Original Research

The role of oxidative stress in high glucose-induced apoptosis in neonatal rat cardiomyocytes

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Abstract

Accumulating evidence has demonstrated that apoptosis plays a critical role in the pathogenesis of diabetic cardiomyopathy. However, the exact molecular mechanisms by which hyperglycaemia induces cardiomyocyte apoptosis are not fully understood. The present study was designed to investigate the role of oxidative stress in high glucose-induced apoptosis in cultured neonatal rat cardiomyocytes. The MTT assay was used to detect the viability of cardiomyocytes exposed to different concentrations of glucose. Oxidative stress was evaluated by measuring intracellular reactive oxygen species with 2′,7′-dichlorofluoresce diacetate staining and by detecting malondialdehyde and superoxide dismutase in the supernatant of culture media. Cardiomyocyte apoptosis was determined by flow cytometry and confocal laser scanning microscopy with Annexin V/PI staining. Our results showed that high glucose can induce oxidative stress and promote apoptosis in neonatal rat cardiomyocytes and the antioxidant can protect against high glucose-induced apoptosis, which suggests that oxidative stress is involved in high glucose-induced cardiomyocyte apoptosis. Furthermore, caspase-3 was found to be activated in the process of high glucose-induced oxidative stress, which subsequently contributes to increased apoptosis in neonatal rat cardiomyocytes. In conclusion, our study demonstrates that oxidative stress is involved in high glucose-induced cardiomyocyte apoptosis via activation of caspase-3.

Keywords: Oxidative stress, high glucose, cardiomyocyte, apoptosis, caspase-3

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Introduction

Diabetic cardiomyopathy has been defined as ventricular dysfunction that occurs independently of coronary artery disease and hypertension. Hyperglycaemia seems to be central to the pathogenesis of diabetic cardiomyopathy and to trigger a series of maladaptive stimuli that result in myocardial fibrosis and collagen deposition.[1] Accumulating evidence has demonstrated that hyperglycaemia-induced apoptosis plays an important role in the pathophysiology of diabetic cardiomyopathy in both animals and humans.[2–4] However, the precise molecular mechanisms by which hyperglycaemia induces cardiomyocyte apoptosis remain unclear.

The production of reactive oxygen species (ROS) has been shown to be increased in streptozotocin-induced diabetic rats.[5–7] Hyperglycaemia-induced oxidative stress is a major risk factor for the development of microvascular pathogenesis in the diabetic myocardium, which results in myocardial cell death, hypertrophy, fibrosis, abnormalities of calcium homeostasis and endothelial dysfunction.[8] The present study was designed to investigate the role of oxidative stress in high glucose-induced apoptosis in cultured neonatal rat cardiomyocytes.

Materials and methods

Primary culture of neonatal rat cardiomyocytes

All experimental procedures were approved by the Animal Care and Ethics Committee of Nanjing Medical University. Rat hearts were surgically removed from 1- to 3-day-old Sprague-Dawley rats obtained from the Laboratory Animals Center of Nanjing Medical University, washed instantly in cold D-Hanks solution, and then minced into 1–3 mm^3 pieces. Thereafter, the minced tissue was subjected to 6–8 cycles of proteolytic dissociation by magnetic stirring (10 min, 37°C) with 0.06% trypsin solution (Gibco, USA). Supernatant from each cycle was pooled and centrifuged. Finally, the cell pellet was resuspended in DMEM supplemented with 20% calf serum (Gibco), 100 U/mL penicillin and 100 mg/mL streptomycin. Selective adhesion was performed after a 1.5 h incubation at 37°C in a humidified atmosphere (5% CO_2 and 95% air) in order to obtain a high purity of cardiomyocytes. Subsequently, 0.1 mmol/L

bromodeoxyuridine (Sigma, USA) was added to the medium for the first 48 h of culture to inhibit the growth of fibroblasts.

Analysis of cell viability

Cardiomyocytes were exposed to different concentrations of glucose (5–50 mmol/L) for 48 h and cell viability was detected by MTT assay. Briefly, cells were seeded into a 96-well plate at a density of 1×10^5/well and incubated with 20 μL of 5 mg/mL MTT solution (Amresco, USA) for 4 h at 37°C following treatment with various concentrations of glucose. Thereafter, 150 μL of dimethyl sulphoxide (Amresco) was added to each well to dissolve the dye crystal formazan and the plate was shaken for 10 min to make sure that all purple crystals were dissolved. The amount of MTT formazan was quantified by determining the absorbance at 490 nm using a microplate reader.

Grouping of the experiments

Cardiomyocytes were randomly divided into three groups: cells cultured in normal culture media (control group); cells exposed to high glucose (30 mmol/L, Sigma; HG group); cells treated with high glucose and antioxidant glutathione (GSH, 250 μmol/L, Sigma; HG + GSH group). After 48 h of treatment, cells were harvested for oxidative stress measurement, apoptosis detection, and caspase-3 activity assay.

Assessment of oxidative stress

Oxidative stress was evaluated by detecting malondialdehyde (MDA) and superoxide dismutase (SOD) in the supernatant of culture media according to the instructions of the detection kit (Jiancheng, China). The change in MDA content reflected the degree of lipid peroxidation in the cell membrane. The activity of SOD indirectly reflected the capacity to remove oxygen free radicals.

Measurement of intracellular ROS

The determination of intracellular ROS production was based on the oxidation of 2′, 7′-dichlorofluoresce diacetate (DCFH-DA). Cardiomyocytes were seeded into a 96-well plate at a density of 1×10^5/well. Following the treatment of glucose and GSH, cells were incubated with DCFH-DA (Beyotime, China) at 37°C for 20 min. The fluorescence was read at 485 nm for excitation and 530 nm for emission in a microplate reader. The amount of emitted fluorescence was correlated with the quantity of intracellular ROS.

Flow cytometric analysis of cell apoptosis

The Annexin V–fluorescein isothiocyanate apoptosis detection kit (BD Pharmingen, USA) was used to determine apoptosis according to the manufacturer's instructions. Briefly, 1×10^5 cells were collected and washed twice with cold PBS. 5 μL of Annexin V and 5 μL of PI were added to the cells, which were resuspended in 500 μL 1 × binding buffer. The cells were gently vortexed and incubated for 15 min at room temperature in the dark, then they were analysed by flow cytometry. Annexin V labelled with a fluorophore could identify cells in the early stage of apoptosis, and PI, a fluorescent nucleic acid binding dye, was responsible for staining cells in the medium and late stages of apoptosis. The apoptotic rate was calculated as the percentage of Annexin V-positive and PI-negative cells divided by the total number of cells in the gated region.

Laser confocal microscopy

Cell slices were prepared by inoculating myocardial cells (1×10^6/mL) onto a 6-hole board with coverslips, then they were stained with Annexin V and PI (BD Pharmingen) and observed under confocal laser scanning microscope. Normal cells could only give off a weak green fluorescence. In the early stages of apoptosis, the cell membranes were stained with Annexin V and gave off a vibrant green fluorescence, while the nucleolus was not stained with PI. The cells in the late stages of apoptosis were highly stained by both Annexin V and PI, therefore, the cell membranes gave off green fluorescence and the nucleolus gave off a red fluorescence.

Caspase-3 activity assay

Caspase-3 activity was measured using an ApoTarget caspase-3 protease assay kit (BioSource International California, USA). Briefly, the culture medium was centrifuged and the supernatant was removed to collect detached cells as a pellet. Attached cells were scraped and combined with the pellet in ice-cold lysis buffer. Samples were centrifuged and the supernatant was collected. Protein concentration was determined with a bicinchoninic acid assay kit (Pierce Rockford, IL, USA). The cell lysate (30 μg) from each sample was combined with 200 μmol/L of DEVD-pNA substrate and diluted with 2 × reaction buffer containing 10 mmol/L of dithiothreitol. After incubation at 37°C in the dark for 1 h, samples were read at 405 nm in a microplate reader.

Statistical analysis

Data are presented as mean ± SD and differences between groups were analysed using one-way analysis of variance (ANOVA) with the SPSS 15.0 (SPSS, Inc., Chicago, IL, USA). Scheffe's *post hoc* test was used to provide specific information on which means are significantly different from each other if the ANOVA was significant. A P value <0.05 was considered statistically significant.

Results

The cell viability was measured by MTT assay and the results are presented in Table 1. Our findings suggested that high glucose inhibits cardiomyocyte viability in a concentration-dependent manner. The lowest effective concentration (30 mmol/L) was chosen as the effective glucose concentration in this experiment.

Oxidative stress was evaluated by detecting MDA and SOD in the supernatant of culture media. Our results showed a significant increase in MDA level and a decrease in SOD activity in the HG group compared to the control group, while in the HG + GSH group, a significant decrease

in MDA level and an increase in SOD activity were found compared to the HG group (Table 2).

The intracellular ROS was detected by DCFH-DA staining and the results are shown in Figure 1. Our findings indicated that high glucose can significantly increase the production of ROS. However, the increased amount of ROS induced by high glucose could be reduced by antioxidant GSH.

Cardiomyocyte apoptotic rate was determined by flow cytometry and the results are shown in Figure 2. The apoptotic rate in the HG group was significantly higher than in the control group, whereas apoptotic rate in the HG + GSH group was significantly lower than in the HG group.

Cardiomyocytes grown on coverslips were stained with Annexin V/propidium iodide and observed under confocal laser scanning microscope. The percentage of apoptotic cells was significantly increased in the HG group, while in the HG + GSH group, the apoptosis percentage was significantly decreased compared with that in the HG group (Figure 3).

Caspase-3 activity was measured using the protease assay kit and the results are shown in Figure 4. Caspase-3 activity in the HG group was significantly higher than in the control group, whereas caspase-3 activity in the HG + GSH group was significantly lower than in the HG group.

Discussion

Diabetic cardiomyopathy is the presence of myocardial dysfunction in the absence of coronary artery disease and hypertension. Left ventricular hypertrophy, metabolic abnormalities, extracellular matrix changes, small vessel disease, cardiac autonomic neuropathy, insulin resistance, oxidative stress, and apoptosis are the most important contributors to diabetic cardiomyopathy onset and progression.[9] Hyperglycaemia is a major aetiological factor in the development of diabetic cardiomyopathy. It increases the levels of free fatty acids and growth factors and causes abnormalities in substrate supply and utilization, calcium homeostasis, and lipid metabolism. Moreover, it promotes excessive production of ROS, which induces oxidative stress leading to abnormal gene expression and faulty signal transduction.[10]

The pathogenesis of diabetic cardiomyopathy is multifactorial. Several hypotheses have been proposed, including autonomic dysfunction, metabolic derangements, abnormalities in Ca^{2+} homeostasis, alteration in structural proteins, and interstitial fibrosis.[11] It has been well documented that apoptosis is involved in the pathogenesis of diabetic cardiomyopathy.[12,13] However, the exact molecular mechanisms by which hyperglycaemia induces cardiomyocyte apoptosis are not fully understood. In the present study, we investigated the role of oxidative stress in high glucose-induced apoptosis in cultured neonatal rat cardiomyocytes. Our findings demonstrated that oxidative stress is involved in high glucose-induced cardiomyocyte apoptosis via activation of caspase-3.

Increased oxidative stress in the diabetic heart is a contributing factor in the development and progression of

Table 1 Effect of high glucose on the viability of cardiomyocytes.[a]

Groups	Glucose concentration (mmol/L)	Absorbance value
Normal glucose	5	0.532 ± 0.031
High glucose	10	0.518 ± 0.037
	20	0.506 ± 0.034
	30	0.473 ± 0.025*
	40	0.445 ± 0.028*
	50	0.409 ± 0.023*

[a]Data are expressed as mean ± SD ($n = 5$).
*$P < 0.05$, vs. normal glucose group.

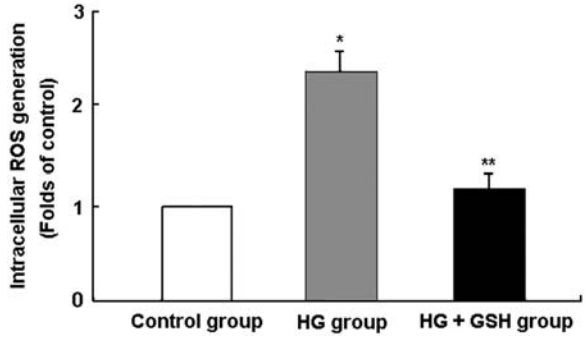

Figure 1 Measurement of intracellular ROS by DCFH-DA staining. The production of ROS was significantly increased in the HG group, while in the HG + GSH group, ROS generation was significantly decreased compared with that in the HG group

Table 2 Assessment of oxidative stress.[a]

	Control group	HG group	HG + GSH group
MDA (nmol/mg pro)	0.42 ± 0.10	0.89 ± 0.18*	0.49 ± 0.15**
SOD (U/mg pro)	165.68 ± 17.42	109.54 ± 22.65*	154.79 ± 20.31**

MDA: malondialdehyde; SOD: superoxide dismutase.
[a]Data are expressed as mean ± SD ($n = 5$).
*$P < 0.05$, vs. control group.
**$P < 0.05$, vs. HG group.

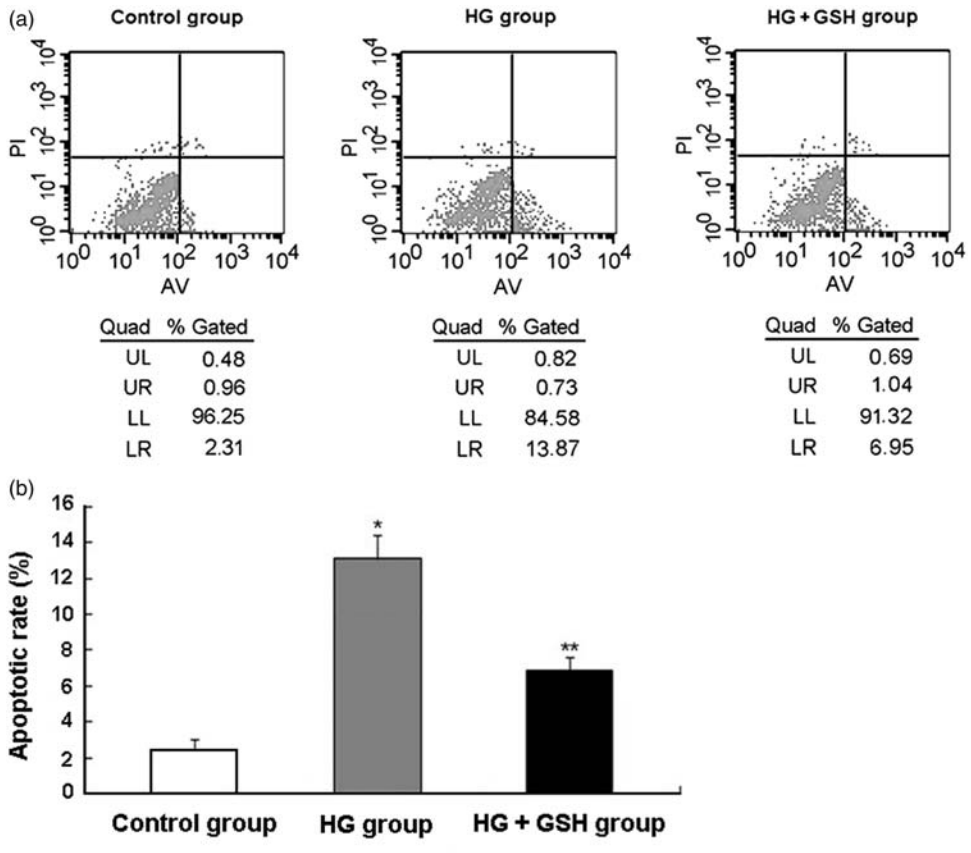

Figure 2 (a) Cardiomyocyte apoptotic rate was determined by flow cytometry with Annexin V/PI staining. (b) The apoptotic rate in the HG group was significantly higher than in the control group, whereas apoptotic rate in the HG + GSH group was significantly lower than in the HG group ($n = 5$). *$P < 0.05$, vs. control group; **$P < 0.05$, vs. HG group

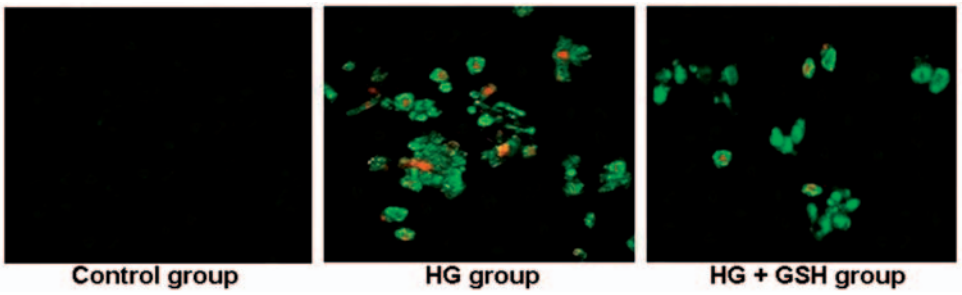

Figure 3 Cardiomyocytes grown on coverslips were stained with Annexin V/PI and observed under confocal laser scanning microscope. The percentage of apoptotic cells was significantly increased in the HG group, while in the HG + GSH group, the apoptosis percentage was significantly decreased compared with that in the HG group. (A color version of this figure is available in the online journal)

diabetic cardiomyopathy.[14,15] Cumulative superoxide-mediated damage or cellular dysfunction occurs when an imbalance exists in ROS generation and degradation pathways. Increased ROS production and impaired antioxidant defenses could both contribute to oxidative stress in diabetic hearts. In the present study, our results showed that high glucose can induce oxidative stress and promote apoptosis in neonatal rat cardiomyocytes and the antioxidant GSH can protect against high glucose-induced apoptosis, which suggests that oxidative stress is involved in high glucose-induced cardiomyocyte apoptosis.

The caspase family proteases are crucial mediators of apoptosis. Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins. Pathways to caspase-3 activation have been identified that are either dependent on or independent

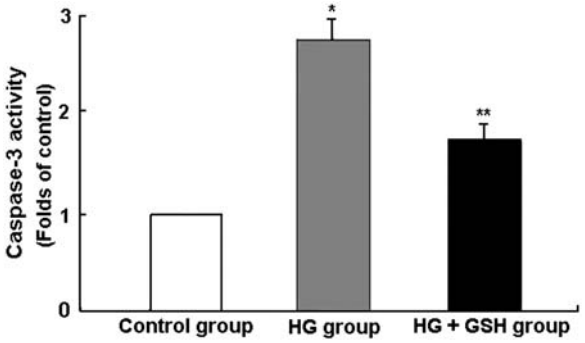

Figure 4 Measurement of caspase-3 activity. Caspase-3 activity in the HG group was significantly higher than in the control group, whereas caspase-3 activity in the HG + GSH group was significantly lower than in the HG group ($n = 5$). *$P < 0.05$, vs. control group; **$P < 0.05$, vs. HG group

of mitochondrial cytochrome c release and caspase-9 function. Caspase-3 is essential for normal brain development and is important in other apoptotic scenarios in a remarkable tissue-, cell type-, or death stimulus-specific manner. Caspase-3 is also required for some typical hallmarks of apoptosis, and is indispensable for apoptotic chromatin condensation and DNA fragmentation.[16] In the present study, caspase-3 was found to be activated in the process of high glucose-induced oxidative stress, which subsequently contributes to increased apoptosis in neonatal rat cardiomyocytes.

In conclusion, our study demonstrates that oxidative stress is involved in high glucose-induced cardiomyocyte apoptosis via activation of caspase-3.

Author contributions: XZ designed the study, performed the experiment, analysed and interpreted the data, and wrote the initial draft of the manuscript. XL contributed to the experimental design, the statistical analysis and the writing of the manuscript.

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