

Original Article

Resveratrol attenuates high glucose-induced cardiomyocytes injury via interfering ROS-MAPK-NF- κ B signaling pathway

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Abstract: Cardiomyocyte inflammatory injury is likely required for cardiomyocytes death under hyperglycemia condition. Resveratrol (Res) is famous for its anti-inflammatory effect. However, there are few reports about the anti-inflammatory effect of Res induced by high glucose in cardiomyocytes. The aim of the present study is to investigate the inflammatory effect of high glucose and the anti-inflammatory effect of Res induced by high glucose in cardiomyocytes. Primary cardiomyocytes were isolated from new born SD rats and high glucose (30 mmol/L) was used as a stimulant for cell injury. Cell viability was assayed by CCK-8 method; protein expression was identified by Western blot or ELISA, respectively. The production of reactive oxygen species (ROS) was observed under a fluorescence microscope. The results indicated that High glucose (30 mmol/L) significantly decreased the cell viability of cardiomyocytes after co-cultivated for 12 h and had a time-dependent manner, and increased IL-1 β , IL-6 and TNF- α secretion in cardiomyocytes. The injury effect of high glucose involved in ROS-MAPK-NF- κ B signaling pathway. For the reason that antioxidant NAC, ERK1/2, p38 MAPK and NF- κ B specific pathway inhibitors was able to abolish the secretion of this inflammatory factors; pretreatment with antioxidant NAC significantly decreased the level of phosphorylated ERK1/2, p38 MAPK and nuclear NF- κ B; pretreatment of PD98059 and SB203580 can significantly decrease NF- κ B level in nuclei. After treatment with Res 20 μ mol/L for 12 h, IL-1 β , IL-6 and TNF- α secretion were markedly decreased, and the phosphorylation of ERK1/2, p38 MAPK and NF- κ B level were also decreased. All the results showed that Res attenuates high glucose-induced inflammatory injury through ROS-ERK1/2/p38-NF- κ B signaling pathway in cardiomyocytes.

Keywords: Resveratrol, high glucose, inflammation, cardiovascular disease, MAPK signaling pathway, NF- κ B signaling pathway

Introduction

Cardiovascular disease (CVD) is the most common cause of mortality and morbidity in the world [1-3]. Diabetes Mellitus (DM) which is one of the common disease of CVD seriously affects human health [4, 5], and diabetic cardiomyopathy (DCM) is an independent complication of DM. Myocardial fibrosis which is most frequently proposed mechanisms can contribute to DCM development [6-8]. Necrosis or apoptosis or both is a wide accepted concept of cardiomyocytes death that can lead to myocardial fibrosis [9, 10]. Research showed that hyperglycemia-induced inflammation and oxidative stress in the heart result in cardiomyocytes death [11-14]. High glucose plays a key role in

cardiomyocytes death and the overproduction of pro-inflammatory cytokines (such as TNF- α , IL-1 β et al.) induced by high glucose, act as a positive feedback mechanism and also stimulate cardiomyocytes apoptosis, which eventually leads to cardiac dysfunction [15-18].

Resveratrol (Res, chemical structure showed in **Figure 6A**), a type of polyphenolic phytoalexin contained abundantly in red grapes and wine, has received considerable attention for its ability of preventing illnesses such as CVD [19, 20], cancer [21-23], and even slowing the aging process [24, 25]. Recently, Res has been proved to have some benefits on DCM [26-28]. However, little is known about the potential mechanisms of Res on high glucose induced cardiomyocytes

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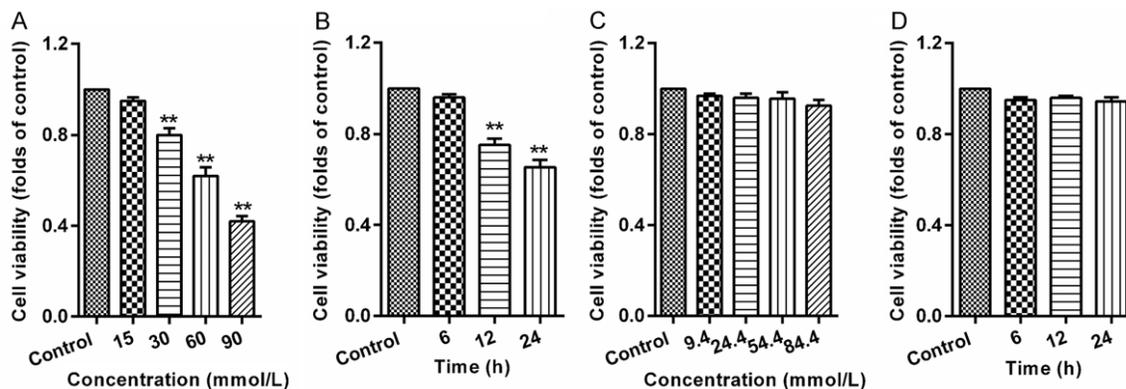


Figure 1. The effect of high glucose treatment on cell viability of cardiomyocytes. A. The cells were incubated with different concentrations of glucose for 12 h; B. The cells were incubated with the concentrations of glucose at 30 mmol/L for 0, 6, 12, 24 h; C. The cells were incubated with different concentrations of mannitol for 12 h; D. Cells were subjected to the concentrations of mannitol at 24.4 mmol/L for 0, 6, 12, 24 h. Results were from six independent experiments and expressed as mean \pm standard errors of mean (SEM), ** $P < 0.01$ vs. control.

injury. Hence, the aim of present study was to investigate the protective effect of Res on high glucose-induced inflammation in cardiomyocytes which mainly focus on the anti-inflammatory effect and ROS, MAPK and NF- κ B signaling pathway.

Materials and methods

Chemicals and animals

Res was from National Food and Drug Testing Institute (Beijing, China). RPMI 1640 Medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Penicillin and streptomycin were from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). CCK-8 was purchased from Invitrogen (Carlsbad, CA, USA). Protein quantification kit was from Beyotime (Jiangsu, China). 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), ERK1/2, phospho-ERK1/2, JNK and phospho-JNK antibodies were obtained from Beyotime (Jiangsu, China). Phospho-p38 and p38 antibodies were from Cell Signaling Technology (Danvers, MA, USA). NF- κ B and Lambin b1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-conjugated secondary antibodies were purchased from the Zhongshan Company (Beijing, China).

Sprague-Dawley (SD) rats (Between 3-7 days old) were provided by the experimental animal center of Beijing. The animal experiment was according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes

of Health Publication No. 85-23, revised in 1996). Primary cardiomyocytes were from new born SD rats according to previous methods [29]. The purity of cultured cardiomyocytes were 98% as evaluated by immunofluorescence staining using cardiac muscle sarcomeric α -actin antibody (Abcam, Cambridge, MA, USA) and showed in [Supplementary Figure 1](#). Cardiomyocytes were kept in the 1% FBS for 12 h prior to the experiment, and then stimulated by high glucose (30 mmol/L). In the Res treatment experiments, Cardiomyocytes were exposed to high glucose (30 mmol/L) for 12 h after pre-treated with the Res for 1 h.

CCK-8 assay

The CCK-8 method was used to detect the cell viability of cardiomyocytes after indicated treatment in 96-well plates. The experimental protocol as follow: After indicated treatment, 10 μ L CCK-8 solution was added to each well and followed by a further 4 h incubation at 37°C. The optical density was measured at 450 nm by a micro-plate reader (Molecular Devices, Sunnyvale, CA, USA). The mean optical density of 6 wells was used to calculate the cell viability percentage.

Enzyme-linked immunosorbent assay (ELISA)

Cardiomyocytes were cultured in 96-well plate and stimulated with high glucose for 12 h. Then, the supernatant was collected and levels of IL-1 β , IL-6 and TNF- α in the supernatant were assayed by ELISA kit specific for rat IL-1 β , IL-6 and TNF- α .

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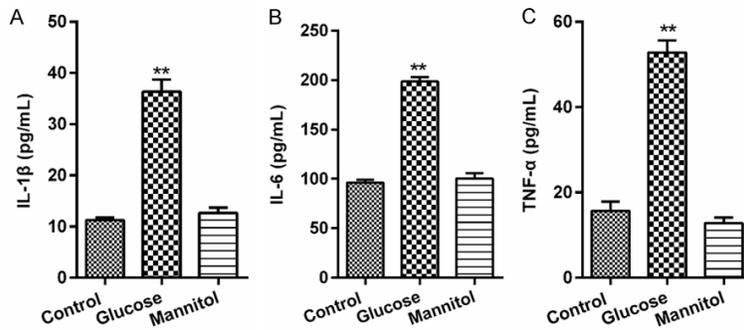


Figure 2. High glucose increases the proteins secretion of IL-1 β , IL-6 and TNF- α in cardiomyocytes. The cells were subjected to high glucose at 30 mmol/L for 12 h, then, the proteins expression of IL-1 β , IL-6 and TNF- α were identified by ELISA, respectively. A-C: IL-1 β , IL-6 and TNF- α proteins expression. Results were from six independent experiments for ELISA, and expressed as mean \pm standard errors of mean (SEM), ** $P < 0.01$ vs. control.

Measurement of intracellular reactive oxygen species (ROS)

The levels of Intracellular ROS were determined by oxidative conversion of cell-permeable DCFH-DA to fluorescent DCF. After indicated treatments, DCFH-DA (10 μ mol/L) in FBS-free DMEM was added to the 6 well plate and the cells were incubated for 20 min at 37°C. Then, the 6 well plate were washed three times with FBS-free DME and DCF fluorescence was measured by using a fluorescent microscope (BX50-FLA; Olympus, Tokyo, Japan). Mean fluorescence intensity (MFI) from six random fields was analyzed using Image J 1.41 software (National Institutes of Health, Bethesda, MD, USA), and MFI was used to represent the amount of ROS.

Western blot

After indicated treatment, the cells were washed twice by ice-cold PBS (pH 7.4) and lysed by lysis buffer supplemented with protease inhibitor cocktail and phosphatase inhibitors (Roche, Basel, Switzerland) 100 μ L per well of a 6-well plate. Concentration of the protein was measured by BCA protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amount of the protein (30 μ g) was loaded, separated by 10% SDS-PAGE, and blotted onto PVDF membrane (0.45 μ m, GE Healthcare, Buckinghamshire, UK). The membranes were incubated with anti-ERK1/2 (1:1500), anti-phospho-ERK1/2 (1:800) or anti-p38 (1:1500), anti-phospho-p38 (1:800), anti-JNK (1:1000), anti-phospho-JNK (1:600), anti-NF- κ B (1:500), antiLamin B1

(1:1000) antibodies at 4°C overnight. After washed three times, the membranes were incubated with the relevant second HRP-conjugated antibodies (1:3000) for 3 h and then the immune complexes were enhanced by chemiluminescence.

Statistical analyses

The results are represented as means \pm standard errors of mean (SEM). Statistical analysis was performed by Steer-Dwass or Mann-Whitney multiple comparison test. This was performed by using Graphpad Prism 6.0 (GraphPad Software, Inc. La Jolla, CA). $P < 0.05$ was considered statistically significant.

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Results

High glucose decrease the cell viability of cardiomyocytes

Figure 1A showed that the cell viability of cardiomyocytes were significantly inhibited by glucose at 30 mmol/L for 12 h and showed a concentration dependent fashion; the result in **Figure 1B** indicated a time-dependent manner when subjected to glucose at 30 mmol/L. However, cells were exposed to mannitol, an osmotic control, did not influence the growth rate of the cardiomyocytes. This suggested that high glucose inhibited cell viability was not a consequence of high osmolarity (showed in **Figure 1C** and **1D**).

High glucose induces inflammatory cytokines expression in cardiomyocytes

The IL-1 β , IL-6 and TNF- α were tested by ELISA kit. As shown in **Figure 2A-C** that IL-1 β , IL-6 and TNF- α proteins in supernate of the DMEM were significantly increased after exposure to glucose at 30 mmol/L for 12 h in compared with control. However, cells were exposed to mannitol at 24.4 mmol/L did not influence the secretion of inflammatory factors of the cardiomyocytes. This suggested that high glucose increased the secretion of IL-1 β , IL-6 and TNF- α was not a consequence of high osmolarity.

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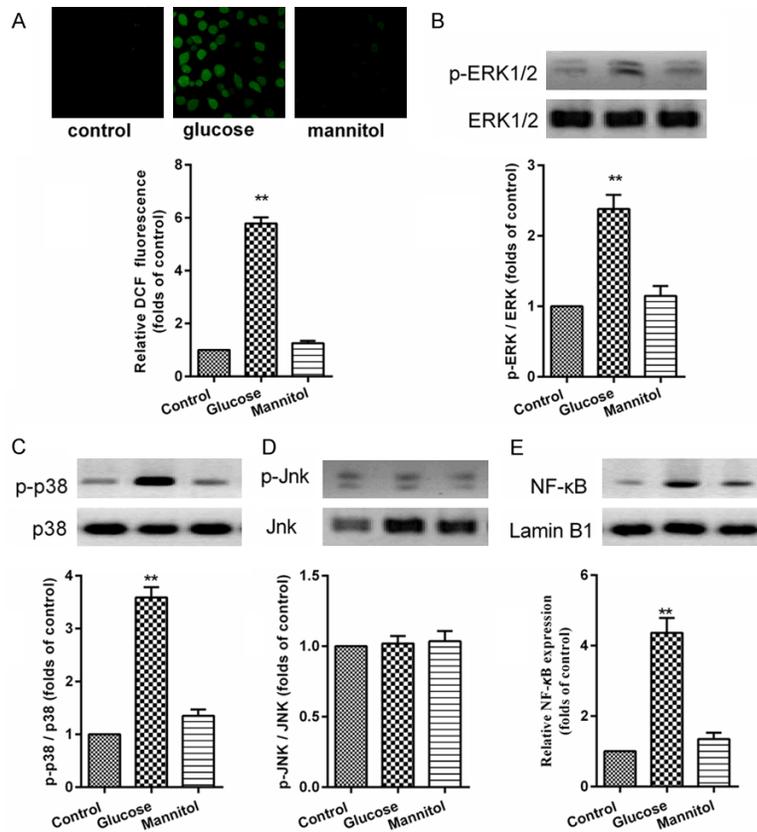


Figure 3. ROS, MAPK and NF- κ B signaling pathway involve in the inflammatory effect of high glucose in cardiomyocytes. The cells were subjected to high glucose at 30 mmol/L or mannitol at 24.4 mmol/L for 12 h, then, the detection of ROS was by laser scanning confocal microscope (A, 600 \times); expression of the phosphorylation of ERK1/2, JNK and p38 and NF- κ B were assayed by Western blot (B-E). Results were from six independent experiments for ROS detection, and three independent experiments for western blot. Data are expressed as mean \pm standard errors of mean (SEM), ** $P < 0.01$ vs. control.

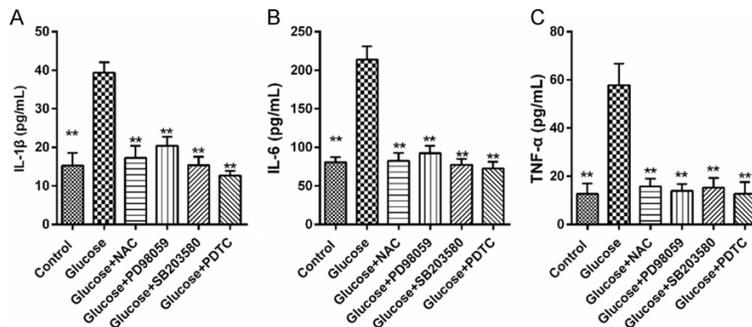


Figure 4. ROS, ERK1/2, p38 and NF- κ B signaling pathway involve in the inflammatory effect of high glucose in cardiomyocytes. The cells were pretreated with antioxidant NAC, ERK1/2, p38 or NF- κ B specific pathway inhibitor respectively for 1 h, and then subjected to high glucose at 30 mmol/L for 12 h, then, the proteins expression of IL-1 β , IL-6 and TNF- α were identified by ELISA, respectively. A-C: IL-1 β , IL-6 and TNF- α proteins expression. Results were from six independent experiments for ELISA, and expressed as mean \pm standard errors of mean (SEM), ** $P < 0.01$ vs. control.

ROS, MAPK and NF- κ B signaling pathway involve in the inflammatory effect of high glucose in cardiomyocytes

As shown in **Figure 3A** that high glucose treatment was able to increase the generation of intracellular ROS in cardiomyocytes. The results from western blot showed that the phosphorylated ERK and phosphorylated p38 proteins expression were increased as well as activating NF- κ B in cardiomyocytes after the stimulation with glucose at 30 mmol/L for 12 h (**Figure 3B, 3C, 3E**). Whereas the phosphorylation of JNK was no obvious change (**Figure 3D**). However, cells were exposed to mannitol at 24.4 mmol/L did not affect the level of the above proteins of the cardiomyocytes. This suggested that high glucose increased the expression of phosphorylated ERK and phosphorylated p38 and activating NF- κ B was not a consequence of high osmolarity.

Further research by ELISA shown that IL-1 β , IL-6 and TNF- α proteins in supernate of the DMEM were significantly decreased after pretreated with PD98059 (ERK1/2 inhibitor) at 1 μ mol/L, SB203580 (p38 inhibitor) at 10 μ mol/L, PDTC (NF- κ B inhibitor) at 100 μ mol/L and antioxidant NAC at 10⁻² mol/L for 1 h. These results indicated that ROS, MAPK and NF- κ B signaling pathway involve in the inflammatory effect of high glucose in cardiomyocytes (**Figure 4A-C**).

The inflammatory effect induced by high glucose via ROS-MAPK-NF- κ B signaling pathway in cardiomyocytes

ROS, MAPK and NF- κ B participate in the expression of

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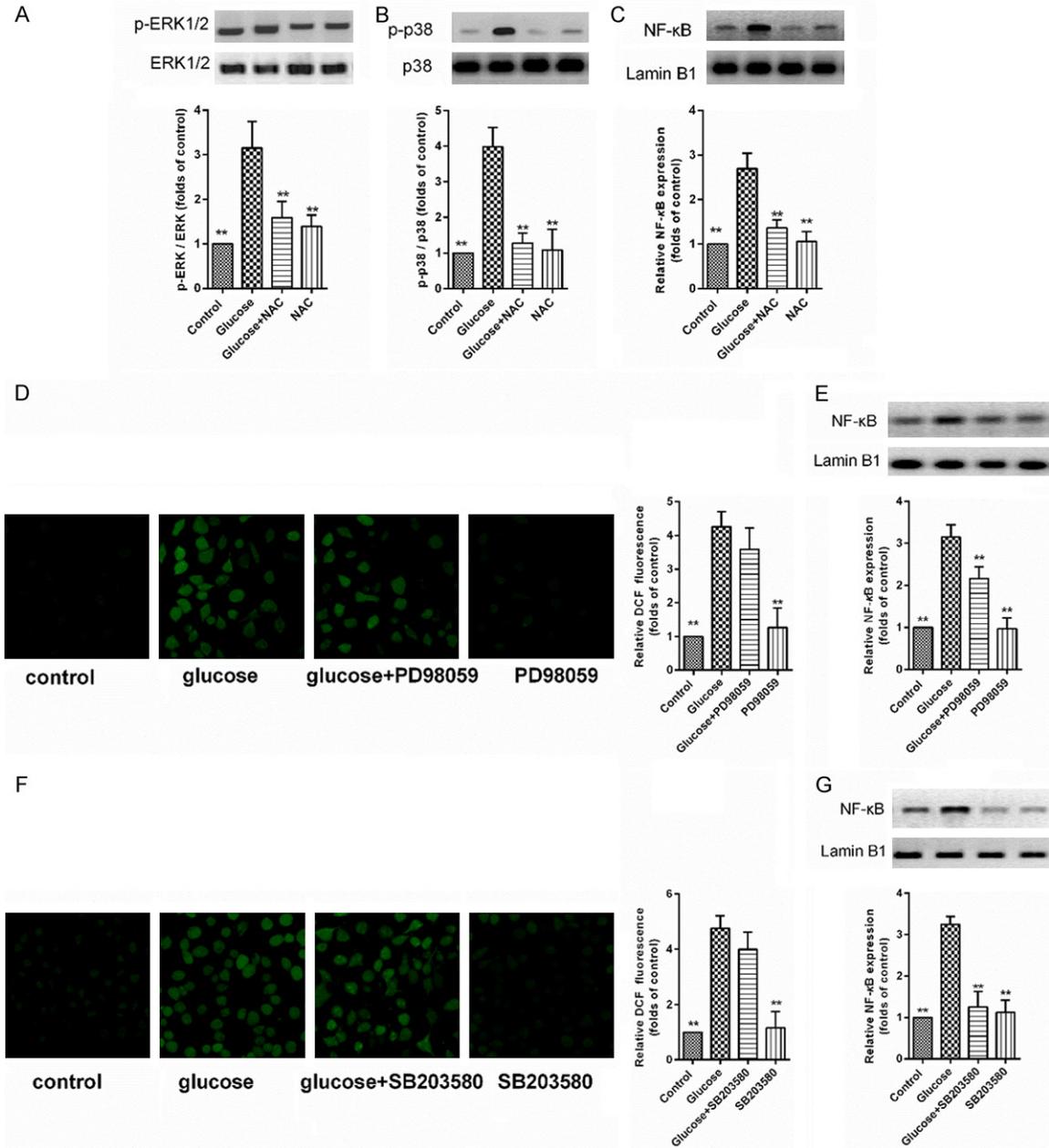


Figure 5. The inflammatory effect induced by high glucose via ROS-MAPK-NF-κB signaling pathway in cardiomyocytes. The cells were subjected to high glucose at 30 mmol/L for 12 h or in combination with the pretreatment of related pathway inhibitor for 1 h, then, the detection of ROS was by laser scanning confocal microscopy (D, F: 600×); expression of p-ERK, p-p38 or NF-κB were assayed by Western blot (A-C, E, G). Results were from six independent experiments for ROS detection and three independent experiments for Western blot and expressed as mean ± standard errors of mean (SEM), **P<0.01 vs. control.

inflammatory cytokines in cardiomyocytes. High glucose-induced inflammation in cardiomyocytes was possibly related to ROS-MAPK-NF-κB signaling. Pretreatment of the cells with 10^{-2} mol/L NAC (antioxidant) for 1 h was able to inhibit the phosphorylation of ERK1/2 and p38 (Figure 5A, 5B) and also decrease the activa-

tion of NF-κB (Figure 5C). This results indicated that ROS are the up-stream of MAPK and NF-κB signaling pathway in the inflammatory effect of high glucose in cardiomyocytes.

Further study by using 1 μmol/L PD98059 (ERK1/2 inhibitor) and 10 μmol/L SB203580

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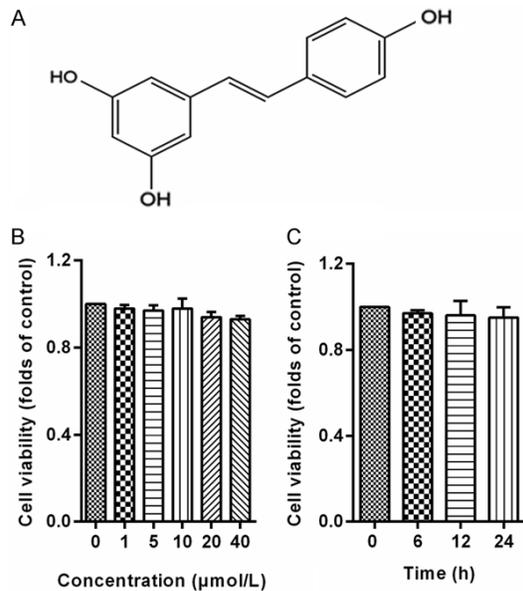


Figure 6. The chemical structure of Res and its effect on the cell viability of cardiomyocytes. A. The chemical structure of Res; B. The cells were incubated with the different concentrations of Res for 12 h; C. The cells were incubated with the concentrations of Res at 20 $\mu\text{mol/L}$ for 0-24 h. Results were from six independent experiments and expressed as mean \pm standard errors of mean (SEM).

(p38 inhibitor) to investigate the role of MAPK in the inflammatory effect of high glucose in cardiomyocytes. Data showed that PD98059 and SB203580 was able to inhibit the level of nuclear NF- κB expression (**Figure 5E, 5G**); however, the expression of ROS was not significantly decreased (**Figure 5D, 5F**). These data showed that the inflammatory effect induced by high glucose via ROS-MAPK-NF- κB signaling pathway in cardiomyocytes.

The effect of Res on the cell viability cardiomyocytes

Figure 6A showed that Res from 0-40 $\mu\text{mol/L}$ did not affect the cell viability of cardiomyocytes, when treatment with Res at concentration of 20 $\mu\text{mol/L}$ for 0-24 h also showed the same manner.

Res attenuates high glucose-induced cardiomyocytes inflammatory damage through ROS-MAPK-NF- κB signaling pathway

As shown in **Figure 7A-C** that IL-1 β , IL-6 and TNF- α proteins in cardiomyocytes were signifi-

cantly decreased in the presence of Res 20 $\mu\text{mol/L}$ for 12 h. The levels of inner ROS as well as the expression of the phosphorylation of ERK1/2 and p38 and the activation of NF- κB were notably decreased (**Figure 7D-G**). These results indicated that the anti-inflammatory effect of Res might be via interfering ROS-MAPK-NF- κB signaling pathway in high glucose-induced inflammation of cardiomyocytes.

Discussion

DCM is a greater risk for cardiovascular morbidity and mortality. Hyperglycemia has been regarded as the primary pathogenic factor of DCM and also directly damages cardiomyocytes [30, 31]. Apoptosis of cardiomyocytes is one of the important outcomes of hyperglycemia-induced inflammation and oxidative stress in the heart [32]. In addition, inflammation with increased cytokine levels in the heart was also found to have an important role in the pathogenic development of DCM [33-36]. High glucose could increase the overexpression of inflammatory cytokines in cardiomyocytes [37, 38]. Nonetheless, the molecular mechanisms of high glucose induced-cardiomyocytes inflammatory response remain largely unknown. In the present study, we found that high glucose could increase the secretion of IL-1 β , IL-6 and TNF- α in cardiomyocytes.

The activation of inflammatory effect via multi pathway, and oxidative damage to cardiomyocytes is of sever in DCM [39, 40]. ROS are both the important second messenger and the direct participant of oxidative stress. The recent researches show that ROS play an important role in high glucose elicited the expression of inflammatory factors. Pro-treatment with antioxidant NAC at 10 mmol/L can significantly inhibit the secretion of IL-1 β , IL-6 and TNF- α in cardiomyocytes.

MAPK and NF- κB signaling also play a pivotal role in inflammation [41-43]. The activation of NF- κB is responsible for the expressions of many inflammatory cytokines. Our result exhibited that MAPK and NF- κB was involved in the expression of IL-1 β , IL-6 and TNF- α protein induced by high glucose, since the selective PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor) and NF- κB inhibitor PDTC significantly

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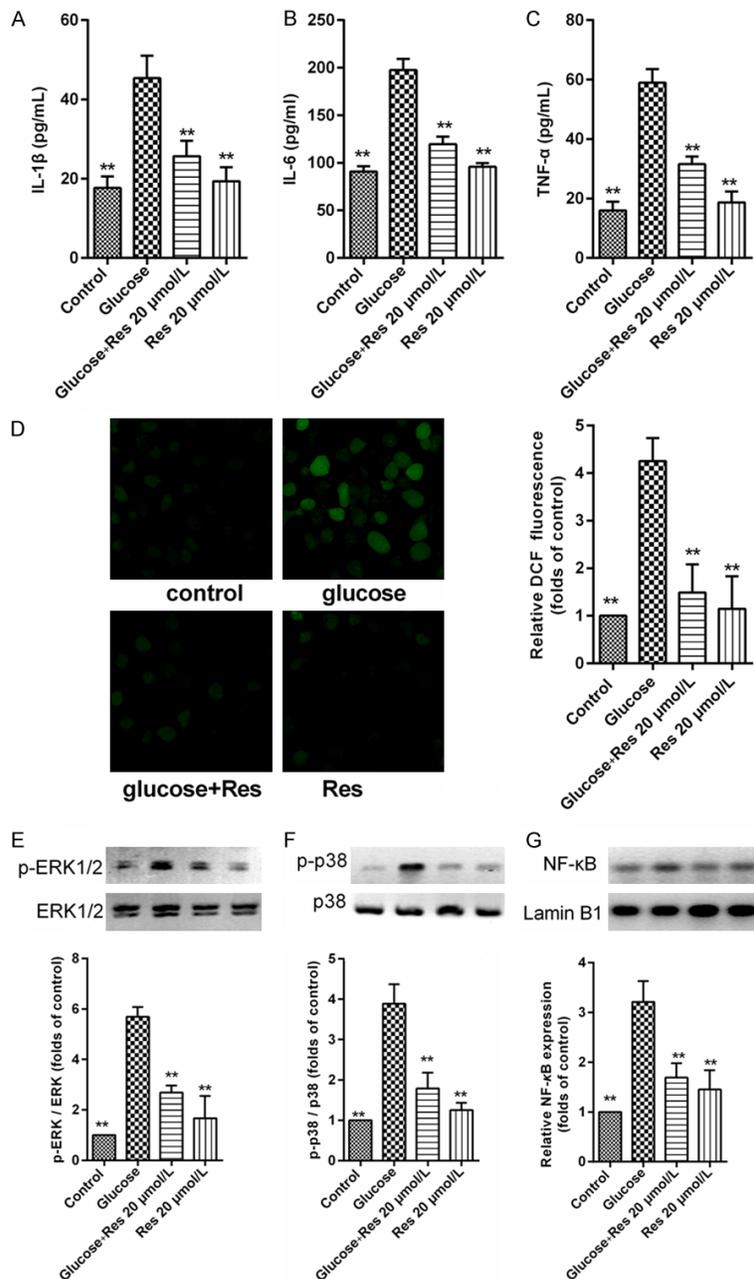


Figure 7. Res decreases protein expression of IL-1 β , IL-6 and TNF- α via ROS-MAPK-NF- κ B signaling pathway in Cardiomyocytes. The cells were subjected to high glucose treatment in the absence or presence of Res at 20 μ mol/L for 12 h. Then, the detection of ROS was by laser scanning confocal microscopy (D: 600 \times); protein expressions of IL-1 β , IL-6 and TNF- α were identified by ELISA and expression of p-ERK, p-38 or NF- κ B were assayed by Western blot. (A-C) IL-1 β , IL-6 and TNF- α protein expression; (D-G) ROS, p-ERK, p-38 and NF- κ B expression. Results were from six independent experiments for ROS detection and three independent experiments for Western blot and expressed as mean \pm standard errors of mean (SEM), **P<0.01 vs. control.

blocked the expression of IL-1 β , IL-6 and TNF- α proteins in cardiomyocytes.

Previous researches have indicated that Res has a various protective effects against inflam-

mation, CVD, aging and cancer. In the present study, we demonstrated that high glucose was able to increase the level of ROS and the anti-inflammatory effect of Res mainly by blocking the expression of ROS. MAPK and NF- κ B signaling pathway is also a target of Res. Our current research also showed that high glucose was able to increase the expression of the phosphorylation of p38 and ERK1/2 and NF- κ B in cardiomyocytes, and the anti-inflammatory effect of Res via mediating MAPK-NF- κ B signaling pathway for the reason that the function of Res is similar to the specific inhibitor of P38, ERK1/2 and NF- κ B signaling pathway.

In conclusion, the present study demonstrates that high glucose induced the expression of IL-1 β , IL-6 and TNF- α protein via ROS-MAPK-NF- κ B signal pathway in cardiomyocytes. And the anti-inflammatory effect of Res might be via interfering ROS-MAPK-NF- κ B signal pathway in cardiomyocytes. These provide the new evidence for the potential inflammatory effect of high glucose and a strategy of dealing with high glucose induced inflammation.

Disclosure of conflict of interest

None.

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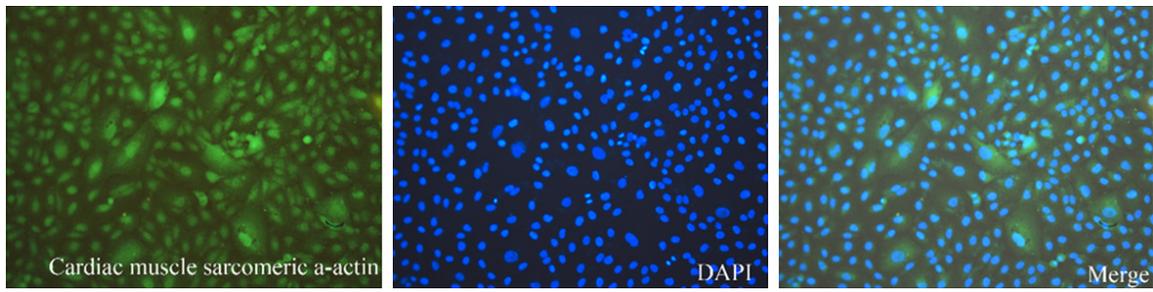
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Supplementary Figure 1. Characterization of primary cultured rats' cardiomyocytes derived from the male Sprague-Dawley rats. The cells were stained with cardiac muscle sarcomeric a-actin and labeled with the Dylight 488 conjugated antibody. The cells were observed at magnification of 200× under a fluorescence microscope.