MicroRNA-34a Regulates High Glucose-induced Apoptosis in H9c2 Cardiomyocytes

Fang ZHAO (赵芳)1*, Bo LI (李波)2, Yin-zhi WEI (卫银芝)1, Bin ZHOU (周斌)1, Han WANG (汪瀚)1, Ming CHEN (陈明)1, Xue-dong GAN (干学东)1, Zhao-hui WANG (汪朝晖)3, Shi-xi XIONG (熊世熙)1
1Department of Cardiovascular Diseases, Zhongnan Hospital of Wuhan University, Wuhan 430071, China
2Department of Oral Radiology, School and Hospital of Stomatology, Wuhan University, Wuhan 430079, China
3Department of Cardiovascular Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China

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Summary: Hyperglycemia is an important initiator of cardiovascular disease, contributing to the development of cardiomyocyte death and diabetic complications. The purpose of the present study was to investigate whether high glucose state could induce apoptosis of rat cardiomyocyte cell line H9c2 through microRNA-mediated Bcl-2 signaling pathway. The expression of miR-34a and Bcl-2 mRNA was detected by using real-time PCR. Western blotting was used to examine the changes in apoptosis-associated protein Bcl-2. Apoptosis of H9c2 cells was tested by using flow cytometry. The results showed that the expression of miR-34a was significantly elevated and that of Bcl-2 was strongly reduced, and apoptosis of cardiomyocytes was apparently increased in the high-glucose-treated H9c2 cells as compared with normal-glucose-treated controls. In addition, we identified Bcl-2 gene was the target of miR-34a. miR-34a mimics reduced the expression of Bcl-2 and increased glucose-induced apoptosis, but miR-34a inhibitor acted as the opposite mediator. Our data demonstrate that miR-34a contributes to high glucose-induced decreases in Bcl-2 expression and subsequent cardiomyocyte apoptosis.

Key words: high glucose; Bcl-2; apoptosis; miR-34a

Diabetes mellitus (DM) is one of the most common chronic diseases threatening the health of millions of people worldwide and the cardiovascular complications are the leading cause of morbidity and mortality in diabetic patients. Patients with DM are at the increased risk for developing coronary artery disease (CAD), hypertension, and heart failure (HF), and the majority of these patients succumb ultimately to heart disease. However, despite the importance of heart disease-promoting comorbidities, ventricular dysfunction and a clinical syndrome of HF can develop independent of underlying CAD, a condition termed “diabetic cardiomyopathy”[1]. Diabetic cardiomyopathy is characterized by myocardial left ventricular dysfunction, both diastolic and later systolic functions, and has been well documented in both humans and animals[2–4].

Hyperglycemia, the primary clinical manifestation of DM, as an independent risk factor, directly causes cardiac damage and leads to diabetic cardiomyopathy. Recent studies have demonstrated that high glucose-induced apoptosis of cardiomyocytes is closely related to diabetic complications[5, 6]. However, the mechanisms by which glucose induces apoptosis of cardiomyocytes are not well defined. Therefore, no therapeutic approach is available for preventing glucose-induced cardiomyocyte toxicity.

MicroRNAs (miRNAs) are endogenous 22 nucleotide non-coding RNAs that anneal to partial complementary sequences in the 3′UTRs of target mRNAs of protein-coding genes to specify translational repression and/or mRNA cleavage[7, 8]. Many miRNA species have served as novel biomarkers, modulators, and therapeutic targets for disease, and they may regulate intracellular metabolism[9–12]. There is increasing expression of miR-34a in the serum samples from diabetic patients[13]. However, the role of miR-34a in glucose-induced cardiomyocyte cell death has not been elucidated.

The purpose of this study was to investigate the potential role of miR-34a in the regulation of high glucose-induced H9c2 cardiomyocyte apoptosis and the possible mechanisms.

1 MATERIALS AND METHODS

1.1 Cell Culture and Treatment

Rat H9c2 cardiomyocyte cell line was obtained from the Cell Culture Center of the Chinese Academy of
Medical Sciences (China). The H9c2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, USA) supplemented with 10% fetal calf serum (GIBCO, USA) at 37°C in an incubator with 5% CO₂. The medium was replaced every 2–3 days, and cells were subcultured or subjected to experimental procedures at 80%–90% confluence. Glucose was obtained from Sigma (USA). The cells were serum-starved overnight prior to all of the experiments. All of the experiments were performed after a 48-h incubation with 5.5 mmol/L D-glucose (normal glucose, NG) or 33 mmol/L D-glucose (high glucose, HG), unless otherwise indicated. The experiments were repeated with at least three different cell preparations in triplicate.

1.2 Transient Transfection of miRNA Mimic or miRNA Inhibitor into H9c2 Cells

H9c2 cells were seeded in 24-well plates at a density of 1×10⁵ per well. After serum starvation overnight, 50 nmol/L miR-34a mimic and the indicated amounts of miR-34a inhibitor (RIBOBIO, China) were transiently transfected into H9c2 cells by Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instructions. Forty-eight h after transfection, cells were used for flow-cytometric analysis, real-time PCR or Western blotting.

1.3 Flow-cytometric Analysis for Apoptosis

Fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) were used to identify apoptotic cells. Double staining with FITC-Annexin V and PI was performed according to the manufacturer’s instructions (BoShiDe, China). The H9c2 cells were harvested and washed twice with phosphate buffer solution (PBS) after the experimental procedures. The cells (1×10⁵) were resuspended in binding buffer, and then FITC-Annexin V and PI were added. The mixture was incubated for 15 min in the dark at room temperature. Cellular fluorescence was then measured by bivariate flow cytometry using a FACScan (Becton Dickinson, USA) and analyzed with CellQuest software (Becton Dickinson, USA). This assay discriminates between intact (FITC+/PI-) and early apoptotic (FITC+/PI-) cells, and late apoptotic (FITC-/PI-) cells.

1.4 Total RNAs Extraction and Real-time PCR

Total RNAs were extracted from cells using Trizol (Invitrogen, USA) according to the manufacturer’s instructions. Only the highly pure RNAs (A₂₆₀/A₂₈₀ in the range of 1.80 to 2.20) were used for downstream assays. Reverse-transcribed complementary DNA was synthesized with the PrimeScript RT reagent kit (TaKaRa, Japan). Real-time PCR for Bcl-2 was performed using the following primers: Bcl-2: forward, 5′-GGTGGTGGAGGAACTCTTCATCA-3′; reverse, 5′-ATGGCCGCTTACGTACTCAG-3′. β-actin was chosen as control and its primers were as follows: forward, 5′-CAGGATGAGGGGCCGACTCATC-3′; reverse, 5′-TAAAGACCCTCTATGCGCAACAGT-3′.

Quantitative real-time PCR was used for microRNA. Mature miRNA measurements were performed using the mirVana qRT-PCR miRNA Detection kit (Ambion, USA) according to the manufacturer’s instructions. U6 was chosen as control for small RNAs. Mature miR-34a primers and U6 snRNA were synthesized by GenScript Co, Ltd (China). The primers were as follows: miR-34a (forward, 5′-TGCCTGAGTCAGGCTTACTAGTG-3′; reverse, 5′-CCAGTGGAGGTTTGCGGAATT-3′); U6 ncRNA (forward, 5′-CTCAGTCGGAGAACAATAG-3′; reverse, 5′-AACCTTTACACGTTTGTTCGTC-3′). Amplification and measurement were performed using an ABI 7900 sequence detection system (Applied Biosystems, USA) under the following conditions: denaturation at 94°C for 4 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 25 s.

1.5 Protein Extraction and Western Blotting Analysis

Cells cultured on 24-well plates were lysed in lysis buffer (Beyotime Institute of Biotechnology, China) on ice for 30 min, and the lysates were clarified by centrifugation at 4°C for 20 min at 10 000 r/min. Proteins were quantified using the bicinchoninic acid (BCA) protein assay (Beyotime Institute of Biotechnology, China). After quantitation of protein concentration, the sample (total of 30 μg protein) was subjected to a 12% SDS-PAGE gel by electrophoresis onto polyvinylidene difluoride membranes (Millipore, USA). The membranes were then blocked with Western blocking buffer (Beyotime Institute of Biotechnology, China) for 2 h and incubated for 12 h at 4°C with anti-Bcl-2 (Santa Cruz, USA) or anti-GAPDH (Cell Signal, USA) antibodies at dilutions of 1:700 and 1:600, respectively. After washings with Tris buffered saline with Tween-20 (TBST) thrice, the membranes were incubated with secondary antibody in TBST solution for 60 min at 37°C, then, washed as above. The immunoreactive bands were detected with enhanced chemiluminescence (ECL) Western blotting substrate (Beyotime, China). Band intensities were quantified by densitometry using Quantity One software (Bio-Rad, USA).

1.6 Statistical Analysis

All quantitative data were reported as X ± s, and analyzed using Independent-Samples T test or ANOVA with Turkey’s test. All statistical analyses were performed using SPSS (Version 17.0) software. A P value of <0.05 was considered significant.

2 RESULTS

2.1 Changes in miR-34a and Bcl-2 Expression and Glucose Exposure-induced Apoptosis of H9c2 Cells

H9c2 cells were exposed to 5.5 or 33 mmol/L glucose for 48 h. Treatment with 33 mmol/L glucose resulted in a significant increase in the apoptosis of H9c2 cells as compared with normal levels of glucose (fig. 1A). Quantitative real-time PCR analysis revealed the expression of miR-34a was increased, and real-time PCR analysis showed that of Bcl-2 was decreased in the high glucose-treated H9c2 cells (fig. 1B and 1C). Western blotting analysis also indicated that the Bcl-2 protein expression was reduced in the high glucose-treated H9c2 cells (fig. 1D).

2.2 Expression of Bcl-2 in H9c2 Cells

miR-34a level was measured in H9c2 cells transfected with miR-34a mimic or miR-34a inhibitor. When 50 nmol/L miR-34a mimic was transfected into cells, the level of miR-34a was increased up to 10-fold. In contrast, transfection of anti-miR-34a decreased miR-34a levels by 30%. These data suggested that transfection of