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MiR-181a affects myocardial ischemia-reperfusion injury in rats via regulating akt signaling pathway

L.-X. ZHANG[1], F. DING[1], C.-Q. WANG[2], Q. BING[3], Z. ZHAO[3], J. WANG[1], L. ZHANG[1]

[1]Department of Gerontology, The Third Hospital of Hebei Medical University, Shijiazhuang, China
[2]Department of Cardiology, The Third Hospital of Hebei Medical University, Shijiazhuang, China
[3]Department of Neuromuscular Disease, The Third Hospital of Hebei Medical University, Shijiazhuang, China

Lixuan Zhang and Fang Ding contributed equally to this work

Abstract. – OBJECTIVE: The aim of this study was to explore the influence of the micro ribonucleic acid (miR)-181a on myocardial ischemia-reperfusion injury (MIRI) in rats by regulating the protein kinase B (Akt) signaling pathway.

MATERIALS AND METHODS: A total of 30 male Sprague-Dawley rats were randomly divided into three groups, including: sham operation group (Sham group), ischemia-reperfusion group (I/R group), and miR group (MiR-181a group). The model of myocardial ischemia-reperfusion was successfully established in rats. The concentration of blood nitric oxide (NO) was detected by the relative kits. Myocardial apoptosis in rats of the three groups was detected using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Furthermore, the expressions of myocardial cell apoptosis-related proteins and tumor necrosis factor-α (TNF-α), and the degree of Akt phosphorylation were determined by Western blotting.

RESULTS: Compared with Sham group and miR-181a group, I/R group exhibited significantly elevated left ventricular end-diastolic pressure (LVEDP) ($p<0.05$). However, the left ventricular end-systolic pressure (LVESP), stroke work (SW), differential pressure (DP), end-systolic pressure-volume relationship (ESPVR), and end-diastolic pressure-volume relationship (EDPVR) significantly decreased in the I/R group ($p<0.05$). In comparison with miR-181a group, the apoptosis index of myocardial cells was remarkably elevated in the I/R group, showing statistically significant differences ($p<0.05$). The protein bands were analyzed using the Quantity One detection software. The results demonstrated that, compared with the Sham group, I/R group showed significantly elevated expressions of cysteine-aspartic protease (Caspase)-3 and TNF-α in rat myocardial tissues ($p<0.05$). However, the protein levels of Akt and endothelial NO synthase (eNOS) phosphorylation and NO in rat myocardial cells were significantly down-regulated ($p<0.05$).

CONCLUSIONS: MiR-181a activates Akt to promote the phosphorylation of its downstream protein eNOS, inhibit the apoptosis of myocardial cells, and alleviate MIRI.

Key Words:
Myocardial ischemia-reperfusion injury, Akt signaling pathway, MiR-181a.

Introduction

Myocardial ischemia exerts a relatively high incidence rate worldwide. Currently, effective clinical treatments include the restoration of perfusion in ischemic myocardium through thrombolytic therapy, coronary-artery bypass grafting, and percutaneous coronary intervention[1,2]. With the progress in cardiac surgery and cardiopulmonary bypass, the myocardial ischemia-reperfusion injury (MIRI) has become an urgent problem to be solved in the medical field. The key to successful surgeries is how to effectively relieve MIRI[3,4]. Lindenblatt et al[5] made experiments in a different direction, and firstly put forward a complete concept on ischemic post-conditioning. They have demonstrated that, before reperfusion, the transient and repeated ischemic circulation and perfusion for ischemic myocardium can mitigate myocardial reperfusion injury well. MIRI is generally inevitable in various reperfusion therapies for cardiomyopathy. As thrombolytic therapy and coronary intervention continue, alleviating ischemia-perfusion injury is particularly important to improve the efficacy of the reperfusion therapy[6,7].

Micro ribonucleic acids (miRNAs) are a type of endogenous non-coding single-stranded RNA molecules. They can participate in the regulation

Corresponding Author: Li Zhang, MD, Ph.D; e-mail: daylight1981@126.com

of post-transcriptional gene expression in animals and plants[8]. So far, multiple miRNAs have been confirmed to exist in plants, animals, and viruses. MiR-181a is a miRNA located on human chromosome 1q32.1. Previous scholars have focused on its role in cancers. Multiple studies have demonstrated that miR-181a is related to the sensitivity of cancer cells to chemotherapy drugs[9]. Meanwhile, it can directly act on target genes and comprehensively regulate the migration, invasion, proliferation, and apoptosis of cancer cells[10,11]. Byrne et al[12] have reported that miRNAs are closely related to the protein kinase B (Akt) signaling pathway in the treatment of myocardial ischemia. Additionally, relevant researches[13] have suggested that the Akt pathway regulates the majority of responses in mammal cells. Furthermore, in ischemia-perfusion (I/R), the activation of the Akt signaling pathway can repress cell apoptosis and activate survival pathways. However, whether miR-181a protects against ischemia-perfusion injury via the Akt pathway remains unknown. Therefore, the aim of this work was to explore the role of miR-181a in myocardial ischemia-reperfusion injury (MIRI) in rats by regulating the protein kinase B (Akt) signaling pathway.

Materials and Methods

Experimental Materials and Grouping

A total of 30 male Sprague-Dawley (SD) rats weighing 0.24-0.26 kg and aged about 7 weeks old were provided by the Animal Core Facility of Jinzhou Medical University. All rats were fed with humidity of 38-50% and temperature of 21-25°C. This study was approved by the Animal Ethics Committee of Hebei Medical University Animal Center. All SD rats were randomly divided into 3 groups (with 10 rats in each group), including: sham operation group (Sham group), ischemia-reperfusion group (I/R group), and miR group (MiR-181a group). For Sham group: during surgery, threading was required without ligation. Meanwhile, 2 mL of normal saline was injected into the abdominal cavity 1 min before the surgery. For I/R group: after 1 h of ligation, reperfusion lasted for 2 h. 10 min before surgery, 2 mL of normal saline was injected into the abdominal cavity. For MiR-181a group: after 1 min of ligation, the reperfusion was conducted for 2 h. 10 min before surgery, 5 mg/kg miR-181a was injected into the abdominal cavity in a bolus manner.

Experimental Instruments and Reagents

Rabbit polyclonal antibodies for cysteine-aspartic protease (Caspase)-3, tumor necrosis factor-α (TNF-α), and phosphorylated protein kinase B (Akt) were purchased from Abcam (Cambridge, MA, USA); terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit, 1.9 F volumetric catheter from Science Instruments (East Oshawa, ON, Canada) and nitric oxide (NO) detection kit from Roche (Roche, Basel, Switzerland).

Establishment of Myocardial Reperfusion Model in Rats

The myocardial reperfusion model was first established in rats according to the following procedure. After anesthesia with an injection of 3% pentobarbital sodium anesthetic into the abdomen, the rats were fixed on an operating table. The trachea was cut open and connected with a respirator to maintain them breathing[13]. A cannula accessed to the left ventricle through the right carotid artery to collect the cardiac function-related parameters. A longitudinal incision was first made at the left sternal margin, with the strongest cardiac apex beat. Subsequently, the subcutaneous tissues were separated using a blunt tool, and the thoracic cavity was cut open to expose the heart. Next, the pulmonary conus from left to right was raised using tweezers. Taken as a reference with the left coronary artery, they were sutured together with a silicone tube at 1.5 mm away from the left edge of the auricle. Additionally, to ensure that there was no blood flow within 1 h of coronary artery ligation, perfusion was continued for 2 h after the release of ligation. Finally, a MIRI model was surgically established in the rats. After ligation, the electrocardiogram showed ST segment elevated by over 0.1 mV and enlarged amplitude of QRS waves, with greyish white myocardium. These characteristics indicated successful blocking. After successful modeling, the cardiac function parameters were collected, and 1 mL of the blood sample was extracted from the carotid artery. Post-operatively, once rats in all groups were executed, the heart was immediately removed[14].

Cardiac Function

After successful modeling, the cardiac function parameters, including end-systolic pressure-volume relationship (ESPVR), left ventricular end-systolic pressure (LVESP), differential pressure (DP),

stroke work (SW) and left ventricular end-diastolic pressure (LVEDP) were measured instantly. Meanwhile, the end-diastolic pressure-volume relationship (EDPVR) was recorded and calculated with a pressure volume catheter.

Detection of Myocardial Cell Apoptosis Via TUNEL Assay

The tissue proteins were first removed using proteinase K working solution. Subsequently, the tissues in each group were added with the corresponding concentration of TUNEL assay solution and 50 mL of TUNEL reaction mixture. After drying the glass, they were incubated at 37°C for 1 h in the dark. The products were then washed with Phosphate-Buffered Saline (PBS) for 3 times, and the number of apoptotic cells was counted under a microscope. After that, the tissues were added with 50 mL of TUNEL reaction mixture and incubated for other 0.5 h as above. After rinsing for 3 times, 50-100 mL of diaminobenzidine (DAB) substrates (Solarbio, Beijing, China) were added, followed by 12-20 min of reaction. Finally, the products were rinsed for 3 times for detection.

Protein Expression of Caspase-3, Akt Phosphorylation and TNF-α Via Western Blotting

After reperfusion, the heart of rats in each group was excised. The myocardial tissues were then lysed to extract the total protein on ice. The concentration of proteins was measured using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Subsequently, 100 g of myocardial tissue proteins were separated by 12% dodecyl sulfate and sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes. After sealing with 5% skim milk, the membranes were incubated with primary antibodies [Akt, endothelial NO synthase (p-eNOS), NOS, Caspase-3, phosphorylated-Akt (p-Akt), and TNF-α] overnight. On the next day, the membranes were washed with TBST for 3 times and incubated with horseradish peroxidase (HRP)-labeled secondary antibodies (diluted at 1:5000) at room temperature for 2 h. The color development reaction was performed by enhanced chemiluminescence (ECL) method. Finally, the brightness value of the bands in each group was analyzed and calculated via Quantity One imaging analyzer.

Determination of NO

After reperfusion, 1 mL of blood was collected from the carotid artery of rats in each group. After centrifugation at 4°C, the supernatant was obtained. The content of NO in each group was determined according to the instructions of the relevant kit (Abcam, Cambridge, MA, USA).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) software was used for all statistical analyses. The Dunnett's test was adopted for paired comparisons, and the *F*-test was employed for inter-group mean comparisons. The data were tested for homogeneity of variance and normal distribution. $p<0.05$ was considered statistically significant.

Results

Cardiac Function

Compared with the Sham group and MiR-181a group, I/R group exhibited significantly lower EDPVR, SW, DP, ESPVR, and LVESP ($p<0.05$). However, LVEDP was remarkably elevated in the I/R group ($p<0.05$) (Table I).

Table I. Comparisons of rat cardiac function parameters among all the groups.

	Sham group (n=10)	I/R group (n=10)	MiR-181a group (n=10)	F	p
LVEDP (mmHg)	7.3 ± 1.0	15.6 ± 1.7*	10.5 ± 0.8#	96.7	<0.001
DP (mmHg)	103 ± 3.2	54.6 ± 4.0*	74.8 ± 3.7#	187.1	<0.001
ESPVR (mmHg/μL)	1.35 ± 0.17	0.98 ± 0.11*	1.24 ± 0.13#	26.5	<0.001
LVESP (mmHg)	110.5 ± 2.5	71.1 ± 3.9*	85.0 ± 3.2#	172.4	<0.001
SW (mmHg/μL)	9372 ± 534	4712 ± 128*	6758.0 ± 296#	109.2	<0.001
EDPVR (mmHg/μL)	0.016 ± 0.0038	0.0016 ± 0.0006*	0.0064 ± 0.0012#	98.5	<0.001

Note: *$p<0.05$, *vs.* Sham group and [#]$p<0.05$, *vs.* I/R group.

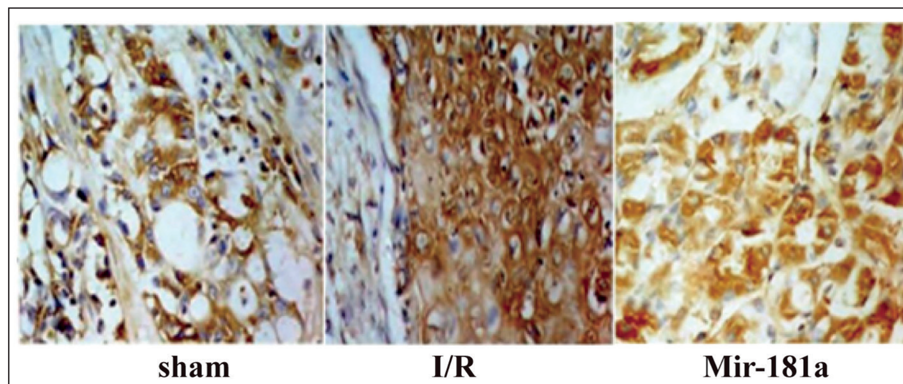

Figure 1. Myocardial cell apoptosis detected by TUNEL staining among all the three groups (magnification × 40).

Myocardial Cell Apoptosis

According to the TUNEL staining results, the normal rat myocardial cells were blue, while the nucleus of the necrotic or apoptotic positive cells was tan (Figure 1). The myocardial cell apoptosis index in the I/R group and MiR-181a group was 29.8-46.8% and 19.8-41.9%, respectively. The difference was statistically significant between the two groups ($p<0.05$).

Protein Expressions of Caspase-3 and TNF-α in Rat Myocardium of Each Group

Compared with the Sham group, the protein expressions of Caspase-3 and TNF-α in myocardial tissues were significantly in I/R group (by 4.6 times and 4.9 times, respectively), with significant differences ($p<0.05$). However, they were remarkably down-regulated in MiR-181a group when compared with the I/R group ($p<0.05$) (Figure 3).

Levels of Akt, NO, and p-eNOS in Rats of Each Group

Compared with the Sham group, the levels of NO, Akt, and p-eNOS were significantly down-regulated in the rat myocardial cells of the I/R group, showing statistically significant differences ($p<0.05$). However, the above indexes were remarkably elevated in both MiR-181a and I/R group ($p<0.05$). The levels of NO and p-eNOS in myocardial cells in MiR-181a group were substantially higher than those in the Sham group, and the differences were statistically significant ($p<0.05$). However, no significant difference was observed in the relative expression level of Akt ($p>0.05$) (Figures 4 and 5).

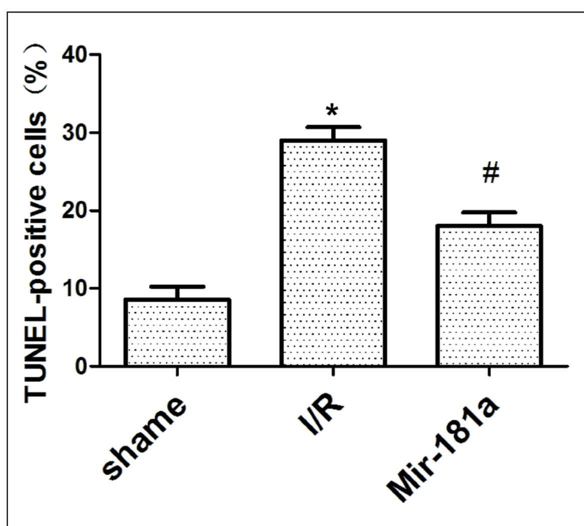

Figure 2. Myocardial cell apoptosis detected via TUNEL staining assay. Note: *$p<0.05$, *vs.* Sham group and $^{\#}p<0.05$, *vs.* I/R group.

Discussion

Currently, cardiac surgery and cardiopulmonary bypass are very common in clinical practice. However, after myocardial reperfusion, these patients may suffer from a series of complications such as arrhythmia, severe contraction,

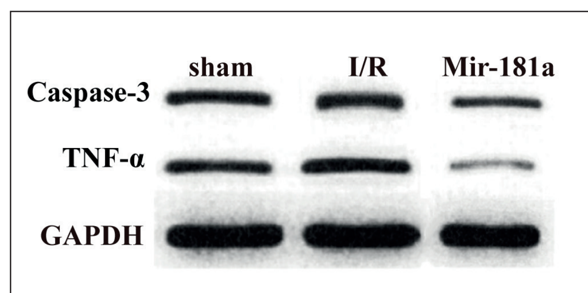

Figure 3. Expressions of Caspase-3 and TNF-α in rat myocardial cells in the rats of all groups.

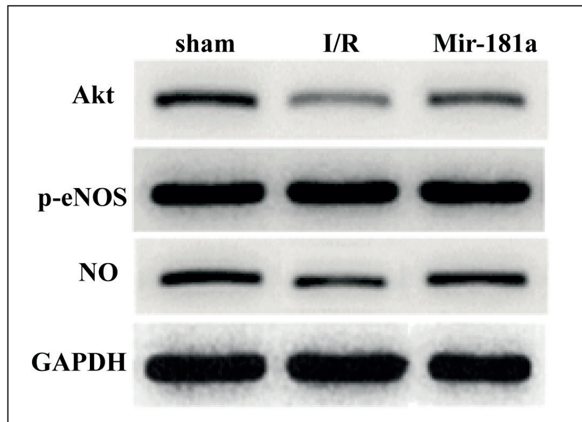

Figure 4. Protein expressions of Akt, NO, and p-eNOS in the rats of all groups.

and diastolic dysfunction, namely MIRIs[14]. As a common clinical complication, I/R causes myocardial cell apoptosis and destroys the normal structure of myocardial tissues. Meanwhile, it also gives rise to decreased cardiac function or mortal arrhythmia[15,16]. According to the results of this study, compared with the Sham group and MiR-181a group, the I/R group showed significantly elevated LVEDP, as well as reduced LVESP, SW, DP, ESPVR, and EDPVR (p<0.05). Moreover, myocardial cell apoptosis index in the I/R group and MiR-181a group was 29.8-46.8% and 19.8-41.9%, respectively. The difference was statistically significant between the two groups (p<0.05). Lu et al[17] have found that miR-181a reduces oxidative stress to alleviate I/R injury. Li et al[18] have demonstrated that miR-181a can protect the myocardium and inhibit the formation of free radicals, thereby repressing the apoptosis of myocardial cells. These results were similar to the findings of the present study, namely miR-181a protected the myocardium by inhibiting the apoptosis of myocardial cells.

In this research, the administration with miR-181a could significantly relieve MIRI at the early stage of myocardial reperfusion. However, our results showed that the I/R group had significantly elevated expression levels of TNF-α and Caspase-3. This verified that MIRI was closely related to the activation of apoptosis pathways. In cardiovascular diseases, miRNAs can regulate target genes, thereby playing important roles in myocardial hypertrophy, necrosis, and apoptosis. They can also participate in each onset of AMI. Since miRNAs keep stable in blood and exhibit remarkably differential expressions under various physiological and pathological conditions, they are considered as ideal biomarkers in blood tests for diseases. Bostjancic et al[19] have pointed out that plasma miR-1, miR-208, and miR-499 serve as underlying biomarkers for predicting AMI. Ke et al[20] have found that in I/R, there are two major signaling pathways related to myocardial cell apoptosis. The first is the intrinsic pathway that mitochondria activate Caspase-3 to induce apoptosis. The second one is the external pathway of Fas ligand or TNF-α. Zhou et al[21] have shown that Caspase-3 and TNF-α are closely correlated with myocardial ischemia. Meanwhile, in I/R, miR-181a suppresses the protein expressions of Caspase-3 and TNF-α in myocardial apoptosis-related pathways. In the present study, the protein expressions of Caspase-3 and TNF-α were the highest in the I/R group. Their protein expressions decreased after treatment with miR-181a, suggesting that miR-181a could inhibit both Caspase-3 and TNF-α in the myocardium.

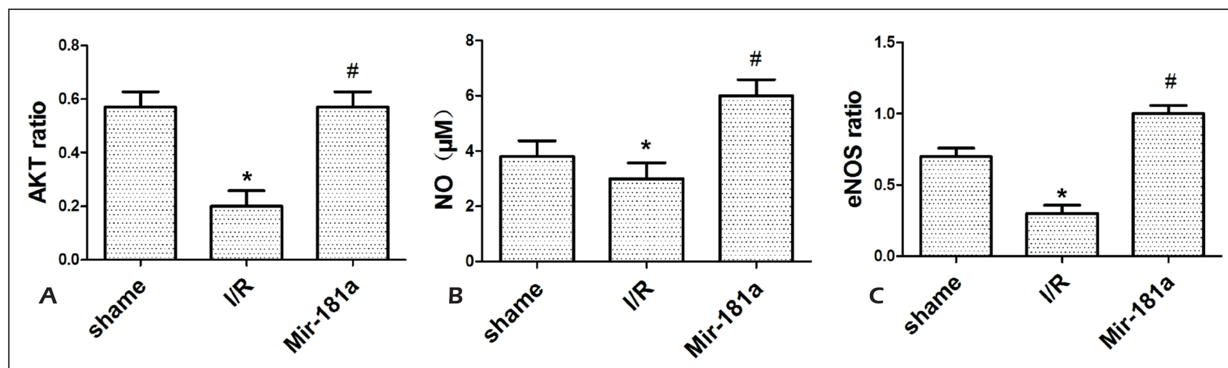

Figure 5. A-C, Levels of Akt, NO, and p-eNOS in myocardial cells of each group determined via Western blotting. Note: *p<0.05, *vs.* I/R group and $^{\#}p$<0.05, *vs.* MiR-181a group.

Compared with the Sham group, the I/R group exhibited significantly decreased levels of NO, Akt, and p-eNOS in rat myocardial cells, and the differences were statistically significant ($p<0.05$). Moreover, these levels significantly increased in both MiR-181a and I/R group ($p<0.05$). The levels of NO and p-eNOS in MiR-181a group were remarkably up-regulated when compared with those in the Sham group ($p<0.05$). These findings proved that miR-181a could inhibit myocardial apoptosis by activating the Akt signaling pathway, so as to protect from MIRI. Currently, the role of the Akt signaling pathway in myocardial I/R has been a research hotspot. It has been found that the inhibition of Akt signals by P13K/Akt inhibitor can accelerate the apoptosis of myocardial cells[22,23]. The phosphorylation of Akt promotes the activation of various target proteins, including p-eNOS[24]. Suchal et al[25] have suggested that the phosphorylation of Akt also mediates the quick response of p-eNOS to stress *in vivo* or *in vitro*. Activated p-eNOS plays an important role in I/R. The present experiment started with the Akt signaling pathway to corroborate the influence of miR-181a on I/R injury. It was discovered that in I/R, miR-181a phosphorylated Akt and promoted the phosphorylation of p-eNOS as well. However, the expression of p-eNOS could up-regulate the concentration of endothelium-derived vascular relaxing factor NO in blood, which regulated the growth, apoptosis, and migration of vascular endothelial cells. Ultimately, this inhibited the apoptosis of myocardial cells.

Conclusions

We found that the activation of Akt by miR-181a accelerates the phosphorylation of its downstream protein p-eNOS, represses myocardial cell apoptosis, and mitigates MIRI.

Conflict of Interests

The authors declare that they have no conflict of interests.

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