MicroRNA-194 participates in endotoxemia induced myocardial injury via promoting apoptosis

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Abstract. – OBJECTIVE: To investigate the expression level of microRNA-194 in myocardial injury induced by lipopolysaccharide (LPS) and its underlying mechanism.

MATERIALS AND METHODS: LPS-induced H9c2 cardiomyocytes injury model was established. The expression level of microRNA-194 at different treatment time points was detected. Survival and apoptosis of cardiomyocytes were detected after overexpression or knockdown of microRNA-194. The target genes of microRNA-194 were predicted by bioinformatics analysis. The relationship between microRNA-194 and target genes was verified by the dual luciferase reporter analysis and Western blot. The effects of microRNA-194 mimics and overexpression plasmid pcDNA3/SIc7a5 on the cardiomyocyte apoptosis were investigated by MTT assay. Expressions of relative genes involved in Wnt/β-catenin pathway during the process of LPS-induced cardiomyocytes injury were detected by qRT-PCR and Western blot.

RESULTS: The expression level of microRNA-194 was increased in LPS-induced H9c2 cardiomyocytes injury model in a time-dependent manner. Overexpressed microRNA-194 directly bound to the target gene SIc7a5 and inhibited its expression. Transfection of microRNA-194 mimics increased apoptosis of H9c2 cells, which was rescued by overexpression of pcDNA3/SIc7a5. MicroRNA-194 was capable of promoting cardiomyocyte apoptosis by activating Wnt/β-catenin pathway.

CONCLUSIONS: MicroRNA-194 promotes cardiomyocyte apoptosis and participates in myocardial injury induced by endotoxemia via activating Wnt/β-catenin pathway.

Key Words: MicroRNA-194, Cell apoptosis, Endotoxemia, Myocardial injury, Wnt/β-catenin pathway.

Introduction

Endotoxemia is a common systemic inflammatory response, and its potential mechanism is still not clear. A large amount of endotoxins released by Gram-negative bacteria lead to infectious shock, which is an important cause of death of endotoxemia patients³-⁵. Lipopolysaccharide (LPS), as a major component of the cell wall of Gram-negative bacteria, can cause endotoxemia in humans and animals. Specifically, LPS strongly stimulates the inflammatory response through activating and releasing cytokines⁴. Heart is one of the most easily damaged organs attacked by endotoxemia. Previous studies have shown that LPS can induce cardiomyocyte inflammation and apoptosis. Importantly, myocardial injury is also an important factor that leads to the death of endotoxemia patients. Additionally, impaired cardiac function can result in refractory hypotension and infectious shock, even multiple organ dysfunction, and failure⁵-⁶. Therefore, exploration of the molecular mechanism of myocardial injury induced by endotoxemia is of great significance.

MicroRNAs (miRNAs) are a class of non-coding RNA molecules, with 18-25 nucleotides in length. Studies⁷ have shown that miRNAs negatively regulate expressions of target genes at the transcriptional or post-transcriptional level. Several investigations⁸ have pointed out that miRNAs can participate in the regulation of multiple biological processes such as cell proliferation, differentiation, apoptosis, and oxidative stress. In recent years, miRNAs are found to be key regulators of cardiovascular function, which may serve as potential targets for the prevention, diagnosis, and treatment of cardiovascular diseases⁹-¹¹. Some studies have reported that microRNA-194 serves as a predictor of heart failure after acute myocardial infarction, affecting the cardiomyocyte apoptosis¹². It has also been found that microRNA-194 is upregulated in the myocardial tissues of patients with degenerative aortic stenosis¹³. Researches¹⁴-¹⁶ have shown that microRNA-194 pro-
motes apoptosis of tumor cells, thereby inhibiting the development of tumors. The damage of cardiac function induced by endotoxemia is closely related to the inflammation and apoptosis of cardiomyocytes. So far, whether microRNA-194 participates in LPS-induced cardiomyocytes injury is still not clear.

We analyzed the expression and possible molecular mechanism of microRNA-194 by constructing the cardiomyocytes injury model induced by endotoxemia. We provide a basis for the gene therapy of myocardial injury induced by endotoxemia, which lays the foundation for the following in vivo studies.

**Materials and Methods**

**Cell Model Construction**

Rat cardiomyocyte cell line H9c2 was cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were placed in a 5% CO2 incubator at 37°C. H9c2 cells were digested with 0.25% trypsin and passaged with 0.02% EDTA twice a week. Culture medium was replaced 3 times a week. Cardiomyocytes were then treated with LPS (10 μg/mL) in serum-free DMEM for 0, 6, 12, and 24 h, respectively.

**Cell Transfection**

Appropriate amount of cells were inoculated in antibiotic-free medium one day prior to transfection. Cells were transfected according to the instructions of Lipofectamine 2000. The liposomes, microRNA-194 mimics, microRNA-194 inhibitor, pcDNA3/Slc7a5 and negative control (NC) (Gepharma) were diluted with serum-free medium respectively, and were mixed for 5-min incubation at room temperature. The mixture was added to corresponding well and the plate was placed in the incubator for 6 h. The serum-free medium was replaced with complete growth medium for the following incubation.

**Cell Viability Detection**

Survival rate of cardiomyocytes was detected by MTT assay. Cells in each group were seeded in 96-well plates at a cell density of 5×10^4/mL. Two days after cells were stably grown, 20 μL of MTT solution at a dose of 5 g/L were added to each well. 4 h later, the supernatant was discarded and 150 μL of dimethyl sulfoxide were added. The optical value was recorded at the wavelength of 490 nm using a microliter plate reader.

**Cell Apoptosis Detection**

Transfected cells in the logarithmic growth phase were seeded in 6-well plates at a density of 1×10^5/mL. Cells were harvested after culturing for 24 h. Annexin V-FITC (Invitrogen, Carlsbad, CA, USA) was used for cell staining for 15 min. Apoptosis was analyzed by a flow cytometer. Each experiment was repeated for three times and the average value was taken.

**Target Gene Prediction**

Target gene prediction was performed by three databases, including TargetScan (http://www.targetscan.org/) miRTarBase (http://www.mirtarbase.mbc.nctu.edu.tw/php/search.php) and miRDB (www.mirdb.org).

**Dual Luciferase Reporter Analysis**

The reporter plasmids containing the wild-type and/or mutant sequences of the 3’UTR of Slc7a5 were constructed. The 3’UTR sequence of Slc7a5 in the rat’s genome was amplified by PCR. Restriction sites of XbaI and NdeI were added at both ends of the sequence via specific primers. The recombinant plasmids were transformed, identified, and sequenced. The resulting plasmids were named as pGL3-Slc7a5-wild and pGL3-Slc7a5-mut, respectively. Cells were plated in 24-well plates one day prior to the transfection. 500 μL of culture medium, reporter plasmids, control plasmids, and transfection solution were mixed in an Eppendorf (EP) tube for 5-min incubation. MicroRNA-194 mimics and NC were added in the 250 μL of the mixed solution, respectively. After 48 h of transfection, luciferase activity was detected using the Dual Luciferase Reporter Assay System. Average value was taken from 4 replicate wells in each group.

**RT-PCR**

Reverse transcription was performed based on the instruction of miScript II RT Kit. Reverse transcribed cDNAs were diluted at a dose of 3 ng/μL. Real-time PCR (RT-PCR) was carried out with a 10 μL system according to the instruction of miScript SYBR Green PCR Kit. The reaction conditions were as follows: 15 min at 95°C and then 40 cycles of 15 s at 94°C, 30 s at 55°C and 30 s at 72°C. U6 or GAPDH was used as the loading control. The primer sequences were listed in Table I.
Western Blotting

Total protein was extracted by using a cell lysate (RIPA) after cells were transfected for 48 h. Protein samples were separated in a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with the blocking solution for 1 h. Primary antibodies (1:1000) were used for incubation at room temperature for 2 h. After washed with Tris-buffered saline and Tween 20 (TBST), corresponding secondary antibodies (1:5000) were used for incubation for 2 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence. Glyceraldehye 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

Statistical Analysis

Statistical Product and Service Solutions (SPSS 22.0, IBM, Armonk, NY, USA) statistical software was used for data analysis. Measurement data were expressed as mean ± standard deviation. Comparison of measurement data was conducted using t-test. Each experiment was repeated for three times. p<0.05 was considered statistically significant.

Results

MicroRNA-194 Was Overexpressed in LPS-Induced Cardiomyocytes Injury Model

The expression level of microRNA-194 was significantly increased in LPS-induced cardiomyocytes injury model in a time-dependent manner (Figure 1A). Transfection of microRNA-194 mimics remarkably increased the expression level of microRNA-194. Transfection of microRNA-194 inhibitor, however, obtained the opposite result (Figure 1B). Results revealed that cell viability was weakened after microRNA-194 overexpressed, while opposite result was observed after microRNA-194 knockdown (Figure 1C). Meanwhile, the overexpression of microRNA-194 increased cell apoptosis (Figure 1D). The above results demonstrated that microRNA-194 exerts a crucial role in LPS-induced cardiomyocytes injury model.

Prediction and Verification of Target Genes of microRNA-194

Three potential target genes, Slc7a5, Bnip2, and Pitpnm2 were screened from TargetScan, miRTarBase and miRDB databases online. Related functional experiments of the three genes were performed. However, qRT-PCR results indicated that only Slc7a5 was found to be differentially expressed (Figure 2A). Therefore, Slc7a5 was selected for the following analysis. Overexpressed microRNA-194 significantly reduced the mRNA (Figure 2B) and protein (Figure 2C) expression of Slc7a5. Further analysis revealed that the binding site of microRNA-194 and Slc7a5 was highly conserved in vertebrates (Figure 2D). Dual luciferase reporter analysis revealed that microRNA-194 was directly bound to the 3’UTR of Slc7a5 (Figure 2E). The above results suggested that microRNA-194 can directly bind to Slc7a5 and inhibit its expression.

MicroRNA-194 Regulated Cell Viability and Apoptosis Via Slc7a5

To explore whether the effect of microRNA-194 on cardiomyocyte apoptosis was regulated by Slc7a5, the plasmid pcDNA3/Slc7a5 without the 3’UTR region of microRNA-194 was utilized. The results showed that cotransfection of pcDNA3/Slc7a5 and microRNA-194 could effectively rescue the decrease of cell viability and the increase of cell apoptosis induced by overexpression of microRNA-194 (Figure 3).
MicroRNA-194 Activated Wnt/β-Catenin Pathway

Expressions of relative proteins in Wnt/β-catenin pathway in LPS-induced cardiomyocytes injury model were detected. Our results indicated that the mRNA (Figure 4A) and protein (Figure 4B) expressions of β-catenin, cyclin D1 and c-myc in H9c2 cardiomyocytes were significantly increased. Similar results were observed after cells were transfected with microRNA-194 mimics, suggesting that microRNA-194 is involved in the activation of Wnt/β-catenin pathway.

Discussion

Recently, the essential roles of miRNAs in toxemia have gradually been recognized. A number of miRNAs are found abnormally expressed in toxemia including microRNA-223, microRNA-297, microRNA-155, microRNA-146 and microRNA-1-132, which were reported to serve as potential biomarkers for diagnosing toxemia17-19. For example, researches have shown that microRNA-214 is aberrantly expressed in ischemic cardiomyopathy and dilated cardiomyopathy, which exerts multiple biological effects20. Further studies have shown that microRNA-214 plays a protective role in cardiomyocyte apoptosis induced by oxidative stress21. These findings demonstrated that miRNAs may participate in the inflammatory response. The imbalance of miRNAs can affect the release of myocardial inflammatory cytokines and the activation of related signaling pathways, thus leading to myocardial inhibition and damage22.

Previous investigations23,24 have demonstrated that cell model and animal model of endotoxemia are constructed by LPS induction. LPS-induced cardiomyocytes injury model was established to investigate the possible mechanism of microRNA-194 in myocardial injury induced by endotoxemia. MicroRNA-194 has been reported to
be associated with the development of cardiovascular disease. Studies have confirmed that microRNA-194 is significantly upregulated in cardiac tissue of endotoxemic rats, suggesting that differentially expressed miRNAs may be associated with myocardial injury. Overexpressed microRNA-194 in the LPS-induced cardiomyocytes injury model was observed. Besides, decreased cell viability and increased apoptosis were found after cells were transfected with microRNA-194.

**Figure 2.** Prediction and validation of target genes of microRNA-194. A, Expressions of candidate target genes, SLC7A5, BNIP2, PITPNM2, in LPS-induced cardiomyocytes injury model. B, The mRNA expression of SLC7A5 after transfected with microRNA-194 mimics. C, The protein expression of SLC7A5 after transfected with microRNA-194 mimics. D, Conserved analysis of the binding sites of microRNA-194 and SLC7A5 in vertebrates. E, Condition of microRNA-194 binding to 3'UTR regions of wild-type and mutant SLC7A5 after transfection of wild-type and mutant plasmids of SLC7A5, respectively (*p<0.05).

**Figure 3.** MicroRNA-194 regulated cell viability and apoptosis via SLC7A5. Cell viability (A) and apoptosis (B) in the control group, microRNA-194 mimics group, pcDNA3/SLC7A5 group, and microRNA-194 mimics + pcDNA3/SLC7A5 group were detected (*p<0.05).
mimics, while opposite results were obtained after microRNA-194 knockdown. The above results suggested that microRNA-194 is correlated to myocardial injury induced by endotoxemia. Furthermore, bioinformatics analysis predicted that Slc7a5 is a target gene of microRNA-194, which was verified by dual luciferase reporter analysis. Of note, overexpression of Slc7a5 in LPS-induced cardiomyocytes injury model could effectively rescue the decreased viability and increased apoptosis induced by overexpression of microRNA-194.

In addition to the excessive release of inflammatory cytokines, cardiomyocyte apoptosis is also the important pathogenesis of myocardial injury induced by endotoxemia. Myocardial inhibition caused by myocardial apoptosis is the leading cause of death in endotoxemia patients. Wnt/β-catenin pathway is a crucial signaling pathway necessary for the development, differentiation and apoptosis of embryonic tissues. Recently, the function of Wnt/β-catenin pathway in cardiovascular development and diseases has gradually been recognized. Wnt/β-catenin pathway has been demonstrated to exert a crucial role in cardiomyocyte hypertrophy, myocardial injury repair and ventricular remodeling. To explore whether Wnt/β-catenin pathway is involved in LPS-induced apoptosis of H9c2 cardiomyocytes in vitro, expressions of relative genes in Wnt/β-catenin pathway were detected. The results showed that Wnt/β-catenin pathway is activated, indicating that microRNA-194 may be involved in LPS-induced H9c2 cardiomyocytes injury via the Wnt/β-catenin pathway. Taken together, our study revealed that microRNA-194 exerts a significant role in LPS-induced apoptosis of H9c2 cardiomyocytes. Overexpressed microRNA-194 decreased the expression level of Slc7a5 and activated Wnt/β-catenin pathway, thus promoting the cardiomyocyte apoptosis. It was suggested that microRNA-194 may be a significant miRNA involved in the cell apoptosis induced by endotoxemia. We provide a theoretical basis for the prevention and treatment of endotoxemia.

Conclusions

We showed that MicroRNA-194 is overexpressed in the cell model of endotoxemia. It promotes cardiomyocyte apoptosis by inhibiting Slc7a5 and activating Wnt/β-catenin pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

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