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miR-93-3p alleviates lipopolysaccharide-induced inflammation and apoptosis in H9c2 cardiomyocytes by inhibiting toll-like receptor 4

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ABSTRACT

Background: miR-93 is recently recognized to perform anti-inflammatory action in the pathological process of cardiomyocytes dysfunction. However, it remains unclear whether miR-93-3p involves in lipopolysaccharide (LPS)-induced inflammation and apoptosis in H9c2 cells. The present study aimed to investigate the functions of miR-93-3p and its target, toll-like receptor 4 (TLR4), in LPS-stimulated cardiomyocytes.

Material and methods: Cell viability was analyzed by CCK-8 assay. AnnexinV-FITC/PI staining and lactate dehydrogenase (LDH) assay were used to evaluate the cell death. The mRNA and protein levels were assayed by RT-qPCR and western blotting, respectively. The targeted gene was predicted by a bioinformatics algorithm and confirmed by a dual luciferase reporter assay.

Results: LDH stimulation resulted in the suppression of cell viability and the increase in apoptosis rate, inflammatory cytokines and LDH levels, while inhibition of TLR4 with TAK-242 or overexpression of miR-93-3p dramatically blocked LPS-induced inflammation and apoptosis in cardiomyocytes. Intriguingly, bioinformatics analysis and experimental data suggested that TLR4 was a direct target of miR-93-3p, which could inhibit TLR4 expression by transfected with miR-93-3p mimics or elevate the expression of TLR4 by transfected with miR-93-3p inhibitors. Overexpression of TLR4 carried out an opposite effect to miR-93-3p and positively regulated LPS-induced inflammation and apoptosis in cardiomyocytes.

Conclusion: miR-93-3p showed the protective effects against LPS-induced inflammation and apoptosis in cardiomyocytes by inhibiting TLR4 expression.

1. Introduction

Cardiovascular diseases (CVDs), such as dilated cardiomyopathy, myocardial infarction, cardiac hypertrophy and heart failure, are now recognized that they may be associated with over-activation of inflammatory response and apoptosis in cardiomyocytes [4,5,21]. Previous studies have found that many detrimental stimuli, including palmitic acid, high glucose, oxidized low-density lipoprotein and lipopolysaccharide (LPS), can trigger inflammation and apoptosis in cardiomyocytes, which contribute to the pathogenesis of CVDs [6,17,22,23,31].

MicroRNA (miRNA) as a class of noncoding RNA is characterized by short and single-stranded RNA (18–25 nucleotides) and have recently emerged as a novel class of post-transcriptional regulators in a variety of biological processes by binding to the 3′-untranslated regions (3′-UTRs) of its target gene, thus inhibiting gene expression [9,10]. Numerous miRNAs have been reported in physiological and pathological processes of the heart [3,28]. In the process of LPS-induced cardiomyocyte apoptosis and inflammatory response, miR-29b and miR-155 are up-regulated [29,37], and miR-99a, miR-145, miR-181b and miR-499 are down-regulated [7,14,15,26]. However, the possible mechanism of miR-93-3p in LPS-induced cardiomyocyte apoptosis and inflammation is unknown.

miRNAs are believed to regulate multiple target messenger RNAs (mRNAs) in different pathologic conditions, however, one gene may be regulated by more than one miRNA [14]. Previous studies have shown that miR-93-3p regulates apoptosis and inflammation through multi-gene targeting, including interleukin-1 receptor-associated kinase 4

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(IRAK4), signal transducer and activator of transcription 3 (STAT3), B-cell lymphoma-2 (Bcl-2) and cyclin E1 [25,34,35]. TLR4 as a direct target of miR-93 contributes to myocardial infarction-associated transcript (MIAT)-regulated cardiac hypertrophy [18]. TLR4 is one receptor for LPS and plays a crucial role in the initiation of inflammatory response by leading to the activation of nuclear factor-kappa B (NF-κB), which accelerates the production of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and IL-6 [24,27]. These inflammatory mediators contribute to the dysfunction, apoptosis and necrosis of cardiomyocytes [13]. However, the association between miR-93-3p and TLR4 in LPS-induced inflammation and apoptosis in H9C2 cardiomyocytes has not been completely clarified. In the present study, a post-transcriptional regulatory mechanism by miR-93-3p was found to regulate LPS-induced inflammation and apoptosis in cardiomyocytes by targeting inhibition of TLR4. miR-93-3p/TLR4 axis highlights a novel mechanism that contributes to the cardioprotective effects in inflammation or apoptosis-induced myocardial damage.

2. Material and methods

2.1. Cell culture

H9c2 cells were purchased from the American Type Culture Collection (ATCC, Bethesda, MD, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), containing 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.), 10% L-glutamine, 0.5% penicillin/streptomycin, with 5% CO2 atmosphere at 37 °C. TAK-242 (an inhibitor of TLR4) and LPS were obtained from Invivogen (San Diego, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

2.2. LDH assay

H9c2 cells were seeded in 96-well plates (1×10^4 cells) and exposed to LPS (10 μg/mL) for 24 h. The plates were centrifuged at 1000*g* for 4 min. The LDH released from the damaged cells and total LDH was subjected to LDH kit (Beyotime Biotechnology, Haimen, China).

2.3. Inflammatory cytokine

Cellular supernatant levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 were detected using the bioactive ELISA assay (Elabscience Biotechnology Co., Ltd., Wuhan, China) with a SpectraMax M5 ELISA plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA), according to the manufacturer's protocol.

2.4. Apoptosis assay

H9c2 cells were incubated with different conditions for 24 h. AnnexinV-FITC/PI kit (Becton, Dickinson and Company, New Jersey, USA) was used to stain cells for 15 min, and then cell apoptosis assay was performed by flow cytometry assay (FACScan, BD Biosciences, San Jose, CA, USA) and analyzed by CELL Quest 3.0 software (BD Biosciences).

2.5. Cell transfection and plasmid constructs

miR-Con, miR-93-3p mimics, scramble and miR-93-3p inhibitors were synthesized by RiboBio (Guangzhou, China) and transfected into H9c2 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientifc, Inc., Waltham, MA, USA), according to the manufacturer's protocol.

A mammalian expression plasmid (pReceiver-M02-ERBB3; GeneCopoeia, Germantown, MD, USA) designed to express the full-length open reading frame (ORF) of rat TLR4 without miR-93-3p responsive 3′-UTR. An empty plasmid was served as a negative control. Overexpressed TLR4 plasmid (vector-TLR4) and control (vector-Con) were transfected into H9c2 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientifc, Inc., Waltham, MA, USA), according to the manufacturer's protocol.

2.6. Luciferase reporter assay

The sequence of miR-93-3p was obtained using online predict software and synthesized by RiboBio (Guangzhou, China). The wild-type (WT) or mutant-type (MUT) 3′-UTR of TLR4 was inserted into the multiple cloning sites of the luciferase expressing pMIR-REPORT vector (Ambion; Thermo Fisher Scientific, Inc.). For the luciferase assay, H9c2 cells (1×10^5) were seeded into 24-wells and co-transfected with luciferase reporter vectors containing the WT or MUT 3′-UTR of TLR4 (0.5 μg) combined with miR-Con or miR-93-3p mimics (100 nM) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The luciferase activity was measured using a luciferase reporter assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

2.7. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. miR-93 was detected using TaqMan® MicroRNA assay (Applied Biosystems, Foster City, USA) followed by manufacturer's instructions. U6 snRNA was used as an endogenous control. cDNA was synthesized by reverse transcription reactions with 2 μg total RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). RT-qPCR was performed by Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific, Inc.) with the TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Inc.). The relative expression levels of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method [20] and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers were used as follows: TLR4: forward 5′-AAGTTATTGTGGTGGTGTCTAG-3′ and reverse 5′-GAGGTA GGTGTTTCTGCTAAG-3′; TNF-α: 5′-AAGCCCGTAGCCCACGTCGTA-3′ and reverse 5′-GCCCGCAATCCAGGCCACTAC-3′; IL-1β: forward 5′-GACCTGGGCTGTCCTGATGA-3′ and reverse 5′-GTGCTGCTGCGAGA TTTGAA-3′; IL-6: forward 5′-TTCCATCCAGTTGCCTTCTTG-3′ and reverse 5′-GAAGGCCGTGGTTGTCACC-3′; GAPDH: forward 5′-GCACCGT CAAGCTGAGAAC-3′ and reverse 5′-TGGTGAAGACGCCAGTGGA-3′.

2.8. Western blotting

Proteins were extracted with radio immunoprecipitation assay (RIPA) buffer (Cat.No: P0013B; Beyotime Institute of Biotechnology, Haimen, China) and blotted as previously described [31]. The primary antibodies of TLR4 (dilution: 1:1000; Abcam, Cambridge, UK) and NF-κB/p65 (dilution: 1: 500; Cell Signaling Technology, Inc., USA) were used to incubate the membranes. Subsequently, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (cat.no: sc-516102; dilution: 1:10,000; Santa Cruz Biotechnology) at room temperature for 2 h and visualized by chemiluminescence (Thermo Fisher Scientific, Inc.). Signals were analyzed with Quantity One® software version 4.5 (Bio Rad Laboratories, Inc., Hercules, CA, USA). β-actin (cat. no. sc-130065; 1: 2000; Santa Cruz Biotechnology) was used to as the control antibody.

2.9. Statistical analysis

Data were presented as mean ± SD. Statistical analysis was performed using IBM SPSS Statistics Version 19.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism Version 7.0 (GraphPad Software, Inc., La