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The mechanism of miR-23a in regulating myocardial cell apoptosis through targeting FoxO3

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Abstract. – OBJECTIVE: Myocardial cell apoptosis represents important pathologic basis of ischemia-reperfusion injury (I/R). MiR-23a is related to myocardial hypertrophy and cardiac remodeling by regulating myocardial cell growth and apoptosis. This study intended to observe the regulating effect of miR-23a in myocardial cell and related target, and investigate its clinical significance to I/R injury.

MATERIALS AND METHODS: The rats were divided into sham group and myocardial I/R group. Myocardial cell cycle and miR-23a expression were tested. H2O2 was applied to treat H9c2 rat myocardial cell to simulate oxidative stress during I/R. The cells were divided into blank group, NC group, miR-23a mimic group, H2O2 group, and miR-23a + H_2O_2 group. ROS content and cell apoptosis were detected by flow cytometry. MiR-23a, FoxO3a, and BIM gene expression were determined by qRT-PCR. FoxO3a and BIM protein levels were measured by Western blot.

RESULTS: Compared with sham group, myocardial apoptosis increased, while miR-23a expression was significantly downregulated in I/R group. H_2O_2 treatment markedly increased ROS levels in H9c2 cells and elevated apoptosis. The overexpression of mMiR-23a effectively reduced cell apoptosis induced by H_2O_2 treatment. H_2O_2 treatment significantly decreased miR-23a expression, while markedly elevated the levels of FoxO3a and BIM. The overexpression of miR-23a apparently impeded the induction of FoxO3a and BIM by H_2O_2.

CONCLUSIONS: The downregulation of miR-23a plays a negative role in oxidative stress and cell apoptosis induced by I/R. The overexpression of miR-23a is of significance to alleviate cell apoptosis through inhibiting FoxO3a and downstream target BIM expression.

Key Words

miR-23a, FoxO3a, BIM, Ischemia-reperfusion, Myocardium, Apoptosis.

Introduction

Timely therapy of coronary artery reperfusion is one of the most effective measures for the treatment of ischemic myocardium and to rescue life[1]. In clinic, thrombolysis, coronary artery ectasia and coronary artery bypass surgery, are commonly used to restore blood reperfusion of ischemic myocardium[2]. Myocardial ischemia reperfusion, however, does not mean instantly restoring normal physiological environment *in vivo*. Ischemic myocardium after blood reperfusion may lead to a more severe myocardial damage, thus inducing ischemia-reperfusion injury (I/R)[3]. I/R aggravates ischemic myocardial dysfunction and structural damage, which seriously impedes myocardial function recovery, harms to the curative effect of ischemic heart disease, and even results in life-ending[4,5]. Myocardial I/R may cause apoptosis and necrosis of myocardial cell, induce ventricular remodeling and cardiac dysfunction, thus giving rise to continuous cardiac function defect[6]. Myocardial cell apoptosis and necrosis are the main pathological mechanisms of I/R, of which apoptosis is the main pattern of myocardial cell damage and occurs earlier[7,8]. Myocardial apoptosis plays an important role in the pathophysiological process of cardiac remodeling after myocardial ischemia. The reduction of myocardial cell apoptosis is of great significance in improving myocardial function after reperfusion and alleviating myocardial remodeling[7]. Molecular abnormal expression and dysfunction may be the pathologic basis of myocardial cell apoptosis after reperfusion and ventricular remodeling. MicroRNAs are a group of endogenous non-coding single-stranded RNAs at the length of 21-23 nucleotides. It exerts the regulatory function behavior by combining the 3'-untranslated regions (3'-UTR) of the target mRNA to suppress translation or degrade the target mRNA[9]. MicroRNAs are closely associated with the development of cardiovascular system and the occurrence of disease, and are involved in the regulation of cell proliferation, differentiation, and apoptosis[10]. It was found that significant changes of miRNAs appeared in brain[11], kidney[12], heart[13], and liver tissues[14] after I/R, suggesting the role of miRNAs in I/R. Although it was reported that miRNAs

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can regulate various development of cardiovascular diseases, the regulating effect of miRNA in oxidative stress injury induced myocardial cell apoptosis still needs to be clarified[10]. MiR-23a is confirmed to be related to myocardial hypertrophy because of its involvement in the regulation of myocardial cell apoptosis[15], whereas its role in I/R-induced myocardial cell apoptosis is still undefined. This study aimed to observe the regulating effect of miR-23a in myocardial cell and related target to investigate its clinical significance to I/R injury.

Materials and Methods

Main reagents and materials

Healthy adult Wistar rats (male) were provided by the Laboratory Animal Center of Harbin Medical University (Harbin, Heilongjiang, China). Rat myocardial cell line H9c2 was from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were bought from Gibco (Grand Island, NY, USA). RNA extraction reagent TRIzol was bought from Invitrogen (Carlsbad, CA, USA). Reverse transcription kit and fluorescence quantitative PCR reagents were bought from Toyobo (Osaka, Japan). Transfection oligonucleotides and miR-23a PCR primers were designed and synthetized by RiboBio (Guangzhou, Guangdong, China). Rabbit FoxO3a and BIM antibodies were got from Abcam (Cambridge, MA, USA).

Wistar rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Fourth Affiliated Hospital of Harbin Medical University.

Rat myocardial I/R model

The rat was anesthetized by intraperitoneal injection of 10% chloral hydrate (3 mg/g). Limb electrocardiogram (ECG) was monitored. Trachea intubation was performed to connect animal auxiliary breathing machine. The breathing ratio was 1:2, the respiratory frequency was 70 rpm, and the tidal volume was 12-15 mL. The chest was opened on the 4th left rib to expose the heart. The left anterior descending coronary artery was searched and blocked by 6-0 non-traumatic suture. ST segment on Q lead arch lifts for 0.1 mV, T wave high, or myocardial color became pale was considered as success. After blocked for 60 min, the suture was removed to perfuse the blood vessel. ST segment fell back represented successful reperfusion.

Grouping

The experimental rats weighted 220-260 g were randomly divided into sham group and I/R group. Sham group, the left anterior descending coronary artery was passed through by 6-0 non-traumatic suture. I/R group, the left anterior descending coronary artery, was blocked for 60 min. The rats were further divided into four subgroups after surgery, as 2 h, 6 h, 12 h, and 24 h after reperfusion, with 5 rats in each group.

Ultrasonic cardiogram

The rat was anesthetized by intraperitoneal injection of 10% chloral hydrate at 24 h after reperfusion to perform ultrasonic cardiogram detection (Philips, Amsterdam, The Netherlands). The end systolic diameter and end diastolic diameter of left ventricular were recorded through left ventricular stort axis view at the papillary muscle level prior to the mitral valve. Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were automatically calculated.

Rat myocardium sampling

The heart was collected after rat was killed. The ventricular tissue supplied by left anterior descending coronary artery was identified and collected. A part was used to extract RNA and protein, while the left was prepared for frozen section.

TUNEL assay

The myocardial tissue was prepared as frozen section to test cell apoptosis by TUNEL kit (Abcam, Cambridge, MA, USA). The section was fixed by 4% paraformaldehyde for 60 min, and washed by phosphate buffered saline (PBS) for twice. Then, the section was treated with PBS containing 0.1% Triton X-100 on ice for 2 min and washed by PBS for twice. Next, the section was added by 50 μL TUNEL detection liquid prepared by TdT enzyme and fluorescent liquid at 37°C for 60 min. At last, the section was read at 488 nm to analyze cell apoptosis.

Rat myocardial cell culture and transfection

Rat myocardial cell H9c2 was seeded in DMEM medium containing 10% FBS and maintained at 37°C and 5% CO_2. The cells were transfected by lipofectamine 2000 and nucleotide sequence in Opti-MEM I medium at 37°C and 5% CO_2 for 4-6 hours. The cells were changed to normal medium and cultured for 48 h. They were divided into five groups. In the blank group, H9c2 cells were normally cultured. In the NC group, the cells were trans-

fected with scramble miRNA (5'-UUCUCCGAACGUGUCACGUTT-3'). In the MiR-23a mimic group, the cells were transfected with miR-23a mimic fragment (5'-AUCACAUUGCCAGGGAUUUCC-3'). In the H_2O_2group, the cells were added with 200 μmol/L H_2O_2 to establish oxidative damage model. In the MiR-23a mimic + H_2O_2 group, the cells were transfected with miRNA-23a mimic fragment and treated with 200 μmol/L H_2O_2. The cells were collected after 24 h for detection.

qRT-PCR

Total RNA was extracted using TRIzol. Its concentration and purity were detected by ultraviolet spectrophotometer and agarose electrophoresis. Then, the RNA was reverse transcripted to cDNA using the following system: 3 μg RNA, 1 μL dNTP (10 mmol/L), 4 μL RT Buffer (5×), 2 μL RT primer (1 μmol/L), 2 μL revertase, 0.5 μL RNase inhibitor, and ddH_2O. Reverse transcription was performed at 16°C for 30 min, 42°C for 15 min, and 85°C for 5 min. Next, cDNA was applied for PCR amplification. The primers used were as follows: miR-23a P_F: 5'-TCACACTATATCACATTGCCAGG-3', miR-23a P_R: 5'-TATGGTTGTTCTGCTCTCTGTCTC-3'. U6 P_F: 5'-ATTGGAACGATACAGAGAAGATT-3', U6 P_R: 5'-GGAACGCTTCACGAATTTG-3'. FoxO3a P_F: 5'-TCGCGCACCAATTCCAAC-3', FoxO3a P_R: 5'-TCGCTGTGGCTGAGTGAGTC-3'. BIM P_F: 5'-ATCTCAGAGCAATGGCTTCC-3', BIM P_R: 5'-ATTCGTGGGTGGTCTTCG-3'. Caspase-3 P_F: 5'-GGACCTGTGGACCTGAAAAA-3', Caspase-3 P_R: 5'-GCATGCCATATCATCGTCAG-3'; β-actin P_F: 5'-GAACCCTAAGGCCAAC-3', β-actin P_R: 5'-TGTCACGCACGATTTCC-3'. The system contained 4.5 μL 2×SYBR Green Mixture, 0.5 μL 5 μm/L primers, 1 μL cDNA, and 3.5 μL ddH_2O. PCR reaction was consisted of 95°C for 5 min, followed by 40 cycle of 95°C×15 s and 60°C×1 min. PCR reaction was performed on ABI ViiA7 (Thermo Fisher Scientific, Waltham, MA, USA). The results were analyzed by Comparative Ct method. U6 and β-actin were used as internal references. Each sample was repeated for three times.

Western blot

Total protein was extracted from cells and quantified using bicinchoninic acid (BCA). The protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. After being blocked in 5% skim milk at room temperature for 60 min, the membrane was incubated in primary at 4°C overnight. After being washed by phosphate-buffered saline and Tween 20 (PBST) twice, the membrane was incubated in secondary antibody at room temperature for 60 min. At last, the membrane was exposed by enhaneed chemiluminescence (ECL) method and scanned to store the data.

DCFH-DA detection of ROS content

DCFH-DA was diluted by serum free medium at 1:1000 to final concentration of 10 μmol/L. Then, the DCFH-DA solution was added to the cells and cultured at 37°C for 20 min. After being washed by serum free medium for three times, the cells were resuspended in 500 μL PBS and detected by flow cytometry at 488 nm.

Cell apoptosis detection

The cells were digested and collected. After being washed by PBS and centrifuged at 1000 g for 5 min, the cells were resuspended in 195 μL Annexin V/FITC solution together with 5 μL Annexin V-FITC and 10 μL PI. After being incubated in the dark for 10-20 min, the cells were detected using flow cytometry (Beckman Coulter, Brea, CA, USA).

Statistical Analysis

Data analysis was performed on SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Measurement data was presented as mean ± standard deviation. The *t*-test was used for data comparison. $p < 0.05$ was considered as statistical significance.

Results

Ultrasonic cardiogram detection results

LVEF and LVFS showed no statistical difference in sham group among different time points ($p > 0.05$). LEVF and LVFS in I/R group were lower than that of sham group at each time point ($p < 0.05$). LVEF and LVFS decreased in an I/R time dependent manner (Table I), suggesting that the cardiac function in I/R rats gradually declined.

I/R triggered myocardial cell apoptosis

The myocardial cell apoptosis in sham group was not obvious. A large number of apoptotic myocardial cells appeared in I/R group, and became increasing in a time dependent manner (Table II), indicating that I/R may trigger myocardial cell apoptosis.

Table I. Ultrasonic cardiogram detection results (%, n=5, Mean ± SD).

Index	Grouping	2h after operation	6h after operation	12h after operation	24h after operation
LVEF	Sham group	81.8±3.1	80.2±2.9	82.9±1.9	83.1±2.8
	I/R group	70.4±2.1	61.8±1.7	55.4±2.0	50.7±1.6
LVFS	Sham group	45.4±3.9	47.3±3.1	46.6±3.7	48.1±4.2
	I/R group	38.2±4.0	33.4±3.0	30.5±2.8	25.7±3.1

Table II. Myocardial cell apoptosis rate comparison (%, n=5, Mean ± SD).

Grouping	2h after operation	6h after operation	12h after operation	24h after operation
Sham group	81.8±3.1	80.2±2.9	82.9±1.9	83.1±2.8
I/R group	70.4±2.1	61.8±1.7	55.4±2.0	50.7±1.6
Sham group	45.4±3.9	47.3±3.1	46.6±3.7	48.1±4.2
I/R group	38.2±4.0	33.4±3.0	30.5±2.8	25.7±3.1

I/R downregulated miR-23a expression in myocardium

Our data by qRT-PCR detection revealed that miR-23a expression in I/R group was significantly lower than that of sham group at each time point. MiR-23a expression in I/R group at 2 h, 6 h, 12 h, and 24 h after operation was 0.81 ± 0.05, 0.72 ± 0.07, 0.64 ± 0.08, and 0.33 ± 0.03 times, respectively, compared with that in sham group ($p < 0.001$) (Figure 1).

MiR-23a overexpression declined cell apoptosis

An important pathogenetic mechanism of myocardial I/R injury is that myocardial cell may produce numerous reactive oxygen species (ROS) after I/R, resulting in oxidative stress damage. In this study, H9c2 rat myocardial cell *in vitro* was treated by H_2O_2 to simulate I/R induced oxidative stress damage. ROS detection showed that its content was elevated in H_2O_2 group and H_2O_2 + mimic group compared with that in blank group, NC group, and miR-23a mimic group, suggesting the successful induction of oxidative stress with H_2O_2 (Figure 2A). The apoptosis rates in blank group, NC group, and miR-23a mimic group were low, and showed no statistical difference ($p > 0.05$). Of note, the treatment of 200 μmol/L H_2O_2 for 24 h induced cell apoptosis, whereas miR-23a mimic transfection significantly reduced cell apoptosis (Figure 2B). It indicated that miR-23a can protect myocardial cell from cell apoptosis due to oxidative damage.

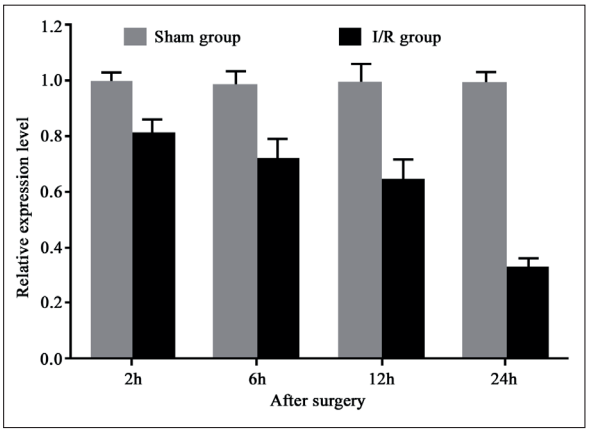

Figure 1. qRT-PCR detection of miR-23a expression at each time point.

MiR-23a targeted FoxO3a to impact rat myocardial cell apoptosis

FoxO3a, a member of the forkhead transcription factor family, plays a critical role in activating target gene transcription and translation and accelerating cell apoptosis regulated by PI3K/AKT signaling pathway. As one of the most important target genes of FoxO3a, BIM plays a vital role in apoptosis signaling pathway associated with FoxO3a activation. FoxO3a expression and function are regulated by miR-23a. Our results observed that H9c2 cell apoptosis was decreased after the transfection of miR-23a mimic. We discussed whether miR-23a affects H9c2 cell apoptosis through specifically targeting FoxO3a. qRT-PCR demonstrated that compared with blank group and NC group, H_2O_2 treatment markedly reduced endogenous miR-23a expression in H9c2 cells ($p < 0.001$), which was

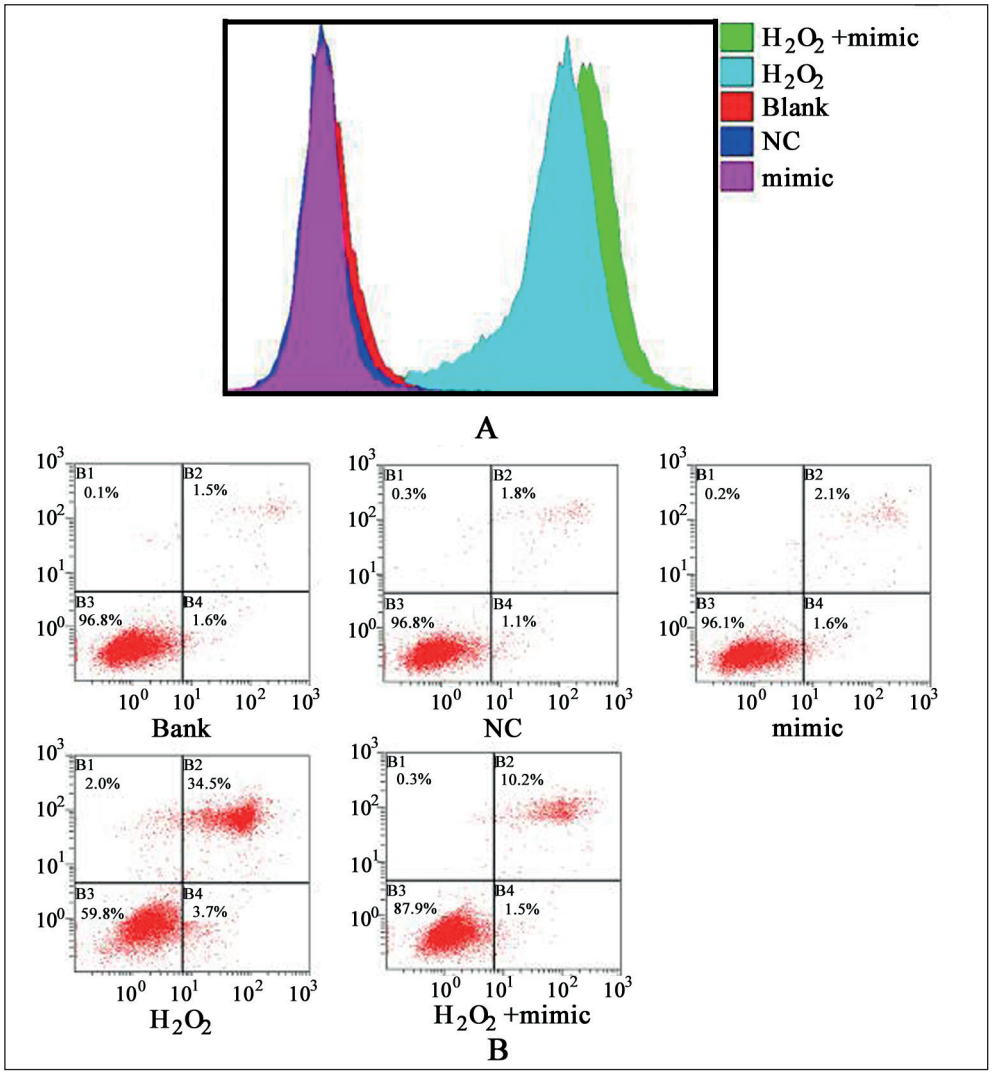

Figure 2. MiR-23a overexpression reduced cell apoptosis. **A**, DCFH-DA detection of ROS content; **B**, Flow cytometry detection of cell apoptosis.

in accordance with I/R-induced miR-23a down-regulation, suggesting that miR-23a declination may play a role in I/R and oxidative damage (Figure 3A). As shown in Figure 3B, compared with blank group and NC group, H_2O_2 treatment apparently enhanced FoxO3a expression ($p < 0.001$), which was opposite to down-regulating effect of H_2O_2 on miR-23a. H_2O_2 treatment may elevate FoxO3a expression through decreasing miR-23a level, revealing that it can affect miR-23a and FoxO3a expression. Compared with single H_2O_2 treatment, miR-23a mimic transfection downregulated FoxO3a gene expression by 44.9% ($p < 0.001$), suggesting that miR-23a mimic may reduce H_2O_2 induced cell apoptosis through downregulating FoxO3a. H_2O_2 treatment not only inhibited FoxO3a gene expression, but also reduced its downstream BIM gene level ($p < 0.001$). BIM gene expression was enhanced after miR-23a overexpression ($p < 0.001$) (Figure 3C). As shown in Figure 3D, H_2O_2 treatment up-regulated FoxO3a and BIM protein levels, while miR-23a weakened such effect. It indicated that miR-23a may alleviate the oxidative damage on myocardial cell apoptosis through targeting FoxO3a and BIM expression.

Discussion

Myocardial infarction induced by hypoxic-ischemia is one of the important causes of

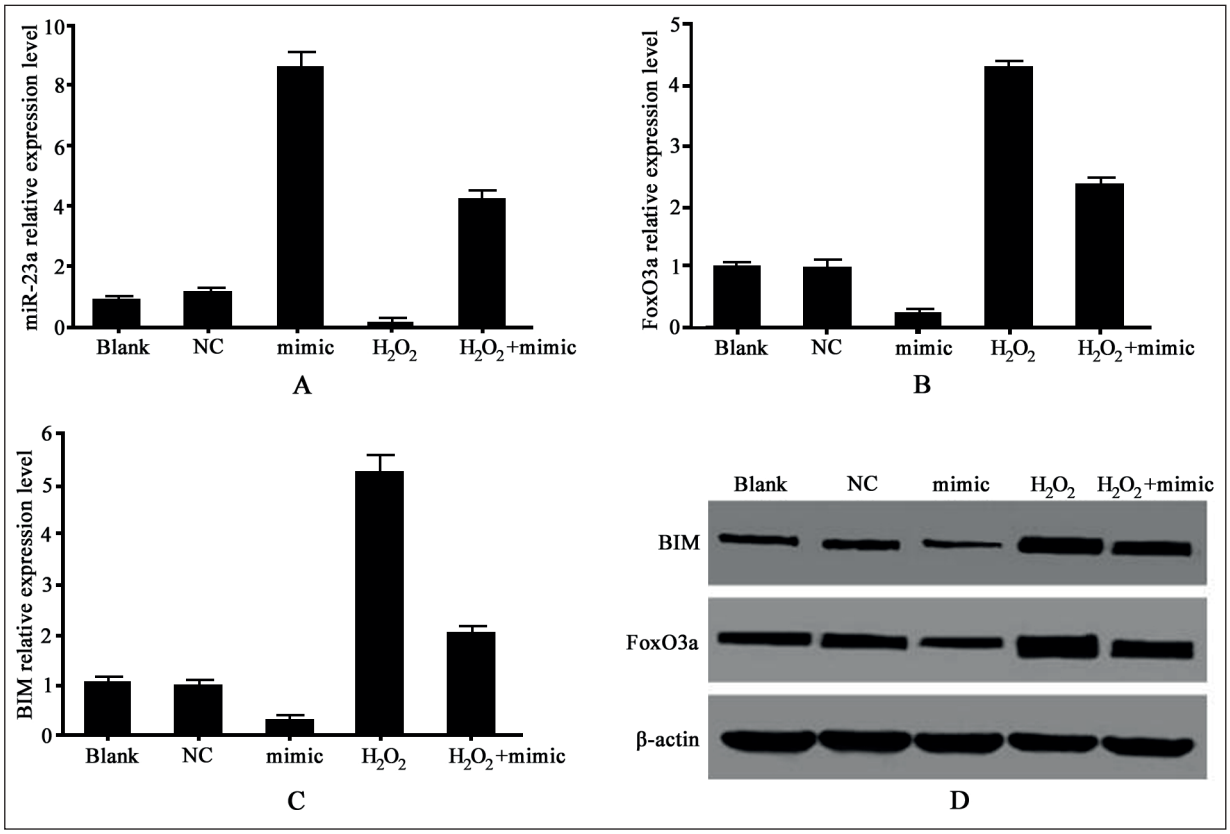

Figure 3. MiR-23a affected BIM through targeting FoxO3a. **A**, qRT-PCR detection of miR-23a expression; **B**, qRT-PCR detection of FoxO3a expression; **C**, qRT-PCR detection of BIM expression; **D**, Western Blot detection of protein expression.

ventricular remodeling, heart failure, and cardiac death[16]. A variety of clinical treatments are usually applied as soon as possible to restore the blood supply for ischemic myocardium, for the purpose of blood reperfusion. Blood reperfusion usually causes I/R injury, which aggravates the organic lesion and dysfunction of ischemic myocardium, such as cardiac fibrosis and heart failure[17]. Mechanisms of I/R injury, early recovery of ischemic myocardial blood supply, and the prevention from I/R injury damage, are key points in ischemic disease treatment[18]. Effective reduction or decrease of apoptosis caused by I/R injury, become the focus of the clinical research[19]. Recently, it was shown that miRNA participates in cardiovascular system development, and the significant role of miRNA in cardiovascular disease was also gradually recognized[10]. Many researches confirmed that abnormal expression and dysfunction of miRNAs are closely related to myocardial fibrosis[20], myocardial hypertrophy[21], myocardial infarction[22], and atherosclerosis[23]. In addition, miRNAs also play an important role in I/R injury and myocardial cell apoptosis[24-27]. It is reported that miR-23a inhibited TGF-β mediated endothelial-mesenchymal transition cells, thereby potentially suppressing myocardial fibrosis[28]. MiR-23a can activate early growth response factor-1 and pituitary tumor transforming factor-1, which promotes myocardial hypertrophy[29]. Van Rooij et al[30] observed that miR-23a was upregulated in the cardiac remodeling process, suggesting its role in promoting myocardial fibrosis. MiR-23a was also confirmed be associated with myocardial hypertrophy in regulation of myocardial cell apoptosis[15], while its role in I/R injury induced myocardial cell apoptosis is still unclear. Our study showed that the apoptosis rate of myocardial cell increased, while miR-23a expression was downregulated in I/R group, indicating the protective role of miR-23a in myocardial cell apoptosis.

Oxidative stress is a state of imbalance, leading to ROS elevation, which is one of the important causes of many diseases in the cardiovascular system[31]. H_2O_2 treatment induces

the production of a large number of ROS in cells within short time, resulting in myocardial I/R injury *in vivo*. Therefore, it has been widely applied in exploring I/R injury mechanism[32,33]. Our study found that H_2O_2 treatment triggered ROS production and induced cell apoptosis, while reduced miR-23a expression. MiR-23a overexpression can alleviate H_2O_2-induced cell apoptosis, revealing the beneficial effects of miR-23a in relieving I/R injury. Moreover, it was also found that the anti-apoptotic effect of miR-23a can effectively reduce the myocardial infarction area and improve blood pump ability in myocardial infarction process[34]. It is worth noting that the anti-apoptotic effect of miR-23a is not always favorable. For example, the anti-apoptotic effect of miR-23a may cause myocardial hypertrophy[15]. FoxO3a is an important member of forkhead transcription factor family that is negatively regulated by PI3K/AKT signaling pathway. AKT can phosphorylate FoxO3a to reduce its affinity to nuclear DNA but it can enhance affinity with the 14-3-3 protein in the cytoplasm. It further transfers FoxO3a from nucleus to cytoplasm, leading to the loss of target gene transcription activity in the nucleus[35]. When the PI3K/AKT signaling pathway activity is weakened and FoxO3a phosphorylation level is decreased, dephosphorylated FoxO3a would enter the nucleus to activate target gene transcription and translation, accelerating apoptosis[16,36]. The main target genes of FoxO3a include proapoptotic gene Bcl-2 interacting mediator of cell death (BIM) and nonspecific cycle-dependent kinase inhibitor (CKI) $p27^{Kip1}$. BIM, the subfamily member of Bcl-2 family containing BH3-only domain structure, plays a critical role in activating programmed cell death and oxidative stress-induced apoptosis[37,38]. The BH3-only domain structure of BIM is the main domain structure to promote apoptosis activity. BIM can activate cell apoptosis through binding with Bax to increase mitochondrial membrane permeability and release cytochrome C[39]. BIM also plays a vital role in FoxO3a-mediated apoptosis signaling pathway. FoxO3a activation upregulates BIM and Bax expression, and enhance cell apoptosis[40]. Moreover, FoxO3a expression and function are targeted regulated by miR-23a[15]. Thus, we investigated whether miR-23a affects H9c2 cell apoptosis in I/R injury by targeting FoxO3a. Our results found that H_2O_2 treatment can increase FoxO3a and downstream BIM expression, whereas miR-23a overexpression decrease FoxO3a and BIM levels to a great extent, and reduce the apoptosis effect caused by H_2O_2.

Conclusions

We showed that miR-23a is of significance to alleviate I/R and oxidative stress and cell apoptosis via the inhibition of FoxO3a and BIM expression.

Conflict of Interest

All the authors declare no conflict of interests.

Acknowledgments

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