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Review Article

Rosuvastatin reduces myocardial ischemia-reperfusion injury by inhibiting miR-155

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Abstract: Background: Rosuvastatin has been reported to protect against myocardial ischemia-reperfusion injury in cardiovascular diseases. MicroRNAs (miRNAs) are widely involved in progression of myocardial ischemia-reperfusion injury. However, the underlying mechanism of rosuvastatin and miRNAs in the myocardial ischemia-reperfusion injury has not been fully explored. Methods: The model of myocardial ischemia-reperfusion injury was established using cardiomyocytes by serum and oxygen deficiency and recovery. The expression of miR-155 was calculated by qRT-PCR. Cell survival and apoptosis were observed by 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2Htetrazolium bromide assay or flow cytometry. Lactate dehydrogenase (LDH), creatine kinase (CK), malondialdehyde (MDA) and superoxide dismutase (SOD) were measured by different assay kits. Results: Cell survival rate and SOD level were inhibited, while apoptosis and the levels of LDH, CK and MAD were enhanced in cardiomyocytes under ischemia-reperfusion compared with those in control group. However, these effects were attenuated by introduction of rosuvastatin. In addition, we found that miR-155 expression was up-regulated in ischemia-reperfusion-treated cardiomyocytes and knockdown of miR-155 alleviated ischemia-reperfusion injury in cardiomyocytes. Moreover, miR-155 level was inhibited by different concentrations of rosuvastatin in ischemia-reperfusion-treated cardiomyocytes. Conclusion: Rosuvastatin reduced ischemia-reperfusion injury in cardiomyocytes by inhibiting miR-155 expression, providing a new point for the myocardial ischemia-reperfusion injury treatment.

Keywords: Rosuvastatin, myocardial, ischemia-reperfusion injury, miR-155

Introduction

Ischemia-reperfusion injury is a tissue damage caused by blood returning to tissue after ischemia or hypoxia [1]. Ischemia-reperfusion injury is a very common occurrence which may be caused by acute mesenteric ischemia, shock, burns, surgery, trauma, etc., which could lead to multi-organic failure and even death. In coronary heart disease patients, although restoration of blood flow to the ischemic heart is the most effective treatment to rescue the cardiac cells and save patients, the reperfusion may also lead to various degrees of myocardial injury which is defined as myocardial ischemia-reperfusion injury. But the underlying molecular mechanism in myocardial ischemia-reperfusion injury is not fully clarified. Therefore, understanding the pathogenesis will shed light on avoiding myocardial ischemia-reperfusion injury in the cardiovascular disease treatment.

Statins are a class of lipid-lowering medications which serve as 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors and ameliorate cardiovascular disease effectively [2]. Rosuvastatin is a member of statins that is primarily used to treat high cholesterol and prevent cardiovascular disease [3, 4]. Previous studies showed that rosuvastatin could alleviate myocardial ischemia-reperfusion injury in the treatment of cardiovascular disease and was associated with inflammatory, immune regulation and preservation of nitric oxide in myocardial ischemia-reperfusion injury [5-8].

MicroRNAs (miRNAs) are endogenous, small non-coding single-stranded RNAs with 18-22 nucleotides in length that regulate mRNA expressions mainly through translational inhibition [9, 10]. Accumulating evidence showed that miRNAs were involved in angiogenesis, inflammation and cellular progression including

cell proliferation, migration, invasion and apoptosis in many diseases [11-15]. Moreover, large number of works declared that many miRNAs are expressed during myocardial ischemia-reperfusion injury, suggesting that miRNAs, such as miR-125a, miR-139, miR-324 and miR-155, may play important roles in myocardial ischemia-reperfusion injury [16, 17]. In this study, we found that the expression of miR-155 was markedly decreased in myocardial ischemia-reperfusion injury by treatment of rosuvastatin. Thus, we speculated that the protective effect of rosuvastatin on myocardial ischemia-reperfusion injury may be related to miR-155.

Materials and methods

Animals and preparation of drug-containing serum

The experiments were approved by the animal care and use committee of Chengde Medical University. Male Sprague Dawley rats with body weight of 250 ± 50 g were purchased from the Model Animal Research Center of Nanjing University. Rats were randomly divided into negative control group, low, medium and high dose of rosuvastatin groups (six rats per group). Rats in the low, medium and high doses of rosuvastatin groups were injected with 2.5 mg/kg, 5 mg/kg, or 10 mg/kg rosuvastatin physiological saline solution through the tail vein, respectively. Rats in negative control group were injected with the same amount of normal saline. After injected for 1~1.5 h, blood was drawn through the femoral artery and serum was obtained at room temperature, centrifuged, sterilized, and inactivated at 56°C for 30 min.

Cell culture and myocardial ischemia-reperfusion injury model

Cryopreserved cardiomyocytes (H9c2) were purchased from ATCC (Manassas, VA, USA). Cardiomyocytes were seeded into DMEM (Invitrogen, Carlsbad, CA, USA) with 10% FBS, and incubated at 37°C with 5% CO_2. For the establishment of myocardial ischemia-reperfusion injury model in vitro, when cell confluence reached up to 80%~90%, cell medium was replaced with DMEM without serum, and incubated for 10 h in an incubator with conditions of 95% N_2 and 5% CO_2 at 37°C. Subsequently, cells were cultured in DMEM containing 10% FBS for 2 h at 37°C with 95% air and 5% CO_2 again. For the control group, the cardiomyocytes were cultured in DMEM with 10% serum under normal conditions. To analyze the potential role of rosuvastatin, cells were incubated with drug-containing serum described above at 37°C with 5% CO_2.

Cell transfection

miR-155 mimic (miR-155), miR-155 inhibitor (anti-miR-155) and their negative control (NC and anti-NC) were purchased from GenePharma (Shanghai, China). The oligos were transfected into cardiomyocytes using Lipofectamine 3000 (ThermoFisher Scientific, L3000015, USA) when the confluence reached up to 70-80% according to the manufacturer's protocol.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol (Invitrogen), and total RNA (1 μg) was transcribed into cDNA using TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and a stem-loop RT primer (Applied Biosystems) according to the manufacturer's protocol. miR-155 expression was normalized to U6 snRNA level. qRT-PCR was performed using SYBR® Green (Promega, Madison, WI, USA) on ABI 7300 System (Applied Biosystems) for 40 cycles. The $2^{-\Delta\Delta Ct}$ method was utilized to calculate the relative expression of miR-155. The primer of miR-155: forward, 5'-ACTAGCACTCACATGGAACAAATGG-3'and reverse 5'-CCAGGTTATGACTAGCACATTAAATGATAG-3'; The primer of U6: forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'.

Measurement of LDH, CK, SOD and MAD

Levels of the lactate dehydrogenase (LDH), creatine kinase (CK), malondialdehyde (MDA) and superoxide dismutase (SOD) in cells were measured using LDH Assay kit, CK Assay kit, MDA Assay kit and SOD Assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions.

Cell survival assay

Cell survival rate was detected by 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2Htetrazolium bromide (MTT) assay. 5×10^3 cells were seeded into 96-well plates and incubated for 48 h at 37°C with 5% CO_2, followed by continued incubation with 10 μl 5 mg/ml MTT phos-

phate buffers for 4 h at 37°C. After discarding the supernatant, 150 ul DMSO was added to each well to fully dissolve the crystals. The absorbance was measured at 450 nm using a microplate absorbance reader (Tecan, Safire II, Switzerland).

Cell apoptosis assay

Cell apoptotic rate was monitored by flow cytometry. After culture for 48 h, cells were collected and resuspended in binding buffer (200 μl), and then stained with Annexin V-FITC (10 μl, BD Pharmingen, San Diego, CA, USA) and propidium iodide (10 μl, BD Pharmingen) for 15 min at room temperature without light. Cell apoptotic rate was detected by flow cytometer (BD Biosciences, San Jose, CA, USA). The experiment was replicated three times and the average value was taken.

Statistical analysis

The data was presented as mean ± S.D. (standard deviation) from three repeated experiments. The analysis of results was shown and plotted using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). All group comparisons were carried out using the Student t-test or one way ANOVA followed by Dunnett's test. The *p* values less than 0.05 was regarded as statistically significant.

Results

Rosuvastatin protected cardiomyocytes from ischemia-reperfusion -induced inhibition of survival and increase of apoptosis

To establish myocardial ischemia-reperfusion injury model, cardiomyocytes were suffered from the insult of ischemia-reperfusion. Compared with control group, ischemia-reperfusion group displayed obvious loss of survival and enhance of apoptosis in cardiomyocytes, suggesting the successful establishment of the model (**Figure 1A** and **1B**). Moreover, to explore the effect of rosuvastatin on ischemia-reperfusion injury, we used three doses of rosuvastatin, 2.5 mg/kg (low), 5 mg/kg (medium) and 10 mg/kg (high) to treat cardiomyocytes respectively. The results showed that with the increase of rosuvastatin dose, the cell survival rate was gradually enhanced in ischemia-reperfusion-treated cardiomyocytes, while the apoptosis rate was gradually decreased. More than that, medium and high doses of rosuvastatin significantly weakened the effect of ischemia-reperfusion on cardiomyocytes survival and apoptosis. However, low dose of rosuvastatin had no significant effect on cell survival and apoptosis.

Rosuvastatin abated effect of ischemia-reperfusion on LDH, CK, SOD and MDA level in cardiomyocytes

To further explore the protective role of rosuvastatin in cardiomyocytes under ischemia-reperfusion, we detected the releases of LDH, CK, MDA and SOD in the media. As shown in **Figure 2A**, **2B** and **2D**, the levels of LDH, CK and MDA were obviously increased in cardiomyocytes after treatment of ischemia-reperfusion compared with those in control group. However, ischemia-reperfusion treatment sharply lowered the level of SOD in cardiomyocytes (**Figure 2C**). Interestingly, after different doses of rosuvastatin treatment, the levels of LDH, CK, SOD and MDA were notably reversed in ischemia-reperfusion-treated cardiomyocytes at medium and high doses of rosuvastatin (**Figure 2A-D**). Low dose of rosuvastatin showed little effect on the levels of LAH, CK, MAD and SOD in cardiomyocytes.

Knockdown of miR-155 decreased ischemia-reperfusion injury in cardiomyocytes

As shown in **Figure 3A**, we found that miR-155 expression was significantly elevated in cardiomyocytes after treatment of ischemia-reperfusion, thus we hypothesized that miR-155 was associated with myocardial ischemia-reperfusion injury. To investigate the function of miR-155 in ischemia-reperfusion-treated cardiomyocytes, cells were transfected with anti-miR-155 or anti-NC. The analysis of transfection efficacy validated that the abundance of miR-155 was effectively reduced in ischemia-reperfusion-treated cardiomyocytes by transfection of anti-miR-155 compared with that in anti-NC group (**Figure 3B**). Then, the results of MTT assay showed that anti-miR-155 transfection obviously improved survival of cardiomyocytes (**Figure 3C**). In addition, down-regulation of miR-155 reduced apoptosis in ischemia-reperfusion-treated cardiomyocytes (**Figure 3D**). More than that, the levels of LDH, CK and MDA was decreased by knockdown of miR-155 (**Figure**

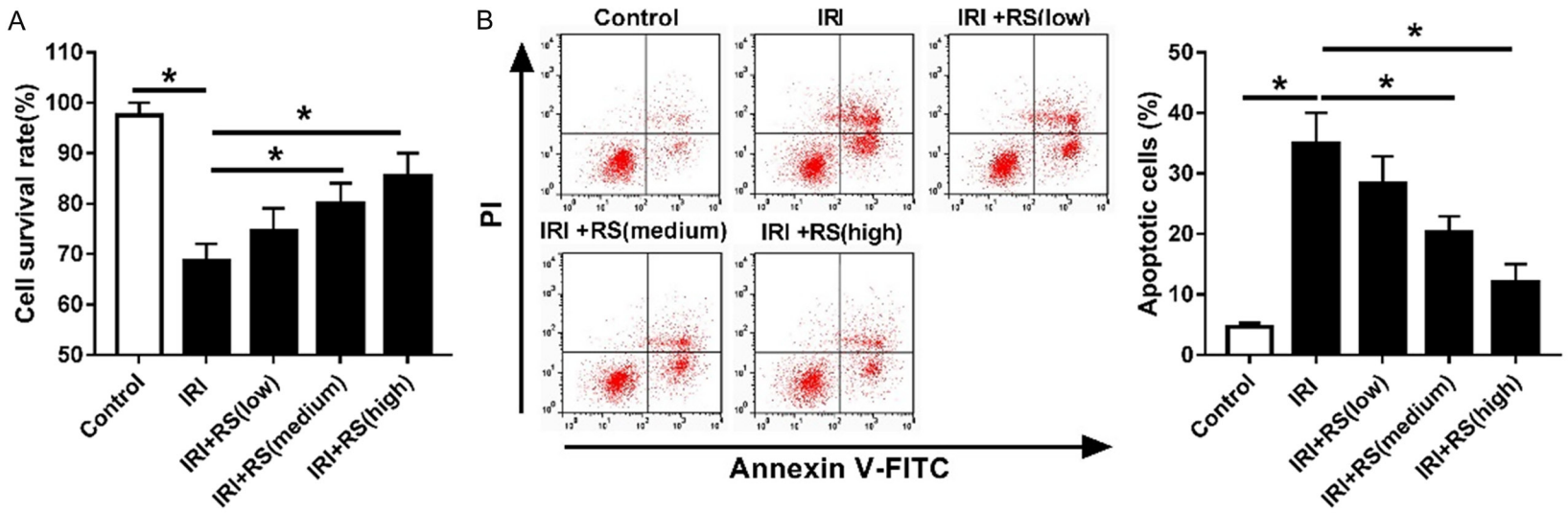

Figure 1. Rosuvastatin attenuated ischemia-reperfusion-induced survival suppression and apoptosis production in cardiomyocytes. cardiomyocytes were treated with ischemia-reperfusion and then incubated with different concentrations of rosuvastatin. Cell survival (A) and apoptosis (B) were detected using MTT assay or flow cytometry in control, IRI, IRI+RS (low), IRI+RS (medium) and IRI+RS (high) groups. IRI: ischemia-reperfusion injury; RS: rosuvastatin. *P<0.05.

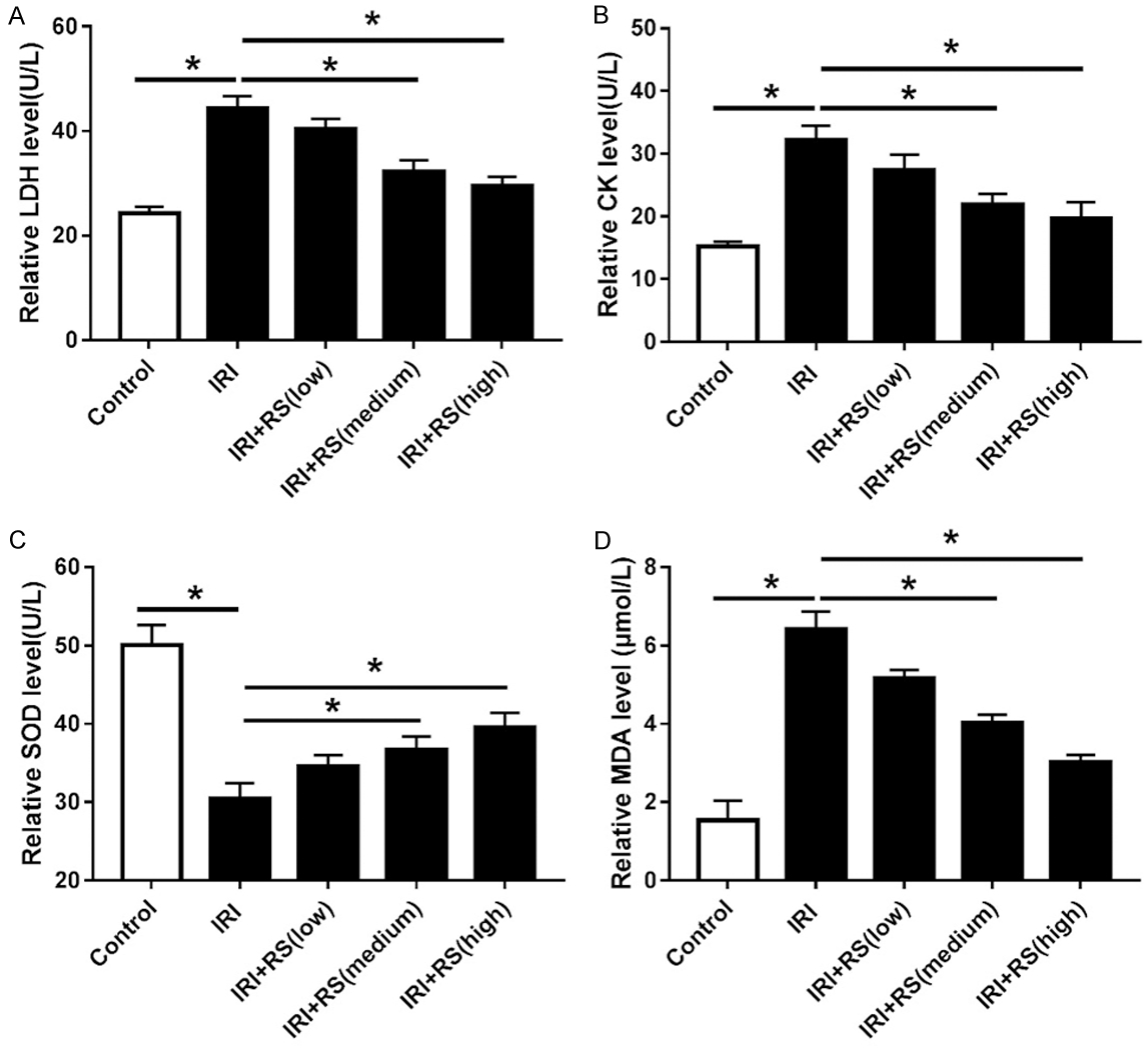

Figure 2. Rosuvastatin impaired effect of ischemia-reperfusion on LDH, CK, SOD and MDA level in cardiomyocytes. The LDH level (A), CK level (B), SOD level (C) and MDA level (D) were detected in control, IRI, IRI+RS (low), IRI+RS (medium) and IRI+RS (high) groups. *P<0.05.

3E, **3F** and **3H**), while the level of SOD was elevated in anti-miR-155-transfected cardiomyocytes (**Figure 3G**).

Rosuvastatin decreased miR-155 expression in cardiomyocytes under ischemia-reperfusion

To further explore the relationship between miR-155 and rosuvastatin in ischemia-reperfusion-treated cardiomyocytes, we detected the miR-155 expression in cardiomyocytes after treatment of ischemia-reperfusion and rosuvastatin. Results revealed that with the increase of rosuvastatin dose, miR-155 expression was gradually decreased in ischemia-reperfusion-treated cardiomyocytes (**Figure 4A**). Furthermore, we used different doses of rosuvastatin to treat cardiomyocytes with high expression of miR-155. The results showed that transfection of miR-155 mimic significantly increased the miR-155 level in ischemia-reperfusion-treated cardiomyocytes, while it was progressively reduced under the medium and high doses of rosuvastatin (**Figure 4B**).

Discussion

Cardiovascular diseases are the leading cause of death in the world, which seriously affect the health of the elderly [18]. Myocardial ischemia-reperfusion injury usually occurs in the treatment of cardiovascular diseases, which is caused by myocardial ischemia and blood supply, leading to metabolic dysfunction and myocar-

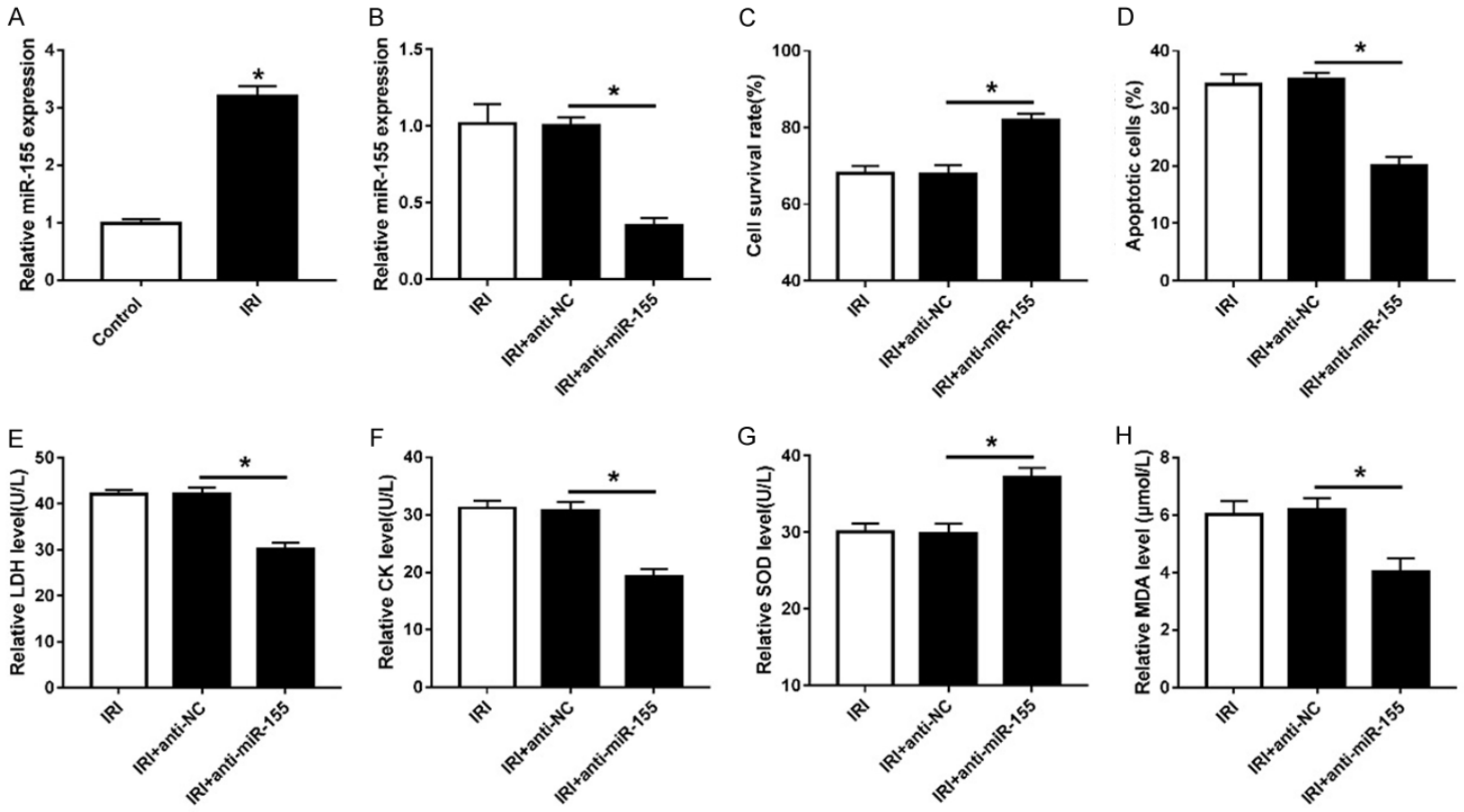

Figure 3. Knockdown of miR-155 promoted cell survival and reduced apoptosis in ischemia-reperfusion-treated cardiomyocytes. (A) The expression of miR-155 was detected by qRT-PCR in cardiomyocytes with or without treatment of ischemia-reperfusion. Cardiomyocytes were transfected with anti-miR-155 or anti-NC and then suffered from ischemia-reperfusion. The expression of miR-155 (B), cell survival (C) and apoptosis (D), levels of LDH (E), CK (F), SOD (G) and MDA (H) were detected by qRT-PCR, MTT, flow cytometry or special assay kit, respectively. *P<0.05.

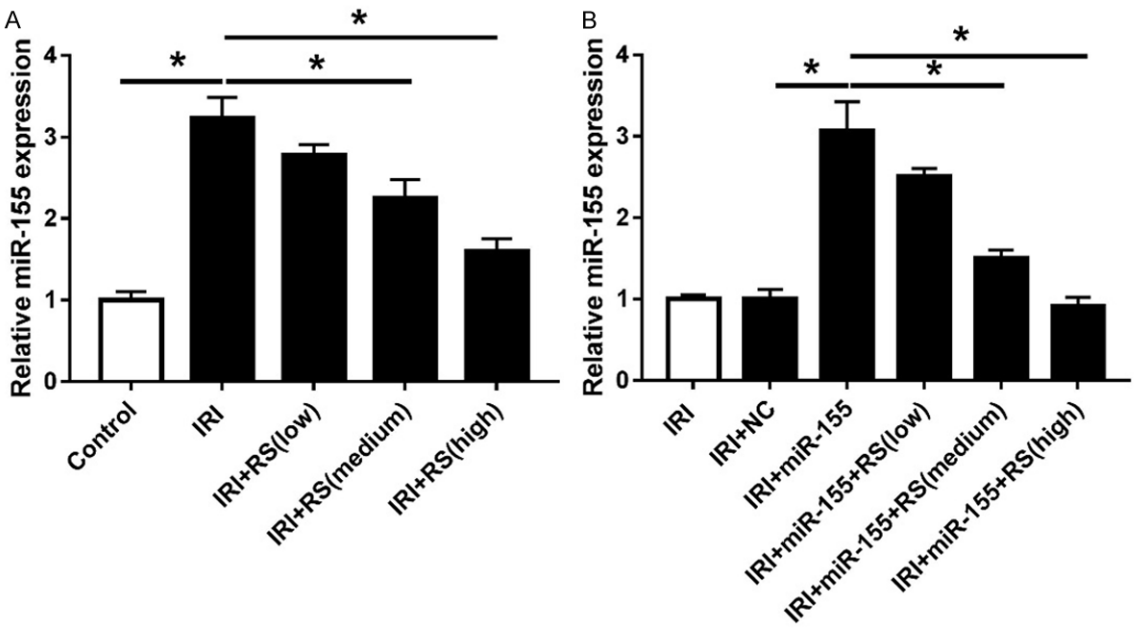

Figure 4. Rosuvastatin decreased miR-155 expression in ischemia-reperfusion-treated cardiomyocytes. A. The expression of miR-155 was detected by qPR-PCR after treatment of ischemia-reperfusion and rosuvastatin. B. The expression of miR-155 was measured in miR-155-transfected cardiomyocytes via qRT-PCR after treatment of ischemia-reperfusion and rosuvastatin. *P<0.05.

dial tissue damage. However, the underlying mechanism of myocardial ischemia reperfusion injury is not completely understood.

Many studies have shown that rosuvastatin is effective in curing myocardial ischemia-reperfusion. For example, rosuvastatin pretreatment was reported to decrease inflammatory and myocardial injury under ischemia-reperfusion condition, which might be mediated through the HMG-CoA reductase pathway [6, 19]. Meanwhile, rosuvastatin pretreatment also increased SOD activity and decreased LDH, CK, MDA and troponin I/T activities with myocardial ischemia-reperfusion condition [20]. Moreover, postconditioning with rosuvastatin reduced the infarct size and the activity of LDH, CK and MDA, increased SOD activity of myocardial ischemia-reperfusion injury by regulating the expression of high mobility group box 1 protein [21]. Meanwhile, rosuvastatin improved systemic and regional hemodynamics by reducing vascular resistance. And the protective effects of rosuvastatin on vascular and cardio cells was known to promote the production of NO in vascular endothelial cells and alleviate myocardial necrosis after ischemia-reperfusion [22, 23]. In our present study, we found that rosuvastatin obviously attenuated ischemia-reperfusion-induced survival suppression, apoptosis induction and levels of LDH, CK, SOD and MAD in cardiomyocytes, suggesting that rosuvastatin decreased myocardial ischemia-reperfusion injury. This also indicated that rosuvastatin might serve as a protective agent for therapeutic intervention of cardiovascular diseases.

MiRNAs are associated with cell growth, immune response and inflammatory injury in cardiovascular diseases. For instance, miR-125a, miR-139 and miR-314 promoted Urocortin protection to reduce myocardial ischemia-reperfusion injury [17]. Besides, miR-126 played the protective effects on myocardial infarction by regulating VEGF-A expression [24]. miR-203 was found to be associated with inflammatory injury in myocardial ischemia-reperfusion injury [25]. Of note, miR-155 was another important biomarker for the diagnosis of cardiovascular diseases. Bao *et al.* suggested that miR-155 and miR-148 reduced cardiac injury of acute viral myocarditis via inhibiting NF-kB pathway [26]. Moreover, miR-155/MMP-16 axis inhibited cell migration in human cardiomyocyte progenitor cells [27]. Various studies reported that miR-155 is widely implicated in the inflammatory response of myocardial tissues and cells.

On the other hand, miR-155 was an important regulatory molecule in ischemia-reperfusion injury. The expression of miR-155 was raised in myocardial ischemia-reperfusion injury and improved the cytokine expression and ROS expression by down-regulating SOCS-1 [28]. Wu *et al.* reported that miR-155/FoxO3a/ARC axis resulted in renal pyroptosis with the renal ischemia-reperfusion injury conditions [29]. In our study, we found that miR-155 expression was enhanced in cardiomyocytes after treatment of ischemia-reperfusion, suggesting that miR-155 might contribute to myocardial ischemia-reperfusion injury. To verify the biological function of miR-155 in ischemia-reperfusion injury, we obtained lowly-expressed cardiomyocytes for the further investigate. The results showed that knockdown of miR-155 promoted survival rate and inhibited its apoptosis. At the same time, anti-miR-155 transfection obviously decreased the level of LDH, CK and MAD and increased the level of SOD in myocardial cells under ischemia-reperfusion condition. These findings suggested that down-regulation of miR-155 attenuated ischemia-reperfusion injury in cardiomyocytes.

Additionally, we observed that the medium and high dose of rosuvastatin could significantly decrease miR-155 expression in ischemia-reperfusion-treated cardiomyocytes, which is also in agreement with a previous study which presented that rosuvastatin reduced the incidence of cardiovascular events by suppressing miR-155/SHIP-1 signaling pathway in patients [16]. According to this, we hypothesized that miR-155 played an important regulatory role in the treatment of rosuvastatin to relieve myocardial ischemia-reperfusion injury. In this study, the medium and high doses of rosuvastatin decreased miR-155 expression in cardiomyocytes transfected with miR-155 mimic, suggesting that rosuvastatin reduces myocardial ischemia-reperfusion injury by inhibiting miR-155 expression.

This study indicated the cardioprotective role of rosuvastatin in vitro. However, there are some limitations in the present work. Functional miRNAs were known to regulate the related targets in varying conditions. However, the potential target of miR-155 participating in this mechanism was absent in this study. Moreover, this study showed little in vivo data on the effect of rosuvastatin. Hence, the promising target and signaling pathway as well as animal experiments should be explored in further study.

In this study, our finding suggested that rosuvastatin reduced myocardial ischemia-reperfusion injury by inhibiting the expression of miR-155, providing a new point for the treatment of ischemia-reperfusion injury in cardiovascular diseases.

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Disclosure of conflict of interest

None.

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