Resveratrol attenuates high glucose-induced oxidative stress and cardiomyocyte apoptosis through AMPK

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ABSTRACT

Background: Diabetic cardiomyopathy (DCM) suggests a direct cellular insult to myocardium. Hyperglycemia-induced oxidative stress and apoptosis have been implicated in the pathogenesis of DCM. NADPH oxidase is a major source of reactive oxygen species (ROS) generation in cardiomyocytes. Resveratrol, a naturally occurring polyphenol, has shown beneficial effects on some cardiovascular complications associated with diabetes.

Objectives: We aimed to examine the role of resveratrol on high glucose-induced NADPH oxidase-derived ROS production and cardiac apoptosis, together with modulation of protein signaling pathways in cardiomyocytes.

Methods: Primary cultures of neonatal rat cardiomyocytes were exposed to 30 mmol/L high glucose with or without resveratrol. Cell viability, apoptosis, superoxide formation, NADPH oxidase activity and its subunits expression, antioxidant enzymes activities, as well as the potential regulatory molecules AMPK, Akt and GSK-3β were assessed in cardiac cells.

Results: Elevated ROS production induced by 30 mmol/L high glucose was inhibited with the addition of resveratrol in primary cultured neonatal rat cardiomyocytes. Consistently, resveratrol markedly suppressed the increased activity of NADPH oxidase and Rac1, as well as the enhanced expression of p67phox, p47phox, and gp91phox induced by high glucose. Lipid peroxidation, SOD, catalase, GSH-px activity and GSH content was reversed in the presence of resveratrol. Moreover, the expression of pro-apoptotic protein Bax was down regulated while anti-apoptotic protein Bcl-2 was up regulated. And cardiac cell injury and apoptosis were markedly rescued by resveratrol. In addition, resveratrol significantly increased phosphorylation of AMP-activated protein kinase (AMPK) at Thr172 in cardiomyocytes exposed to high glucose. Compound C, the pharmacologic inhibitor of AMPK, could mostly abrogate roles of resveratrol on cardiomyocytes in high glucose. In contrast, Akt and GSK-3β were little influenced by resveratrol.

Conclusions: Our data demonstrated that resveratrol protected cardiomyocytes against high glucose-induced apoptosis through suppression NADPH oxidase-derived ROS generation and maintenance endogenous antioxidant defenses. And the protective effects of resveratrol are mostly mediated by AMPK related pathway.

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1. Introduction

Cardiovascular disease is the primary cause of morbidity and mortality in diabetic patients. The increased risk of macrovascular and microvascular injuries contributes to the high incidence of cardiovascular disease, such as coronary heart disease, hypertension and ischemia in diabetes (Forbes and Cooper, 2013). In addition to these widespread vasculature defects, both clinical and experimental studies have demonstrated the existence of a specific diabetic cardiomyopathy, in which alterations at the level of the cardiomyocyte are distinct. 'Diabetic cardiomyopathy' was first described as a distinct entity by Rubler et al. (1972). It is characterized by early impairments in diastolic function, accompanied by the development of cardiomyocyte hypertrophy, myocardial fibrosis and cardiomyocyte apoptosis (Schilling and Mann, 2012).

The pathological cellular and molecular mechanisms underlying diabetes-induced cardiac damage are complicated and multifactorial. Increased reactive oxygen species (ROS) derived from hyperglycemia is the key contributor in the development and the progression of diabetes-induced cardiac complications (Ansley and
Once these studies have clearly demonstrated that ROS production exceeds the capacity of antioxidant defense systems (Bonnetfont-Rousselot et al., 2000). Once ROS production exceeds the capacity of antioxidant defense systems, overwhelmed free radicals can damage DNA integrity, membrane lipids and protein function by oxidation and lead to functional abnormalities, apoptosis or necrosis (Wold et al., 2005). NADPH oxidase is one of the main sources responsible for myocardial ROS production in diabetes (Kuroda and Sadoshima, 2010). It transfers reducing equivalents from NADPH or NADH to oxygen, which results in superoxide anion (O$_2^-$) generation. The enzyme complex consists of membrane subunits such as gp91phox and p22phox, and cytosolic subunits such as p40phox, p47phox, p67phox, Rac1, etc. (Cave et al., 2006). Sustained activation of NAD(P)H oxidase may further impair antioxidant defenses via depletion of intracellular NADPH (Gao and Mann, 2009). Given the key role of oxidative stress on the pathogenesis of diabetic heart, there is growing interest in antioxidants used as a compensatory therapeutic approach.

Resveratrol (Res) is a natural polyphenol present in many plant-based foods and beverages commonly consumed in our diets, including peanuts, cranberries, blueberries and grapes (Li et al., 2012). As a non-flavonoid polyphenolic compound, resveratrol has been demonstrated to be potent antioxidants and free radical scavengers (Lorenz et al., 2003). It possess beneficial effects in various cardiovascular disease animal models, including hypertension, atherosclerosis, ischemic heart diseases, and heart failure (Dolinsky and Dyck, 2011). Moreover, resveratrol treatment significantly decreased the blood glucose level in STZ-treated type 1 diabetic animals (Huang et al., 2010). Indeed, previous studies have shown that resveratrol alleviated diabetes-induced cardiovascular system disorders via different endogenous signaling pathways including oxidative stress/antioxidant defense system and glucose/insulin metabolism (Turan et al., 2012). These studies have clearly demonstrated the potential beneficial effects of resveratrol in the pathogenesis of diabetic cardiomyopathy. However, it is not clear whether these effects were due to direct cardioprotective effects of resveratrol on the cardiomyocytes, or secondary to improved glycemic control by resveratrol.

In the present study, primary neonatal rat cardiomyocytes have been isolated and cultured. We aim to investigate the direct role of resveratrol on cardiomyocytes when exposed to high glucose, as well as the potential modulation of protein signaling pathways.

2. Materials and methods

2.1. Reagents

Dulbecco’s modified eagle medium (DMEM), fetal bovine serum (FBS) and collagenase type II were purchased from Gibco (Los Angeles, CA, USA). Cell Counting Kit-8 was purchased from Dojindo (Kumamoto, Japan). LDH, MDA, catalase, GSH-px, GSH assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). SOD assay kit was purchased from BestBio (Shanghai, China). Annexin V-FITC and PI kit was purchased from Sigma (St. Louis, MO, USA). Bcl-2, Bax, p67phox, p22phox, plasma-AMPKα (Thr172) and AMPKα, plasma-Akt (Ser473) and Akt, plasma-GSK-3β (Ser9) and GSK-3β were purchased from Cell Signaling Technology (Boston, MA, USA), gp91phox, p47phox and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Resveratrol and other reagents were purchased from Sigma (St. Louis, MO, USA) unless noted otherwise.

2.2. Animals

Sprague-Dawley rats (1–3 days old) was supplied by the Center of Experimental Animal in Hubei University of Science and Technology and conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH publication no. 85–23, revised 1996). All experimental procedures were approved by the Committee of Experimental Animals of Hubei University of Science and Technology.

2.3. Primary culture of neonatal rat cardiomyocytes and experimental treatment

Neonatal rat cardiomyocytes were isolated according to previously described methods with little modification (Kovacic et al., 2003). The cardiomyocytes were cultured in DMEM supplemented with 10% FBS (Gibco, USA), 100 U/mL penicillin and 100 ng/mL streptomycin at 37 °C in a humidified atmosphere (5% CO$_2$ and 95% air). The cells were exposed to normal medium (5 mmol/L D-glucose), high glucose (30 mmol/L D-glucose), or mannitol (5 mmol/L D-glucose plus 25 mmol/L mannitol) for 24–48 h with or without resveratrol (50 μM unless noted otherwise). Compound C 10 μM was applied in the medium 1 h before high glucose and resveratrol treatment.

2.4. Assessment of cell viability

Cell viability was performed with Cell Counting Kit-8 (Dojindo, Japan) in 96-well plates according to the manufacturer’s instructions.

2.5. Biochemical analysis

Lactate dehydrogenase (LDH) was determined on its ability to catalyze the reversible oxidation–reduction reaction between lactic acid and pyruvic acid. MDA was measured by thiobarbituric acid reacting substances (TBARS) method. Superoxide dismutase (SOD) activity was determined based on its ability to inhibit the oxidation of oxymine by O$_2^-$ produced from the xanthine/xanthine oxidase system. Catalase activity was measured by employing hydrogen peroxide to generate H$_2$O and O$_2$. Glutathione peroxidase (GSH-px) activity was determined by its ability to specifically catalyze glutathione reduction reaction to hydrogen peroxide, and reduced glutathione (GSH) content was based on the formation of GSH–o-phthalaldehyde. The colorimetric analysis was using the automatic microplate reader (Biotek synergy2, VT, USA). Protein concentration was determined by the coomassie blue staining. The detailed procedures followed the instructions in the corresponding assay kits.

2.6. Intracellular ROS measurement

Intracellular superoxide anions were measured using the dihydroethidium (DHE) fluorescence probe and high performance liquid chromatography (HPLC) assay. The cardiomyocytes were washed three times with cold DPBS after the experiment procedures. The cells were incubated with 10 μM DHE for 30 min at 37 °C. After washing with cold DPBS twice to remove free DHE, the images of cardiomyocytes were captured and analyzed immediately under an inverted fluorescence microscopy (Olympus IX71, Japan).

Furthermore, the generation of intracellular ROS was assessed by HPLC (SHIMADZU, LC-20AD, Japan) using the DHE fluorescent probe with an emission wavelength of 596 nm and an excitation wavelength of 490 nm (Zielonka et al., 2009). Briefly, the
cells were incubated in DMEM containing 10 μM DHE for 30 min, then harvested and permeabilized with 0.1% Triton X-100 in DPBS. Protein concentration was assessed using the Bicinchoninic Acid (BCA) protein assay kit (Beyotime, China). After that, 100 μL of the cell lysate was added into equal volume of 0.2 mol/L HClO4 in methanol and placed on ice for 2 hours to allow protein precipitation. After incubating on ice, the mixture was centrifuged at 20,000 g for 30 min at 4 °C. One hundred microliters of the supernatant was transferred into equal volume of 1 mol/L potassium phosphate buffer (pH 2.6). The samples were centrifuged at 20,000 g for 15 min at 4 °C. Finally, the supernatant was subjected to the HPLC then ran the analysis.

2.7. In situ detection of apoptotic cells by TUNEL assay

To estimate apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed on cardiomyocytes using an in situ apoptosis detection kit (Roche, Germany) according to the manufacturer’s instructions. In brief, the cardiomyocytes were washed twice with DPBS after the experiment procedures, then fixed with 4% paraformaldehyde at room temperature away from light for 30 min, and then incubated with proteinase K for 15 min in 37 °C after that, cells were placed in 3% H2O2 for 15 min at room temperature. Cells were washed several times in PBS in every experimental procedure. Next, cells were treated by TUNEL detection kit. 3, 3′-diaminobenzidine (DAB) was used as the staining agent followed by hematoxylin staining the cell nuclei. Images were captured under a fluorescence microscopy (Olympus BX53, Japan) and the TUNEL apoptosis rate (%) = number of TUNEL – positive cardiomyocytes/total number of cardiomyocytes.

2.8. Annexin V and phosphatidylinositol (PI) binding staining

The assay was performed with an Annexin V–FITC Apoptosis Detection Kit according to the manufacturer’s instructions (Best Bio, China). Briefly, following incubation with the appropriate drugs, 1 × 106 cells were collected by centrifugation, then they were resuspended in 500 μL 1 × binding buffer, after that, 5 μL Annexin V-FITC and 5 μL propidium iodide (PI) were added into the solution, cells were gently vortex and incubated with them for 15 min at 37 °C in the dark. Annexin V-FITC and PI-stained cells were analyzed by FACScan flow cytometer (Becton Dickinson, USA).

2.9. Determination of rac1 activity

Rac1 activity was measured according to the manufacturer’s protocol (Rac1 Activation Assay Kit; Millipore, USA). The cells were washed twice with cold DPBS. The proteins in cardiomyocytes were extracted by 1 × MLB (Mg2+/lysium/wash buffer) with protease inhibitor cocktail. The cell homogenates were spun at 12,000 rpm for 15 min and the supernatants were saved for active Rac1 pulldown assay and total Rac1 content. Fifteen microliter PKA-PBD agarose beads were then added into each sample and incubated overnight at 4 °C with gentle agitation. Agarose beads conjugated with the active Rac1 were centrifuged and collected. The active Rac1 and total Rac1 content was measured by western blot assay.

2.10. Measurement of NADP+/NADPH ratio

The NADP+ and NADPH were measured by using EnzyFluo™ NADP+/NADPH assay kit (Bioassay Systems, CA, USA) according to the manufacturer’s protocols. It was based on a glucose dehydrogenase cycling reaction, in which the formed NADPH reduces a probe into a highly fluorescent product. The fluorescence intensity of this product, measured at λex/em = 530/585 nm, is proportional to the ratio of NADP+/NADPH of the sample.

2.11. Immunohistochemical measurement of Bcl-2 and Bax

After being carefully rinsed in PBS, the three groups of cardiomyocytes were fixed with 4% paraformaldehyde at room temperature for 30 min, and permeabilized with 0.1% Triton X-100 for 10 min. Cells were then inactivated by endogenous peroxidase with 3% H2O2 for 15 min in the dark, and blocked cross-reactivity with 5% BSA for 30 min at room temperature. Cells were washed three times in PBS in every experimental procedure. After that, the cells were incubated with rabbit anti Bcl-2 (1:300, Abcam, USA) and rabbit anti Bax (1:200, Abcam, USA) at 4 °C overnight. After washing with PBS, the cells were incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG (Goodbio, China) for 45 min at room temperature. The reaction was visualized with DAB, counter-stained with hematoxylin, dehydrated in ethanol, and coverslipped. Images (>×400 magnification) were captured. The staining was quantified with Image-Pro Plus v6 analysis software.

2.12. Western blot analysis

Cells cultured on 6-well plates were plated in modified RIPa buffer on ice for 30 min, and then the lysates were clarified by centrifugation at 12,000 rpm for 15 min at 4 °C. Proteins in the supernatant were quantified using the BCA protein assay. Equal protein was used for Western blot using the following antibodies: phospho-AMPKα, AMPKα, p67phox, p47phox, gp91phox, p22phox, phospho-Akt (Ser473), Akt, phospho-GSK-3β (Ser9), GSK-3β, Bcl-2, Bax (Cell Signaling Technology, USA) and β-actin (Santa Cruz Biotechnology, USA). The membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were developed by ECL kit (Pierce Biosciences, USA).

2.13. Statistical analysis

Data were presented as mean ± SEM. Statistical analysis was performed with a one-way analysis of variance (ANOVA) followed Student–Newman–Keuls test using SPSS 10.0 statistical software. Differences were considered to be significant at P<0.05.

3. Results

3.1. Resveratrol increased cell viability in cardiomyocytes exposed to high glucose

To investigate the role of resveratrol on cell survival, we firstly examined the viability of primary cultured neonatal rat cardiomyocytes incubated in 5–50 mM/L D-glucose up to 72 h. As shown in Fig. 1, cell viability was significantly inhibited when glucose concentration increased to 30 mM/L (P<0.05, Fig. 1A) and when incubated time exceeded 24 h in 30 mM/L D-glucose (P<0.05, Fig. 1B). Mannitol (30 mM/L) was used as osmotic control and could not mimic the effects of 30 mM/L D-glucose. Resveratrol was then applied to primary cultured neonatal cardiomyocytes in 30 mM/L D-glucose glucose for 24 h. As shown in Fig. 1C and D, the addition of resveratrol 50 and 100 μM significantly increased cell viability and decreased LDH release into the culture media (P<0.05). Lactate dehydrogenase (LDH) is an oxidoreductase enzyme which is present in cytosol of animals and plants. It is a fairly stable enzyme and has been widely used to evaluate the presence of damage and toxicity of tissue and cells. The released LDH into the culture media occurs with the loss of plasma membrane integrity, a process most often associated with necrotic cell death. Thus, these results indicated that resveratrol could rescue cardiomyocytes from high glucose induced injury. In the following experiments, 30 mM/L D-glucose was used to induce high glucose (HG) circumstance, and 50 μM resveratrol was applied unless noted otherwise.
3.2. Resveratrol prevented oxidative stress in primary cultured neonatal rat cardiomyocytes exposed to high glucose

The intracellular level of ROS in myocardial cultures was first estimated by DHE, a fluorescent probe which served as an index of the level of ROS production. As shown in Fig. 2A and B, the intensity of red fluorescence was markedly enhanced in cardiomyocytes exposed to high glucose in contrast to control (P < 0.05 vs control). Application of resveratrol 50 μM could significantly inhibit intracellular ROS generation (P < 0.05 vs HG). The dihydroethidium fluorescence was further quantified using HPLC assay. As shown in Fig. 2C, ROS level significant increased in cells exposed to high glucose, and it was reversed in the presence of resveratrol (P < 0.05).

Next, we explored whether the NADPH oxidase is contributed to the increased ROS production. NADPH oxidase is an enzyme complex consisting of a lot of essential subunits such as Rac1, p47phox, p67phox, gp91phox and p22phox. As shown in Fig. 3A and B, Rac1 activity and the expression of p47phox, p67phox and gp91phox were markedly increased induced by high glucose in cardiomyocytes (P < 0.05 vs control). In contrast, resveratrol supplementation significantly reversed the alteration of Rac activity and the subunit expression (P < 0.05 vs HG). These results suggested that resveratrol down-regulated NADPH oxidase, which lead to suppress ROS generation in cardiomyocytes exposed to high glucose.

Third, the redox status was further assessed by the endogenous antioxidant enzymes using biochemical measurement (Fig. 4A–E). It is known that under physiological conditions, SOD enzymes degrade O$_2^•$ to form the more stable species, H$_2$O$_2$ and O$_2$. H$_2$O$_2$ is further catalyzed to water by the antioxidants GSH-px and catalase. Glutathione exists in reduced (GSH) and oxidized (GSSG) states. Reduced glutathione (GSH) is a major tissue antioxidant that provides reducing equivalents for the glutathione peroxidase (GSH-px) catalyzed reduction of lipid hydroperoxides to their corresponding alcohols and hydrogen peroxide to water. Decreased GSH is an indication of oxidative stress. Our results have shown increased formation of MDA in cardiomyocytes exposed to high glucose, accompanied with markedly inhibited activities of antioxidant enzymes SOD, catalase and GSH-px, as well as GSH content in cardiomyocytes exposed to high glucose (P < 0.05 vs control). Resveratrol 50 μM markedly inhibited lipid peroxidation and up-regulated SOD, catalase and GSH-px activities, and increased GSH content (P < 0.05 vs HG).

3.3. Resveratrol prevented high glucose-induced apoptosis in cardiomyocytes

Apoptotic cells were detected by both TUNEL staining and flow cytometry. As shown in Fig. 5A and B, apoptotic positive cells, which...
stained in brown by TUNEL staining, were much higher in HG group in contrast to control. And compared to high glucose group, resveratrol treatment significantly prevented high glucose-induced myocardial cell apoptosis.

We also detected apoptotic cells using the dual staining approach with PI and Annexin V by flow cytometry. PI (−)/annexin (+) cells represent the early apoptotic populations and PI (+)/annexin (+) cells represent late apoptotic populations. Results are shown in Fig. 5C, both PI (−)/annexin (+) and PI (+)/annexin (+) cells were significantly increased in high glucose compared with control (P < 0.05 vs control). And resveratrol 50 μM significantly decreased the number of apoptotic cells in high glucose (P < 0.05 vs HG).

3.4. Resveratrol regulated the expression of Bax and Bcl-2 in cardiomyocytes exposed to high glucose

It is well known that apoptosis is regulated by series of apoptotic related proteins. Among them, Bax is regarded as a pro-apoptotic protein while Bcl-2 as an anti-apoptotic marker for apoptosis. We further investigated whether resveratrol suppressed apoptosis through
both regulatory proteins. As indicated with the immunohistochemical staining (Fig. 6A and B), enhanced expression of Bax, but inhibited expression of Bcl-2 was presented in high glucose group in contrast to control (P < 0.05 vs control). Remarkably, resveratrol significantly down regulated Bax-2 expression and up regulated Bcl-2 in the rat neonatal cardiomyocytes exposed to high glucose (P < 0.05 vs HG). This result was further confirmed by western bolt analysis for Bax and Bcl-2 expression (Fig. 6C).

3.5. Influence of resveratrol on Akt/GSK-3β and AMPK pathway in cardiomyocytes exposed to high glucose

The Akt/GSK-3β pathway plays an important role in regulating cellular survival, proliferation and apoptosis. As shown in Fig. 7A, both phosphorylation of Akt and GSK-3β proteins were significantly lower in rat neonatal cardiomyocytes exposed to high glucose, but neither Akt nor GSK-3β phosphorylation was affected in the
presence of resveratrol. It was suggested that Akt/GSK-3β pathway was not involved in prevention of high glucose-induced rat neonatal cardiomyocytes apoptosis by resveratrol. However, as shown in Fig. 7B, resveratrol could markedly enhance the phosphorylated AMPKα in rat neonatal cardiomyocytes (P < 0.05 vs HG), which was also inhibited when cells were exposed to high glucose (P < 0.05 vs control). Compound C (10 μM), a pharmacologic inhibitor of AMPK, could block those effects mentioned earlier.

In parallel with that, resveratrol failed to attenuate the NADPH oxidase and Rac1 activities, as well as the subunit expression of p47phox, p67phox and gp91phox in the presence of Compound C (Fig. 8A–C, P < 0.05). Moreover, the expression of Bax and Bcl-2 regulated by resveratrol was also blunted when AMPK was inhibited by Compound C (Fig. 9A, P < 0.05). As a consequence, the rescued role of resveratrol on apoptotic cell death was abolished when resveratrol applied was combined with Compound C (Fig. 9B and C, P < 0.05).

4. Discussion

As the hallmark sign of diabetes, hyperglycemia acts as one of the central drivers of the metabolic, functional and structural alterations in the diabetic heart. Abnormalities in glucose control itself is sufficient to trigger an array of maladaptive processes (Robertson et al., 2004; Yao and Brownlee, 2010), which contribute to the development and progression of diabetic cardiomyopathy (Fang et al., 2004). In the present study, when primary cultured neonatal rat cardiomyocytes were exposed to 30 mmol/L high glucose over 24 h, cell injury and apoptosis were markedly increased. It is consistent
with previous reports that cardiomyocyte apoptosis is correlated with blood glucose levels (Fiordaliso et al., 2000). As the most well-known form of programmed cell death, apoptosis is essential for maintaining tissue homeostasis under normal physiology. Increased myocardial apoptosis causes loss of contractile units, which is closely associated with decreased contractility, cardiac remodeling, hypertrophy, and dysfunction in heart (Dhalla et al., 2014; Foo et al., 2005). Evidence from animal and human samples now indicates that cardiomyocyte apoptosis plays an important causal role in the development of diabetic cardiomyopathy (Cai et al., 2002; Kuethe et al., 2007). Elevated apoptosis indicates a poor cardiovascular outcome (Bernecker et al., 2003). In our study, when resveratrol was applied to the cells exposure to high glucose, the apoptotic cell injury and death were greatly rescued. Since apoptosis is tightly

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**Fig. 8.** Inhibition AMPK reversed the roles of resveratrol on NADPH oxidase activity and subunit expression in primary cultured neonatal rat cardiomyocytes exposed to high glucose. A: Decreased Rac1 activity by resveratrol was blunted in the presence of Compound C (10 μM). B: Decreased expression of NADPH oxidase subunits p47phox, p67phox and gp91phox by resveratrol was reversed in the presence of Compound C (10 μM). C: Inhibited NADPH activity by resveratrol was abrogated in the presence of Compound C (10 μM). Resveratrol: 50 μM. *P < 0.05 vs control group. #P < 0.05 vs HG group. &P < 0.05 vs HG + Res group. n = 6.

**Fig. 9.** AMPK inhibition exacerbated apoptosis in primary cultured neonatal rat cardiomyocytes exposed to high glucose. A: Compound C (10 μM) abolished the regulation of resveratrol on Bax and Bcl-2 expression. B: Representative images of apoptotic cardiomyocytes stained by TUNEL (magnification = 400×, bar is 20 μm). Rescued myocardial apoptosis by resveratrol was exacerbated with the addition of Compound C (10 μM). C: Quantitative analysis for TUNEL staining. Resveratrol: 50 μM. *P < 0.05 vs control group. #P < 0.05 vs HG group. &P < 0.05 vs HG + Res group. n = 6.
controlled by a series of regulatory proteins, we further checked the expression of pro-apoptotic proteins Bax and anti-apoptotic proteins Bcl-2. As expected, the increased ratio of Bax/Bcl-2 has been reversed by the addition of resveratrol in the cardiomyocytes exposed to high glucose.

Increased oxidative stress is the key contributor in the development and progression of diabetic cardiomyopathy. The membrane-bound enzyme complex NADPH oxidase is one of the major sources of ROS in cardiovascular cells. It comprises multiple protein components that must be assembled in the cell membrane for the enzymes to become active. Varied subunits of NADPH oxidase are found expressed in various cell types (Drummond et al., 2011) and are inducible in certain pathologies (Ritchie et al., 2007). In the present study, elevated subunits expression of NADPH oxidase p67phox, p47phox, gp91phox and Rac1 activity was induced by high glucose in cardiomyocytes, as well as enhanced NADPH oxidase activity. High glucose also impaired the endogenous antioxidant defense system, as indicated with the suppressed SOD, catalase and GSH-px activity, lower GSH content and increased lipid peroxidation. Resveratrol has been demonstrated to be potent free radical scavengers. It can quench reactive oxidative species such as hydrogen peroxide, superoxide and peroxynitrite (Holthoff et al., 2010; Kovacic and Somanathan, 2010). In the present study, resveratrol supplementation reversed high glucose-induced apoptosis through inhibiting NADPH-derived ROS production and alleviating the reduction of cardiac antioxidant enzyme activities. And these effects were possibly mediated by AMPK related signaling pathway. Our results further confirmed the therapeutic potential for resveratrol in the treatment of diabetic cardiovascular diseases.

Authors' contributions

GS, YQ participated in the design of the study and conducted experiments, analyzed data, performed the statistical analysis and wrote draft of the manuscript; KZQ, CHG have been involved in performing experiments; WJL planned experiments and was involved in revising manuscript; LC conceived of the study, designed and coordinated the study, wrote manuscript. All authors read and approved the final manuscript.

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Taken together, using the primary cultured neonatal rat cardiomyocytes, we have demonstrated that resveratrol prevented high glucose-induced apoptosis through inhibiting NADPH-derived ROS production and alleviating the reduction of cardiac antioxidant enzyme activities. And these effects were possibly mediated by AMPK related signaling pathway. Our results further confirmed the therapeutic potential for resveratrol in the treatment of diabetic cardiovascular diseases.


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