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MiRNA-1 promotes pyroptosis of cardiomyocytes and release of inflammatory factors by downregulating the expression level of PIK3R1 through the FoxO3a pathway

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Abstract. – OBJECTIVE: Pyroptosis can be seen in both physiological and pathological processes, and whether it plays a positive or negative role has not been fully clarified, especially in the study of cardiology, which deserves more attention. A model of cardiomyocyte Hypoxia/Reoxygenation was established to detect MicroRNA (miRNA) expression.

MATERIALS AND METHODS: The C57BL/6 mice and H9c2 cells were respectively treated with Ischemic/Reperfusion (I/R) and Hypoxia/Reoxygenation (H/R). Then, the concentrations of interleukin-1β (IL-1β) and IL-18 were detected in serum and culture medium supernatant, as well as the expression levels of FOXO3, Caspase1, and PIK3R1.

RESULTS: In I/R group, the concentrations of IL-1β and IL-18 in the supernatant of H9c2 cell culture solution were significantly higher compared with the control group. Consistent with the supernatant concentrations of IL-1β and IL-18, IL-1β, and IL-18 were also increased at a high level in I/R group at the transcriptional level. Furthermore, the *in vitro* experiments showed that FOXO3 and Caspase1 were significantly upregulated in myocardial cells with the treatment of I/R group compared to sham group. Concerning pyroptosis, the expression of PIK3R1 was dramatically decreased and the expression of miRNA-1 was significantly increased in H9c2 cells.

CONCLUSIONS: MiRNA-1 promoted pyroptosis of cardiomyocytes and release of inflammatory factors by downregulating the expression level of PIK3R1.

Key Words:
Pyroptosis, Cardiomyocyte, MiRNA-1, PIK3R1.

Introduction

Programmed cell death is an important process in the growth and development of multicellular organisms[1]. In the 1990s, several studies[2,3] involving infectious diseases have reported that the original macrophages of mice infected with bacteria were massive dead and their contents were released in large quantities as the cells collapsed. Further researches have shown that such programmed death of macrophages caused by exogenous infection is characterized by the release of interleukin, and other small molecules leading to the occurrence of inflammatory response, which is different from the characteristic of classical programmed death which does not trigger inflammatory response in the body. Later, Fink et al[4] named this particular programmed phenomenon of cell death pyroptosis. Pyroptosis is a kind of programmed cell death which is similar to apoptosis in morphology and accompanied by inflammatory response. Activation and release of a large number of inflammatory factors is its characteristic[5,6]. Interleukins (ILs) are a class of cytokines produced by and acting on a variety of cells. They play an important role in a series of processes such as the maturation, activation, proliferation, and immune regulation of immune cells. Researches have shown that IL-1β and IL-18 are cytokines produced by monocytes, endothelial cells, and other cells in response to endogenous and exogenous injury[7]. Characteristic inflammatory factors are eventually activated and released in the pyroptosis pathway. During the process of I/R, the pre-IL-1β and pre-IL-18 were cut and activated by upstream Caspase-1. The activation of IL-1β and IL-18 activated neutrophils[8], NKT cells and other immune cells, triggering inflammatory response and causing injury or death in living cells. This process elicits a chemotactic effect on the surrounding macrophages and further induces cell death[9]. Martinon et al[10] have shown that pyroptosis plays an important role in the occurrence and development of various diseases. Therefore, it is of great significance to further investigate the regulatory factors in the process of pyroptosis. Our work will provide a good understanding of pathogenesis and novel therapy of disease.

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MicroRNAs (miRNAs) are a type of short non-coding RNA consisting of 21-23 nucleic acids. At the initial transcriptional stage, miRNAs are form primordium miRNAs (primer miRNAs), which are composed of thousands of nucleic acids. RNA polymerase II is involved in this process in the nucleus. Then, the primer miRNA is processed by Drosha to form an immature miRNA consisting of about several hundred nucleic acids. Pre-miRNAs are eventually transferred from the nucleus to the cytoplasm *via* nuclear output factors expression, and further processed by Dicer into mature miRNAs[11]. Mature miRNA can interact with mRNA (messenger RNA) 3' terminal non-translation region (3' UTR), which plays a role in gene silencing by inhibiting mRNA translation or directly degrading mRNA.

Forkhead box-containing protein, class O (FoxO) transcription factors play important regulatory roles in cell cycle, metabolism, DNA repair, apoptosis, resistance to oxidative stress, and longevity[12]. FoxO3 a family member promotes the expression of target genes such as FasL, Bim, p130, and Cdkn1b/p27, leading to apoptosis. After being phosphorylated by a kinase, FoxO3a transferred out of the nucleus, entered the cytoplasm, bound to protein 14-3-3, and lost its pro-apoptotic activity, which was confirmed[13].

Various miRNAs are related to pyroptosis in I/R, the association between miR-1 and pyroptosis has not been reported. We hypothesized that the changes of miR-1 may affect I/R *in vivo* and H/R *in vitro* through the pyroptosis process. In this study, we demonstrated that miR-1 may serve as a new intervention target to improve cardiomyocytes pyroptosis injury.

Materials and Methods

Cell Culture

H9c2 cell (Cell Culture Center, Shanghai, China) lines were treated with high glucose Dulbecco's Modified Eagle's Medium (DMEM; Life Technology, Wuhan, China) containing 10% fetal bovine serum (FBS; Life Technology, Wuhan, China) at 37°C, 5% CO_2, pH value 7.2-7.4. Under the condition of aseptic constant temperature culture, the cells showed adherent growth. When the concentration of FBS drew to about 1%, H9c2 cells fused quickly. The cells were cultured according to the above method, the medium was changed every other day, and the cell state was observed. When the degree of cell fusion reached to 90%, we passed the cells.

Cell Processing

The H9c2 cells in the control group were cultured in normal environment (5% CO_2, 21% O_2, 74% N_2), and the H9c2 cells in the H/R group were cultured in a 37°C thermostatic incubator for 24 h (5% CO_2, 1% O_2, 94% N_2) in hypoxic culture, and then H/R cells were moved to an environment with normal oxygen concentration for reoxygenation for 12 h at 37°C (5% CO_2, 21% O_2, 74% N_2).

Mouse Modeling and Grouping

Forty C57BL/6 mice (6-8 weeks) were numbered one by one and divided into 4 groups according to the random number table method: control group, I/R group, I/R + miRNA-1 inhibitor group, and I/R + miRNA-1 inhibitor + AAV-si-PIK3R1 group, in which mice in the latter two groups were pre-treated with miRNA-1 inhibitor, and si-PIK3R1 was administered within 30 min after surgery. The I/R modeling method was as follows: the left anterior descending coronary artery was ligated for 30 min, and then reperfused for 4 h to establish an animal model of myocardial ischemia reperfusion. This investigation was approved by the Animal Ethics Committee of People's Hospital of Rizhao Animal Center.

Enzyme-Linked Immunosorbent Assay (ELISA)

The prepared standard liquid was successively added into a row of wells with the concentration gradient, each well was 100 μL, and the sample to be tested was added into the well. After sample addition, we sealed the plate and moved it in the 37°C thermostat for 90 min. 100 μL biotin-labeled mouse anti-IL-1β antibody (Elabscience, Wuhan, China) and anti-IL-18 (Elabscience, Wuhan, China) were added into the other wells, and the plate was sealed with a membrane and then put into 37°C for 60 min. In addition to the blank control well, 10 μL peroxidase-labeled antibody was added to the other wells, and the plate was sealed with a membrane and then placed in a 37°C incubator for 30 min. Adding 90 μL color rendering solution to all wells and put the plate into 37°C stand in the thermostat for 30 min away from light. 100 μL termination solution was added to all wells, and the absorbance of each hole at 450 nm wavelength was measured in the microplate reader (Bio-Rad, Hercules, CA, USA). The absorbance of the sample and the standard material in each well was recorded with the zero well or blank control well as the reference value, according to the concentration of the standard material. Then,

we draw the standard curve for the absorbance, and finally used the absorbance of the sample to find out the corresponding concentration in the standard curve[14].

Real Time-quantitative Polymerase Chain Reaction (RT-qPCR)

The system was established according to the reagent and dosage (Thermo Fisher Scientific, Waltham, MA, USA). After the solution was fully mixed, then it was centrifuged at 5000 rpm for 15 s. Cycle conditions were as follows: 5 min at 95°C, (95°C 10 s-60°C 20 s-72°C 20 s-78°C 20 s) 40 cycles. The above procedure obtained the cycle number of the amplification curve of the reference gene and the targeted gene respectively. The $2^{-\Delta\Delta Ct}$ method was used to calculate fold amplification. Primers used were shown in Table I.

Western Blot

The H9c2 cardiomyocytes of each group were collected and placed on ice to convert to ice protein. The protein concentration was measured with a bicinchoninic acid (BCA) kit (Jiancheng, Nanjing, China), and a corresponding volume of sample was taken based on the measured concentration. Then, after 1.5 h electrophoresis, 1 h transfer to the polyvinylidene difluoride (PVDF, Thermo Fisher Scientific, Waltham, MA, USA) membrane, and 2 h blocking, we used the diluted primary antibody to incubate at 4°C overnight. The next day, we selected the corresponding secondary antibody, incubated it at room temperature for 1h, and finally reacted with enhanced chemiluminescence (ECL) (Yifei Xue Biotechnology, Nanjing, China) and exposed in the darkroom. The optical density of the target band was analyzed using AlphaEaseFC software processing system (Witec, Littau, Switzerland). The antibodies we use are as follows: PIK3R1 primary antibody (Abcam, 1:1000, Cambridge, MA, USA), FOXO3 primary antibody (Abcam, 1:1000, Cambridge, MA, USA), Caspase1 primary antibody (Abcam, 1:2000, Cambridge, MA, USA), GAPDH primary antibody (1:1000, ProteinTech, Rosemont, IL, USA)

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 21.0 (IBM, Armonk, NY, USA). The data obtained were expressed by mean ± SD (standard deviation). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Test level $p<0.05$ was considered to be of statistical significance.

Results

Hypoxia/Reoxygenation can Induce Pyroptosis of H9c2 Cells

We found that the concentrations (pg/mL) of inflammatory factor IL-1β and IL-18 in supernatant of H9c2 cardiomyocyte culture solution of rats were increased in H/R group (Figure 1A and 1B). We detected that the expression of IL-1β and IL-18 in the H/R group were also significantly higher than those in the control group at transcriptional level (Figure 1C and 1D). Western Blot

Table I. Real time PCR primers.

Gene name	Forward (5'>3')	Reverse (5'>3')
MiRNA-1	AGGGGGTGGAATGTAAAGAAG	GCGTTGTGTTGTGTTGTGTT
MiRNA-199	GGGACAGTAGTCTGCACAT	GAGAGGAGAGGAAGAGGGAA
MiRNA-126	AGGGCGCATTATTACTTTTGG	GTTGTGGTTGGTTGGTTTGT
MiRNA-30	GGGCTTTCAGTCGGATGTT	GTTGTGGTTGGTTGGTTTGT
MiRNA-133	AGGGAGCTGGTAAAATGGAA	GTTGTTGGTTGGTTGGTTGT
MiRNA-873	GGGGCAGGAACTTGTGAG	GTGTGGTGTGGTATGGTGTG
MiRNA-15	UAGCAGCAUAAUGGUUUGUG	GTTGTGGTTGGTTGGTTTGT
IL-18	AACGAATCCCAGACCAGAC	AGAGGGTAGACATCCTTCCAT
IL-1β	ACAACCGCAGTAATACGGAGCA	TGTGCTCTGCTTGAGAGGTGCT
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

qRT-PCR, quantitative Reverse Transcription-Polymerase Chain Reaction.

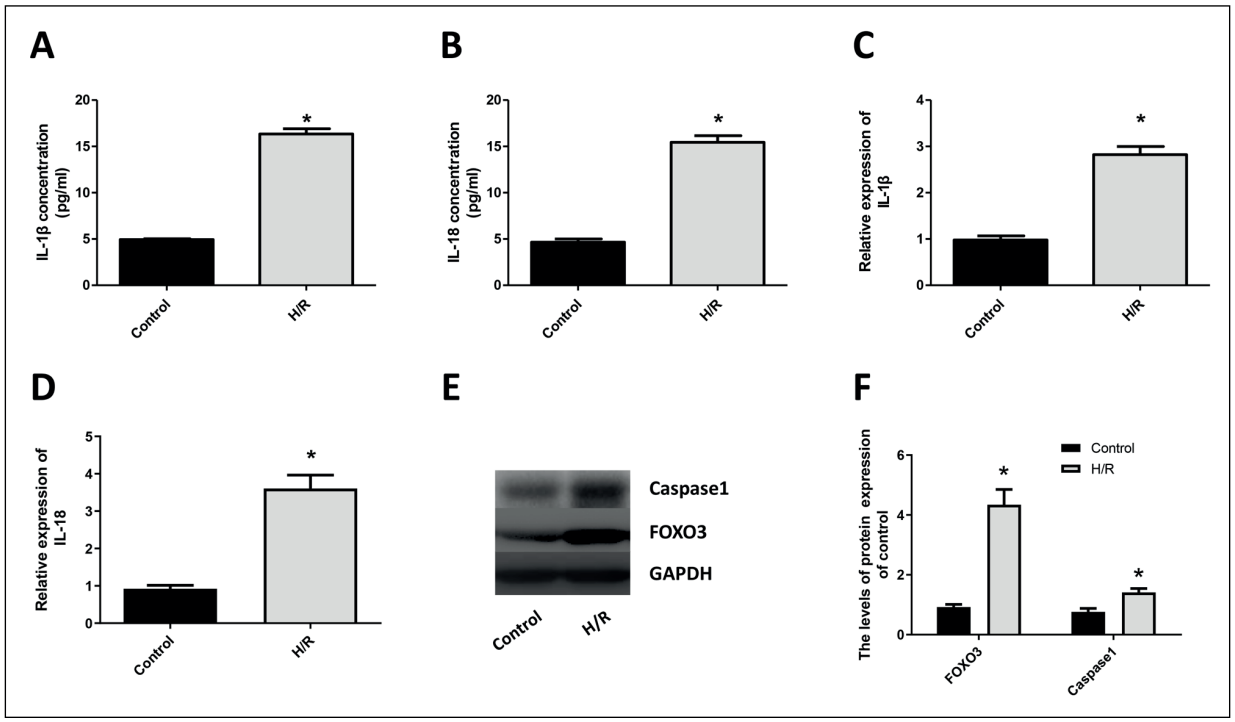

Figure 1. Hypoxia/Reoxygenation can induce pyroptosis of H9c2 cells. **A**, and **B**, Concentration (pg/mL) of inflammatory factor IL-1β and IL-18 in supernatant of H9c2 cardiomyocyte culture solution. **C**, and **D**, Expression of IL-1β and IL-18 in the H/R group detected by qRT-PCR. **E**, and **F**, Expression level of pyroptosis related protein FOXO3 and Caspase1 in H9c2 cells. ("*" indicates statistical difference from the control group $p<0.05$).

results showed that the expression level of FOXO3 and Caspase1 in H9c2 cells in the treatment of H/R was also higher compared with control group (Figure 1E and 1F).

MiRNA-1 Regulates PIK3R1 Expression in H9c2 Cells

We measured the expression of common miRNAs in cardiovascular diseases. *In vitro,* cell culture experiments showed that the expression levels of miRNA-1, mirRNA-15, miRNA-199, miRNA-126, miRNA-133 in H9c2 cells were higher than those in the control group. However, the expression of miRNA-30 and miRNA-873 was not significantly different from that in the control group (Figure 2A). Bioinformatics analysis revealed that there are conserved miRNA-1 binding sites in the 3' untranslation region (3'-UTR) of PIK3R1. Compared with the control group, the expression level of miRNA-1 in H9c2 cells in both H/R group and H/R+mimics group was significantly up-regulated. However, the H/R induced upregulation of miRNA-1 was abolished by the transfection of miRNA-1 inhibitor in H9c2 cells (Figure 2B). In addition, the expression levels of PIK3R1 in untransfected cells and cells transfected with miRNA-1 mimics were significantly lower than that in the control group after H/R treatment, while PIK3R1 expression level in H9c2 cells pre-transfected with miRNA-1 inhibitors was dramatically higher than that in the mimics transfection group (Figure 2C and 2D).

MiRNA-1 Affects Pyroptosis of H9c2

Our results showed that the inflammatory factors IL-1β and IL-18 secreted by H9c2 cells transfected with miRNA-1 inhibitors were significantly lower than those in the H/R group after H/R treatment. In contrast, the inflammatory factors IL-1β and IL-18 secreted by H9c2 cells transfected with miRNA-1 inhibitors and mimics were significantly higher than those of the control group and only transfection inhibitor group (Figure 3A and 3B). Under the treatment of H/R, the expression of FOXO3 and Caspase1 were significantly reduced in the H9c2 cells transfected with miRNA-1 inhibitor, compared with the H/R group. At the same time, transfection of inhibitors and mimics in the H9c2 cells treated with H/R, pyroptosis related proteins FOXO3 and Caspase1

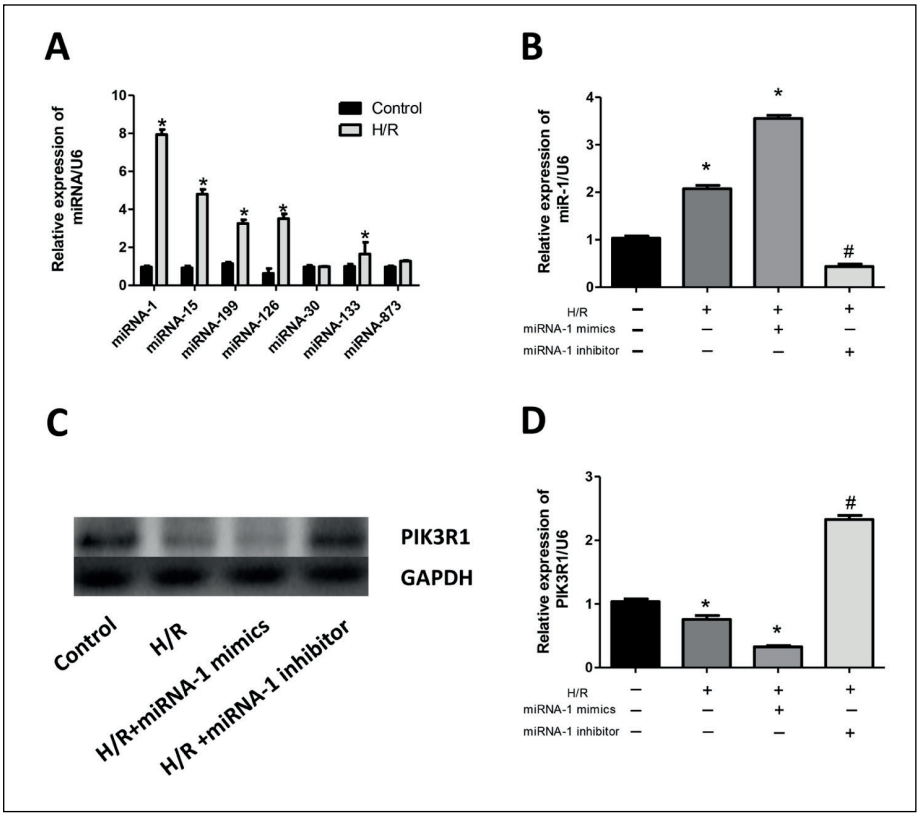

Figure 2. Regulation of miRNA-1 on PIK3R1 expression in H9c2 cells. **A**, MiRNAs expression in pyroptosis H9c2 cells. **B**, Expression of miRNA-1. **C**, and **D**, Effect of miRNA-1 on the expression of PIK3R1 in H9c2 cells. ("*" indicates statistical difference from the control group $p<0.05$ and "#" indicates statistical difference from the miRNA-1 mimics group $p<0.05$).

expression level were significantly higher than that of control group and only transfection inhibitor group (Figure 3C and 3D).

MiRNA-1 Affects Pyroptosis of Cardiomyocytes by Regulating PIK3R1 Expression

After I/R treatment, the expression level of PIK3R1 in myocardium in mice injected with only miRNA-1 inhibitor was up-regulated compared with that in the I/R group. Injection with both the inhibitor and AAV-siPIK3R1 attenuated the inhibition of miRNA-1 induced up-regulation of PIK3R1 in the myocardium of mice treated with I/R (Figure 4A and 4B). The inhibition of miRNA-1 suppressed the concentrations of IL-1β and IL-18 were significantly compared with the I/R group. After I/R treatment in mice treated with both inhibitor and AAV-siPIK3R1, the concentration (pg/mL) of the myocardial inflammatory factor IL-1β and IL-18 were significantly higher than that of the control group and the inhibitor only group (Figure 4C and 4D). The up-regulation of FOXO3 and Caspase1 expression was abolished by the miRNA-1 inhibitor in the myocardium of mice treated with I/R compared with the I/R group. But, the expression level of FOXO3 and Caspase1 were higher in the mice treated with the inhibitor and AAV-siPIK3R1 than that in the control group and the inhibitor only group (Figure 4E and 4F).

Discussion

Several studies referring pyroptosis and diseases have demonstrated that pyroptosis is closely related to cardiomyopathy, atherosclerosis, aneurysms, infectious diseases, and organ dysfunction caused by thermal injury and other diseases. Hence, pyroptosis may be an indispensable part of the innate immune response of the body[15]. Researches have demonstrated the role of pyroptosis in the process of many diseases and a large number of studies have expounded the molecular mechanism related to pyroptosis. Nevertheless, there are still few reports focusing on the regulatory mechanism of pyroptosis. In our research, it was found that the expression level of PIK3R1 in myocardial cells with pyroptosis was significantly changed[16], which proved that PIK3R1 may be an important regulatory factor affecting pyroptosis[17].

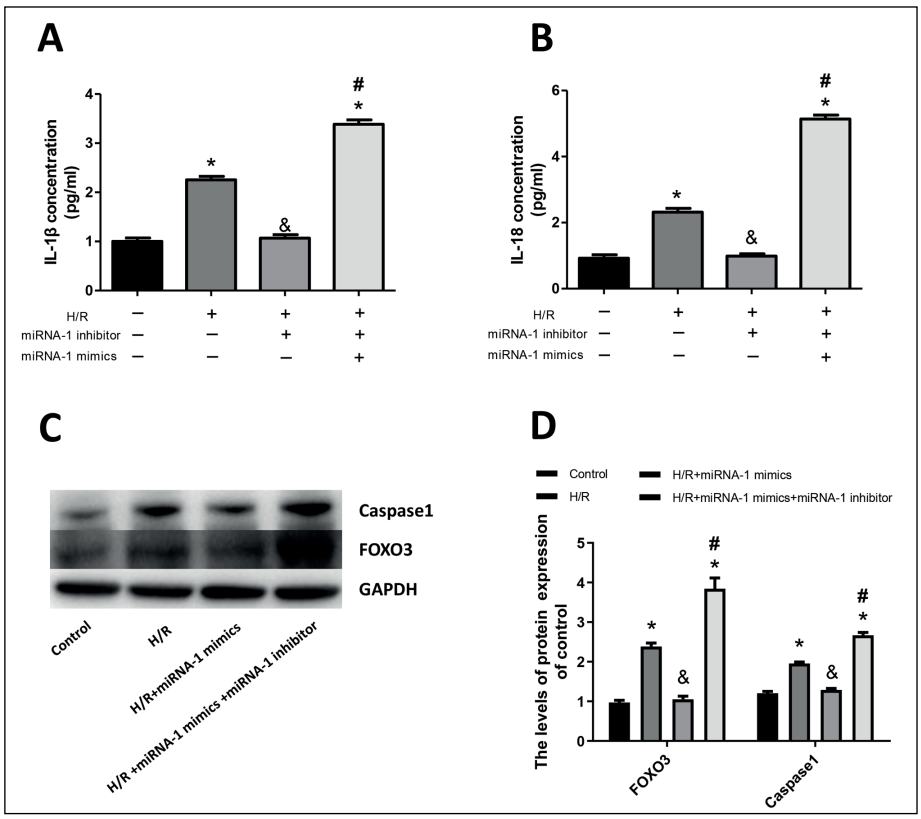

Figure 3. MiRNA-1 affects pyroptosis of H9c2 cells. **A**, and **B**, Concentration (pg/mL) of the inflammatory factor IL-1β and IL-18. **C**, and **D**, Expression of FOXO3 and Caspase1 detected by WB. ("*" indicates statistical difference from the control group $p<0.05$, "#" indicates statistical difference from the miRNA-1 inhibitor group $p<0.05$ and "&" indicates statistical difference from the H/R group $p<0.05$).

In addition, through the detection of miRNA related to cardiovascular diseases, we found that there were significant changes in the expression levels of multiple miRNAs under the condition of cardiomyocyte pyroptosis[11]. Among them, the expression level of miRNA-1 was significantly changed. Currently, there was no relevant research on the relationship between PIK3R1 and pyroptosis, as well as the role of miRNA-1 in the pathogenesis of pyrotosis[18]. We firstly investigated the potential role of miRNA-1 in cardiomyocyte pyroptosis by reperfusion of myocardial ischemia *in vivo* and hypoxia of myocardial cells *in vitro*. It was found that there were significant changes in PIK3R1 and miRNA-1 expressions during cardiomyocyte pyroptosis induced by reoxygenation[19], suggesting that PIK3R1 and miRNA-1 may be related to the occurrence and development of pyroptosis, which laid a foundation for subsequent studies. Under the treatment of I/R, pyroptosis was caused by activated inflammatory factors, which served as a bridge connecting between cardiac ischemia reperfusion with pyroptosis. Therefore, interventional regulation of pyroptosis pathway and targeted inhibition of inflammatory factors may provide a new therapeutic method for alleviating I/R.

Several studies have shown that miRNA plays an important role in cell proliferation, differentiation, metabolism, apoptosis, immune response and other biological processes. Among them, researches on cardiovascular diseases have found that many miRNAs are closely related to myocardial damage in cardiovascular diseases, such as miRNA-1, miRNAs-206, miRNA-873. We showed that the expression of PIK3R1 in cardiomyocytes with pyroptosis was significantly decreased, which suggested that PIK3R1 might be closely related to pyroptosis. Subsequent experiments indicated that miRNA-1 can significantly down-regulate the expression level of PIK3R1 in cardiomyocytes, however, FOXO3 and Caspase1 were key proteins in cell pyroptosis[20,21], and inflammatory factor IL-1β and IL-18 were significantly increased[22]. After PIK3R1 expression was up-regulated, the effect of promoting pyroptosis and the release of inflammatory factors disappeared. Such results suggested that miRNA-1 could down-regulate PIK3R1 expression and promote pyroptosis of cardiomyocytes, indicating that the

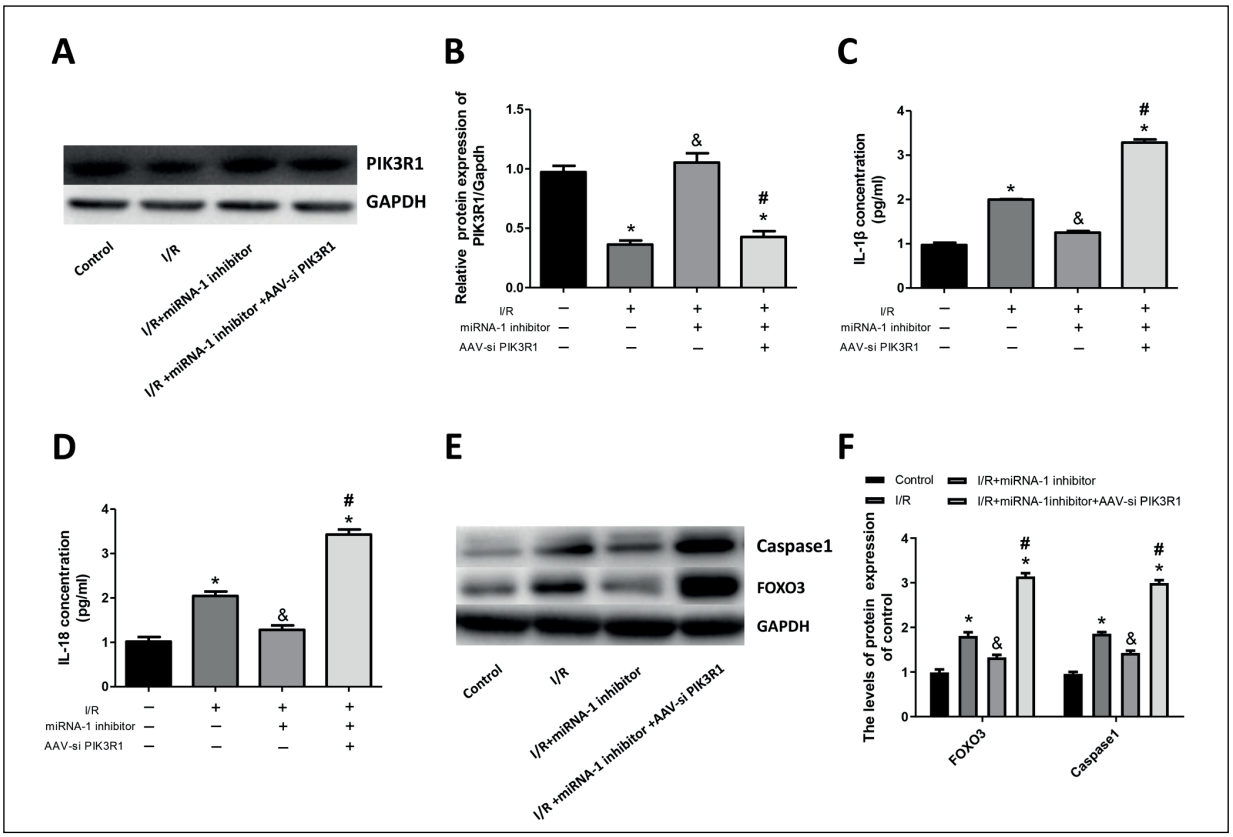

Figure 4. MiRNA-1 affects pyroptosis of cardiomyocytes by regulating PIK3R1 expression. **A**, and **B**, Expression of PIK3R1 detected by WB. **C**, and **D**, Concentration (pg/mL) of the inflammatory factor IL-1β and IL-18. E, and F, Expression of FOXO3 and Caspase1 detected by WB. ("*" indicates statistical difference from the control group $p<0.05$, "#" indicates statistical difference from the I/R + miRNA-1 inhibitor group $p<0.05$ and "&" indicates statistical difference from the I/R group $p<0.05$).

miRNA-1-PIK3R1 pathway may be an important molecular mechanism of cardiomyocyte pyroptosis.

Conclusions

In summary, miRNA-1 aggravated pyroptosis of cardiomyocytes and elevated release of inflammatory factors by down-regulating the expression level of PIK3R1. The PIK3R1 pathway may be an important molecular mechanism in the process of cardiac pyroptosis.

Conflict of Interests

The authors declare that they have no conflict of interests.

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