microRNA-99a Reduces Lipopolysaccharide-Induced Oxidative Injury by Activating Notch Pathway in H9c2 Cells

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SUMMARY

microRNA-99a (miR-99a) is recently recognized as a key regulator in various cancers and cardiovascular diseases. In the present study, we sought to investigate the effects of miR-99a in rat cardiomyocyte H9c2 cells against oxidative injury induced by lipopolysaccharide (LPS).

MTT assay, reactive oxygen species (ROS) assay, flow cytometry and lactate dehydrogenase (LDH) assay were respectively used to explore viability, ROS levels, apoptosis, and cell death in H9c2 cells. Quantitative PCR (qRT-PCR) was performed to confirm the expression of miR-99a. Western blot was performed to determine the expression of Notch pathway factors.

LPS could significantly suppress viability and increase cell death, apoptosis, and ROS level (P < 0.05). However, miR-99a could significantly increase the viability and decrease apoptosis and ROS level of H9c2 cells (P < 0.05). Overexpression of miR-99a could activate a Notch pathway and regulate the expression of B-cell CLL/lymphoma 2 (BCL2) and cleaved caspase 3.

Our study found that overexpression of miR-99a could attenuate LPS-induced oxidative injury in H9c2 cells, possibly via a Notch pathway. These findings suggest that miR-99a may be a key factor in cardiomyocyte oxidative injury and could be a new therapeutic strategy for cardiovascular diseases. (Int Heart J 2017; 58: 422-427)

Key words: Cell death, Apoptosis, Reactive oxygen species (ROS), Cardiovascular diseases

Cardiovascular diseases, such as myocardial infarction, are considered one of the most important risk factors for disease mortality in modern society. Risk factors are associated with unhealthy lifestyle, which include stress, cholesterol, hypertension, excess consumption of alcohol, and lack of regular physical activity.1,2 The risk of cardiovascular diseases increases with the increased production of reactive oxygen species (ROS) in the organism.3,4 Therefore, oxidative stress increases the damage to the cardiovascular system, which may aggravate myocardial apoptosis, contributing to the pathogenesis of myocardial stunning, infarction, and possibly to the genesis of arrhythmias.5,6

microRNAs (miRNAs), the endogenous non-protein-coding RNAs of about 22 nucleotides, are widely expressed in various tissues and organs of eukaryotes, regulating the degradation and translation of mRNAs by binding to the 3'-untranslated region of target mRNAs.6,7 At present, several studies have shown that miRNA plays important roles in the development and progression of malignant tumors and cardiovascular diseases.6,8 For example, microRNA (miR)-208b and miR-499 in the plasma indicate myocardial damage.9,10 miR-214 has protective effects on myocytes by relieving H2O2-induced injury.11 Therefore, miRNA therapeutics hold great promise in treating cardiovascular diseases.12

Tumor suppressor miR-99a has been reported to be frequently deregulated in several types of human cancers.13,14 It inhibits cancer cell proliferation and migration and promotes apoptosis through regulating its mRNA targets.15,16 However, in a model of myocardial infarction, miR-99a has been revealed to inhibit cardiomyocyte apoptosis, suggesting a cardioprotective role for miR-99a.17 These studies lead us to speculate about the role of miR-99a in oxidative injury during cardiovascular disease, however, little has been reported in previous studies.

In the present study, we sought to investigate the effects of miR-99a on oxidative injury of cardiovascular diseases in rat cardiomyocyte H9c2 cells. Oxidative injury was induced by lipopolysaccharide (LPS), and an miR-99a mimic or inhibitor was transfected. MTT assay, lactate dehydrogenase (LDH) release, apoptosis assay, and ROS detection were performed to...
assess changes in cellular oxidative injury. Factors of the Notch signaling and apoptosis were also detected to reveal the potential mechanism of miR-99a. This study will provide fundamental information and potential therapeutic strategies for oxidative injury during cardiovascular diseases.

METHODS

Cells and LPS treatment: The rat cardiomyocyte-derived cell line H9c2 was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Sijiqing, Hangzhou, China). The cells were treated in serum-free medium supplemented with 10 μg/mL LPS (Sigma-Aldrich, Boston, MA, USA) for 18 hours. Untreated cells served as the control group.

Plasmid construction and transfection: Mature miR-99a mimic (5'-AACCC AUAGA UCUGU UUG UG-3'), inhibitor (5'-CACCAAU AUUU CCGG UU-3') and the negative control (NC) were designed and synthesized by GenePharma (Shanghai, China). Cell transfection was performed using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol. At 48 hours post transfection, the cells were collected for further analysis.

MTT assay: Cell viability was measured by MTT assay using an MTT Cell Proliferation Assay Kit (ATCC, Manassas, VA, USA) according to the manufacturer’s protocol after the cells were treated and transfected. The MTT assay was added to each well of 96-well plates, and the cells were cultured for 4 hours, and then washed twice in phosphate-buffered saline (PBS). Formazan solution was then added and the cells were incubated with gentle shaking until the particles had dissolved. Optical density (OD) was measured at 570 nm.

ROS assay: ROS was determined by flow cytometry using 2,7-dichlorofluorescein diacetate (DCFH-DA) (Jiancheng, Nanjing, China). The cells were seeded into a 6-well plate after treatment, washed twice with PBS, and incubated in serum-free culture medium containing 10 μM DCFH-DA for 20 minutes at 37°C in the dark. The cells were then washed with PBS and digested by trypsin (Gibco). All samples were centrifuged and the supernatant was removed. The cells were resuspended to 500 μL PBS and the fluorescent intensities were measured using a flow cytometer (488 nm excitation, 521 nm emission, Beckman Coulter, Brea, CA, USA).

Apoptosis assay: Flow cytometry analysis was performed to quantify the apoptotic cells by using an Annexin V-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) Apoptosis Detection Kit (Biosea Biotechnology, Beijing, China). The cells (1 x 10^6 cells/well) were seeded in 6 well-plates, washed twice with cold PBS, and resuspended. The adherent and floating cells were combined and treated according to the manufacturer’s protocol and measured with a flow cytometer (Beckman Coulter) to identify the apoptotic cells (FITC-positive and PI-negative).

LDH assay: LDH assay was performed to assess cell death after LPS treatment and transfection. Cells were cultured in fresh medium and treated according to the manufacturer’s protocol for an LDH Cytotoxicity Assay Kit (Beyotime, Shanghai, China). The culture medium was collected to detect LDH release. OD was measured at 490 nm.

Quantitative PCR (qRT-PCR): Total RNA was isolated from transfected cells by TRizol reagent (Invitrogen) and treated with DNase I (Promega, Madison, WI, USA). Reverse transcription was performed using a One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Shiga, Japan). miR-99a was quantified by qRT-PCR on a LightCycler 480 (Roche, Basel, Switzerland) using the forward primer 5'-ACAGT CGAGA TGGGA TACCCT ACCCC TTCGG CAGCA CA-3' and reverse primer 5'-CTCGT GAGGT CGAGT GGCCG A-3'. Data were calculated by the 2^(-ΔΔCt) method normalized by U6 (forward primer 5'-CTCCG TTCCG CAGCA CA-3' and reverse primer 5'-AAGGC TTCAC GAATT TGCGT-3').

Western blot: The cell protein was separated using RIPA Lysis Buffer (Beyotime) supplemented with protease inhibitors (Roche). The proteins were quantified using a BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). GAPDH antibody was purchased from Sigma; Rabbit-anti-mouse primary antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). After the protein samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes, they were blocked with 5% skim milk and then incubated overnight in primary antibody (1:1000) at 4°C. The membranes were washed in PBS and incubated in horseradish peroxidase-conjugated secondary antibody (1:2000, Santa Cruz) for 1 hour at room temperature. Immobilon Western Chemiluminescent HRP Substrate (Millipore, Boston, MA, USA) was added to develop the signals, which were analyzed using Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

Statistical analysis: All experiments were repeated three times. The results of multiple experiments are presented as the mean ± SD. Statistical analyses were performed using SPSS 19.0 statistical software (IBM, New York, NY, USA). P values were calculated by one-way analysis of variance (ANOVA). P < 0.05 was considered to indicate a statistically significant difference.

RESULTS

LPS induces oxidative injury in H9c2 cells: Research has shown that LPS may lead to oxidative injury in cells, so we estimated LPS-induced changes in cell viability, cell death, and ROS level in H9c2 cells. Treatment of the cells with LPS for 18 hours significantly decreased cell viability when detection was performed at 2 and 3 days after treatment (P < 0.05 and P < 0.01, Figure 1A). LDH release was markedly elevated by LPS (P < 0.001, Figure 1B), suggesting increased cell death. In addition, LPS could significantly increase the percent of apoptotic cells (FITC-positive and PI-negative, Figure 1C), with significant difference between groups (P < 0.05, Figure 1D). It also elevated ROS level (P < 0.05, Figure 1E). These results suggested that LPS induced oxidative injury in H9c2 cells.

miR-99a attenuates LPS-induced oxidative injury in H9c2 cells: miR-99a level was altered in H9c2 cells by cell transfection and then verified by qRT-PCR (Figure 2A). The results showed that the expression level of miR-99a was significantly increased by mimic compared with that of the control group (P < 0.05). On the contrary, transfection of miR-99a inhibitor significantly decreased the miR-99a expression level compared...
with the control group ($P < 0.05$). The successful transfection was further verified by Western blot detecting targets of miR-99a (Figure 2B): insulin like growth factor 1 receptor (IGF1R) and mechanistic target of rapamycin (mTOR), whose protein levels can be inhibited by miR-99a according to previous reports.\textsuperscript{20,21} Consistently, miR-99a mimic suppressed and its inhibitor promoted IGF1R and mTOR protein levels, suggesting that miR-99a level was effectively changed in H9c2 cells, which could be used in further experiments.

As shown in Figure 3A, cell viability was significantly decreased by miR-99a inhibitor ($P < 0.05$), while no significant change was induced by miRNA-99a mimic ($P > 0.05$). After LPS treatment, however, miRNA-99a mimic significantly increased cell viability ($P < 0.05$), while si-miRNA-99a did not have an obvious effect ($P > 0.05$). miR-99a mimic suppressed LDH release from H9c2 cells with or without LPS treatment ($P < 0.05$ and $P < 0.001$, Figure 3B), and miR-99a inhibitor increased LDH release in both cases ($P < 0.001$), suggesting that miR-99a might inhibit H9c2 cell death. Cell apoptosis exhibited similar changing patterns (Figure 3C): miR-99a

![Figure 1](image1.jpg)

**Figure 1.** Lipopolysaccharide (LPS) successfully induces oxidative injury in rat cardiomyocyte H9c2 cells. H9c2 cells were treated with LPS for 18 hours. **A:** Cell viability determined by the MTT assay at 0, 1, 2 and 3 days post LPS treatment. **B:** Lactate dehydrogenase (LDH) release reflecting cell death. **C:** Cell apoptosis determined by flow cytometry after fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining. **D:** Percent of apoptotic cells (FITC-positive and PI-negative) based on flow cytometry results. **E:** Level of reactive oxygen species (ROS). Error bars indicate mean ± standard deviation. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.**
miR-99a regulates Notch pathway and apoptotic factors: A number of studies have reported the participation of a Notch pathway in regulating cell oxidative injury and apoptosis, so this study detected factors in this pathway to explore the regulatory mechanism of miR-99a in H9c2 cell oxidative injury. Both Notch1 and Notch2, as well as their activated forms, were promoted by miR-99a mimic and suppressed by miR-99a inhibitor. Jagged 1 (JAG1) showed the same trend with Notch1 and Notch2. Apoptotic factors B-cell CLL/lymphoma 2 (BCL2) and caspases 3 were also detected. BCL2 was suppressed by LPS treatment, but miR-99a mimic induced BCL2 and miR-99a inhibitor had the opposite effects. Although caspase 3 level did not change markedly, the cleaved caspase 3 was induced by LPS treatment, and suppressed by miR-99a mimic. Taken together, miR-99a might activate a Notch pathway and regulate key apoptotic factors in H9c2 cells.
miR-99a regulates the Notch pathway and apoptotic factors in rat cardiomyocyte H9c2 cells. After treatment with lipopolysaccharide (LPS) for 18 hours, H9c2 cells were transfected with miR-99a mimic or miR-99a inhibitor and the negative control (NC). At 48 hours post transfection, protein levels of Notch1/2, activated Notch1/2, jagged 1 (JAG1), B-cell CLL/lymphoma 2 (BCL2), caspase 3 and cleaved caspase 3 were determined by Western blot analysis.

Discussion

Cardiovascular diseases represent the main cause of human morbidity and mortality. The common risk factors for developing atherosclerotic cardiovascular disease, namely hypertension, hyperlipidemia, diabetes mellitus, tobacco use, and family history, are associated with a pathophysiological vascular phenotype. Increasing evidence suggests that oxidative stress, which results from an excessive generation of ROS, has a key role in the pathogenesis of cardiovascular diseases. Moreover, several studies have demonstrated that miRNAs regulate posttranscriptional gene expression and control cardiac contractility, regeneration, fibrosis, and hypertrophy. Therefore, we sought to investigate the effects of miR-99a in H9c2 cells against oxidative injury induced by LPS.

It has been reported that ROS is generated at an accelerated level in the post-ischemic myocardium. ROS may induce cell apoptosis since it functions as signal transduction intermediates to induce transcription factor activation and gene expression. In the present study, we demonstrated for the first time that miR-99a played a critical role in LPS-induced cardiomyocyte oxidative injury. It effectively promoted LPS-suppressed cell viability, and suppressed LPS-induced cell apoptosis and ROS level, which reflected the attenuated oxidative injury in H9c2 cells. These findings are consistent with a former study on the protective role of miR-99 against cardiomyocyte apoptosis. Thus, miR-99a has protective effects against cardiomyocyte oxidative injury.

This study also analyzed the potential mechanism of miR-99a in regulating oxidative injury by detecting the Notch pathway. In mammals, the Notch pathway consists of receptors Notch 1 to Notch 4 and multiple ligands including JAG1. It has been demonstrated that the Notch pathway plays a critical role in suppressing cell apoptosis, and regulating cardiovascular development and vascular diseases. In the present study, we found that overexpression of miR-99a activated both Notch1 and Notch2, and promoted JAG1 expression, suggesting the activated Notch pathway. Existing studies have reported that the Notch pathway facilitates the control of ROS and protects cardiomyocytes and hepatocytes. Thus, the results of this study support that regulation of the Notch pathway may be one potential mechanism of miR-99a in attenuating cardiomyocyte oxidative injury.

Moreover, overexpression of miR-99a could promote the expression of BCL2 and suppress activation of caspase 3, both of which are key regulators of the apoptotic cascade. BCL2 constitutes a critical control point in apoptosis, controlling the release of cytochrome c from mitochondria. Overexpression of BCL2 prevents apoptosis, while activated caspase 3 promotes apoptosis of cardiomyocytes. Some miRNAs could have an impact on these apoptotic factors to regulate cell apoptosis. In our study, miR-99a was capable of promoting BCL2 and suppressing the activation of caspase 3, supporting that miR-99a inhibits cell apoptosis in LPS-treated H9c2 cells. This result also implies that miR-99a regulates these apoptotic factors, and that more detailed mechanisms may be uncovered in further research.

Conclusion: In conclusion, this study reports the protective role of miR-99a against LPS-induced oxidative injury in H9c2 cells. miR-99a activates the Notch pathway, promotes the expression of BCL2, and inhibits the activation of caspase 3, which may one of the potential mechanisms of its functions. All of these findings suggest that miR-99a may be a new therapeutic strategy to attenuate oxidative injury in cardiovascular diseases.

References


