), whereas overexpression of p-Akt may successfully withstand ERS-induced apoptosis and protect the myocardium against hyperglycaemia-induced dysfunction (59). In addition, ROS-stimulated mitogen-activated protein kinase (MAPK) pathways, including the p38 MAPK, ERK1/2 and JNK signaling pathways, are involved in HG-induced injuries, and suppression of these signaling pathways may also significantly mitigate HG-induced cytotoxicity, apoptosis, overproduction of ROS and dissipation of MMP (13). Based on these reports, the cardioprotective function of Ang-(1-7) may be associated



with the regulation of ERS and MAPK pathways. To confirm this hypothesis, further investigation is required.

In summary, Ang-(1-7) protects H9c2 cardiomyoblasts against HG-induced cytotoxicity, cell apoptosis, oxidative stress, mitochondrial damage and inflammation, and PI3K/Akt signaling pathway activation may play a key role in the protective function of Ang-(1-7). These conclusions offer a basis for further studies on the cytoprotective effect of Ang-(1-7) against diabetic cardiovascular complications, in order to identify novel methods for the prevention of hyperglycaemia-induced cardiomyocyte injury.

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